IN-VIVO AND *IN-VITRO* CHOLESTEROL REMOVAL BY LACTOBACILLI AND BIFIDOBACTERIA

A thesis submitted for the degree of Doctor of Philosophy

By

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B. Sc. Hons (Food Technology), M. Sc. (Food Technology)

2006

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DEDICATED TO THE

LIONG, LOKE AND WONG FAMILIES

I. Abstract

Four strains of *Lactobacillus acidophilus*, eleven strains of *L. casei* and five strains of *Bifidobacterium* were studied for their acid and bile tolerance. Possible mechanisms of cholesterol removal were also examined. All strains showed varying levels of tolerance at pH 2.0 for two hours. *L. acidophilus* ATCC 4962, *L. casei* ASCC 290, *L. casei* ASCC 292, *B. longum* BB536 and *B. infantis* 17930 were more acid tolerant than the other strains studied. All strains could tolerate the presence of bile, with greater tolerance to cholic acid and oxgall, however the strains showed inhibition by taurocholic acid. All strains were able to assimilate cholesterol at varying levels ranging from 4.17 to 32.25 μg/mL. Cholesterol assimilation patterns suggested that its removal was associated with growth of the organisms. Cholesterol removed by dead- and resting cells ranged from 0.79 to 3.82 mg/g of dry weight, indicating the possibility of removal of cholesterol via binging to cellular surface. Fatty acid methyl esters, as quantified using gas chromatography, showed changes of hexadecanoic-, octadecanoic-, total saturated-, and unsaturated acids in cells grown in the presence of cholesterol compared with those grown without cholesterol, suggesting that cholesterol from the medium was incorporated into the cellular membrane.

Similar strains of *Lactobacillus* and *Bifidobacterium* were also evaluated for their bile salt deconjugation ability, bile salt hydrolase activity (BSH) and co-precipitation of cholesterol with deconjugated bile. Results showed that more cholic acid was liberated from the deconjugation of sodium glycocholate than sodium taurocholate. BSH activity indicated that substrate specificity was also more towards glycine-conjugated bile compared to taurine-conjugated bile. Most strains of lactobacilli and bifidobacteria exhibited highest total BSH activity (1.60-1.99 U/mL for lactobacilli and 0.87-1.39 U/mL for bifidobacteria) in bile salt mixture (2.8 mM sodium glucocholate and 1.2 mM sodium taurocholate) than individual conjugated bile. Co-precipitation of cholesterol with cholic acid was observed from deconjugation of both conjugated bile, and increased rapidly with decreasing pH levels below 5.0, with maximum co-precipitation at pH 1.0.

Based on these preliminary experiments, we selected the more acid and bile tolerant strains (tolerated pH 2.0 for more than 120 mins and 0.3% bile) with best cholesterol removal ability from each of *L. acidophilus*, *L. casei* and *Bifidobacterium* for subsequent optimization. *L. casei* ASCC 292, *L. acidophilus* ATCC 4962 and *B. infantis* ATCC 17930 were grown in the presence of six prebiotics, namely, sorbitol, mannitol, maltodextrin, high-amylose maize, fructooligosaccharide (FOS), and inulin, in order to determine the best combination of inoculum size (probiotic) and best concentration of prebiotics for removing the highest level of cholesterol.

First-order models showed that the combination of *L. casei* ASCC 292, FOS, and maltodextrin was the most efficient for the removal of cholesterol, while *L. acidophilus* ATCC 4962 in the presence of mannitol, FOS and inulin was best for cholesterol removal. *B. infantis* ATCC 17930 in combination with sorbitol, inulin and maltodextrin were significant for removal of cholesterol ($P < 0.05$). Optimum experimental regions were developed using the steepest ascent. Subsequent second-order polynomial regression model estimated that the optimum condition of the factors for cholesterol removal by *L. casei* ASCC 292 was 1.71% (wt/vol) inoculum size, 4.95% (wt/vol) FOS, and 6.62% (wt/vol) maltodextrin, while that for *L. acidophilus* ATCC 4962 were 2.64% w/v inoculum size, 4.13% w/v mannitol, 3.29% w/v FOS and 5.81% w/v inulin. Similarly, optimum cholesterol removal (52.18 μg/mL) was achieved from inoculum size of *B. infantis* ATCC 17930 at 2.70% (w/v), sorbitol at 6.30% (w/v), maltodextrin at 4.60% (w/v) and inulin at 8.60% (w/v). Validation experiments showed that the response surface method was reliable for developing the model, for optimizing factors, and for analysing interaction effects.

Analyses of growth, substrate utilization, growth yield, mean doubling time, and short-chain fatty acid (SCFA) production by the use of quadratic models indicated that cholesterol removal was growth associated and was encouraged by higher growth and substrate utilization rates. The production of organic acids also appeared to be growth associated and highly influenced by the types and concentrations of prebiotics. In addition, the production of lactic and acetic acids was relatively sensitive to the end-product fermentation of maltodextrin, sorbitol, FOS and inulin. Increased production of lactic acid also showed cessation of growth of the organisms, indicating inhibition of growth at high concentration of lactic acid (approximately beyond 109 mmol/L). Increased concentration of FOS contributed to the increased production of propionic acid, while mannitol and maltodextrin exhibited a positive correlation on the production of formic acid.

We subsequently evaluated the hypocholesterolemic effects of the optimized synbiotics using rats kept on a high-cholesterol diet (1% cholesterol). *L. casei* ASCC 292, *L. acidophilus* ATCC 4962 and *B. infantis* ATCC 17930 were evaluated in the presence of all prebiotics combined as well as with individual prebiotics.

Three synbiotics were given to male Wistar rats ($n = 6$) including *L. casei* ASCC 292 and fructooligosaccharides (FOS) (LF), *L. casei* ASCC 292 and maltodextrin (LM) and *L. casei* ASCC 292, FOS and maltodextrin (LFM). The control group had no probiotic or prebiotics. The effect of the synbiotic on intestinal microflora and concentration of organic

acids was also investigated. The spleen, liver and kidney were analysed to determine the presence of lactobacilli which may indicate translocational property of the probiotic. The LFM diet lowered serum total cholesterol and triglycerides levels, while the LM diet increased serum HDL-cholesterol level. The LFM diet decreased the population of staphylococci, bacteroides, *E. coli* and total coliforms in most bowel segments, possibly contributed by increased concentration of lactic acid in those segments.

Similarly, four diets namely *L. acidophilus* ATCC 4962 and fructooligosaccharide (LF), *L. acidophilus* ATCC 4962 and mannitol (LM), *L. acidophilus* ATCC 4962 and inulin (LI) and *L. acidophilus* ATCC 4962 and all three prebiotics (LFMI) were evaluated in rats. The LFMI diet reduced serum total cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol levels by 32.40%, 32.51% and 42.95%, respectively. The LI diet decreased the pH values in the intestines resulting in decreased population of total aerobes, staphylococci, *Escherichia coli*, coliforms and bacteroides.

Rats were also given diets containing *B. infantis* ATCC 17930 namely *B. infantis* and sorbitol (BS), *B. infantis* and maltodextrin (BM), *B. infantis* and inulin (BI), and *B. infantis* and all three prebiotics (BSMI). Rats on the BM diet decreased total serum cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol level compared to the control, possibly due to increased production of propionic acid. Diet BS, BM and BI increased the population of *Bifidobacterium* in the cecum and colon, accompanied by increased concentration of acetic acid. These led to decreased counts of total aerobes, *Escherichia coli* and bacteroides in those intestinal segments. There was no *Lactobacillus* and *Bifidobacterium* detected in the spleen, liver and kidney of all synbiotics studied, indicating no occurrence of translocation.

The synbiotic containing *L. acidophilus* ATCC 4962, mannitol, FOS and inulin showed the most promising hypocholesterolemic effects in rats, and thus was used for further evaluation on plasma lipid profiles and red blood cell (RBC) membrane properties in hypercholesterolemic pigs. Twenty four White male Landrace pigs were randomly allocated to four treatment groups for 8 weeks (n = 6). Treatment factors were the supplementation of synbiotic (with and without) and dietary fat (5% and 15%).

Plasma lipoprotein profiles were measured using commercial assay kits while RBC properties were studied using Wright's stain, commercial kits and fluorescent anisotropy. The supplementation of synbiotic reduced serum total cholesterol ($P = 0.001$), triacylglycerol ($P = 0.002$) and LDL-cholesterol ($P = 0.045$) for both dietary fat. Although pigs given the

high-fat diet remained hypercholesterolemic after the experimental period, pigs on the low-fat diet supplemented with the synbiotic achieved normal levels after 8 weeks, while those on the control diet remained hypercholesterolemic. A higher concentration of esterified-cholesterol in HDL of pigs supplemented with synbiotic than the control regardless of dietary fat ($P = 0.036$) indicated that cholesterol was reduced in the form of cholesteryl-esters (CE). Reduced concentration of CE ($P < 0.001$) and increased concentration of triacylglycerol ($P = 0.042$) in LDL of pigs on synbiotic suggested that LDL-cholesterol was reduced via the hydrolysis of smaller and denser LDL particles. The deformity in RBC of pigs on the high-fat diet was more prevalent that those given the low-fat diet. Despite this, pigs without synbiotic showed higher occurrence of spur cells than the pigs given synbiotic, as supported by the higher cholesterol:phospholipid ratio in RBC ($P = 0.001$). Also, fluorescence anisotropy that targeted the apolar, polar and interfacial regions of phospholipids in RBC of pigs was significantly lower ($P < 0.001$) when pigs were supplemented with synbiotic compared to those without supplementation for both dietary fat content, indicating reduced membrane rigidity and improved fluidity.

II. Certificate

Dr. Nagendra P. Shah (M. Sc., Ph. D.)
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CERTIFICATE

This is to certify that the thesis entitled "*IN-VIVO* AND *IN-VITRO* CHOLESTEROL REMOVAL BY LACTOBACILLI AND BIFIDOBACTERIA" submitted by Liong Min Tze in partial fulfilment of the requirements for the award of Doctor of Philosophy in Food Technology at Victoria University is a record of bonafide research work carried out by her under my personal supervision and the thesis has not been previously formed the basis for the award of any degree, diploma or other similar title.

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Werribee, Australia
(Professor N. P. Shah)
Thesis supervisor

Date:

III. Acknowledgement

I would like to thank my principal supervisor, Professor Nagendra P. Shah for his ongoing support throughout my studies. His continuous diligence and patience over these years are genuinely appreciated. He has also granted me the opportunity to broaden my career in the academic field to which I extend my sincere gratitude. With his motivation, I have obtained the dynamism to overcome the complicated and subdued hurdles for the completion of this degree.

I would also like to thank my co-supervisor, Professor Frank Dunshea for all his contribution on my studies. His methodical and comprehensive planning had ensured a systematic, assistance-assured and solution-oriented research experience to which I have gratefully gained knowledge of. His intellectual input, unbiased and accessible nature had alleviated the complications encountered from the field work and beyond, to which I am indeed indebted.

I am grateful to Victoria University, the Australian government and the University of Science, Malaysia for their financial support that had enabled me to complete my degree and the privileged opportunities to broaden my knowledge via international conferences.

I wish to thank the laboratory manager, Mr. Dale Tomlinson and all the laboratory technicians, especially Mrs. Ira Prasathya, Mr. Joe Pelle, Ms. Marianne Tellbach, Mrs. Stacey Lloyd, Mrs. Min Thi Nguyen, Mrs. Charmine DiQuattro and Mr. Michael Rogerson for all their technical help and also their patience with my persistent chemical and instrumental demands.

I am also extremely thankful to my colleagues and friends for their on-going support; Lydia Ong, Anne Lise Tang, Jamie Cheong, Ninoska Donaires, Mark Pellegrino, Sudinna Hewakapuge, Rupika Liyanaarachchi, Shruti Harnal, Wun Keat Ding, Bogdan Zisu, Thanut Amatayakul, Paul Capela, Umi Purwandari, Santosh Kumar, Yogesh Mistry, Natalie Nemaza, Osaana Donkor, Daniel Otieno and Thuy Pham. They have all eased the pain encountered throughout this thorny journey and indeed have been more than friends.

Finally, the most special thanks to my parents, husband and sister for their continuous support and never-failed daily phone calls that gave me strength and willpower when I needed them most.

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IV. Publications and Awards

Journal Publications

1. Liong, MT and Shah, NP. 2005. Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties. **Food Res Int** 38: 135-142.
2. Liong, MT and Shah, NP. 2005. Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. **J Dairy Sci** 88: 55-66.
3. Liong, MT and Shah, NP. 2005. Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. **Int Dairy J** 15: 391-398.
4. Liong, MT and Shah, NP. 2005. Acid and bile tolerance and the cholesterol removal ability of bifidobacteria strains. **Biosci Microflora** 24: 1-10.
5. Liong, MT and Shah, NP. 2005. Optimization of cholesterol removal by probiotics in presence of prebiotics using response surface methodology. **Appl Environmen Microbiol** 71: 1745-1753.
6. Liong, MT and Shah, NP. 2005. Optimization of growth of *Lactobacillus casei* ASCC 292 and production of short-chain fatty acid in the presence of fructooligosaccharide and maltodextrin. **J Food Sci** 70: M113-M120.
7. Liong, MT and Shah, NP 2005. Optimization of cholesterol removal, growth and fermentation patterns of *Lactobacillus acidophilus* ATCC 4962 in presence of mannitol, FOS and inulin: a response surface methodology approach. **J Appl Microbiol** 98: 1115-1126.
8. Liong, MT and Shah, NP. 2005. Production of organic acids from fermentation of mannitol, FOS and inulin by a cholesterol removing *Lactobacillus acidophilus* strain. **J Appl Microbiol** 99: 783-793.
9. Liong, MT and Shah, NP. 2005. Roles of probiotics and prebiotics on cholesterol: the hypothesized mechanisms. **Nutrafoods** 4: 45-57.
10. Liong, MT and Shah, NP. 2006. Synbiotic effects of *Lactobacillus acidophilus* ATCC 4962, fructooligosaccharide, mannitol and inulin on serum lipid profiles, intestinal microflora population and intestinal organic acids production in rats. **LWT Food Sci Technol** (In review).
11. Liong, MT and Shah, NP. 2006. The application of response surface methodology to optimize removal of cholesterol, and to evaluate growth characteristics and production of organic acids by *Bifidobacterium infantis* ATCC 17930 in the presence of prebiotics. **Int J Probiotics Prebiotics** 1: 41-56.

12. Liong, MT and Shah, NP. 2006. Effects of *Lactobacillus casei* ASCC 292, fructooligosaccharide and maltodextrin on serum lipid profiles, intestinal microflora and organic acids concentration in rats. **J Dairy Sci** 89: 1390-1399.
13. Liong, MT and Shah, NP. 2006. Sorbitol, maltodextrin, inulin and *Bifidobacterium infantis* modify serum lipid profiles, intestinal microbial population and organic acids concentration in rats. **Int J Probiotics Prebiotics** (In press).
14. Liong, MT, Dunshea, FR and Shah, NP. 2006. Effects of a synbiotic containing *Lactobacillus acidophilus* ATCC 4962, FOS, mannitol and inulin on serum lipid profiles and morphology of red blood cells in hypercholesterolemic pigs on high- and low-fat diets. **Am J Clin Nutr** (In review).

Chaired Oral Presentations

1. Liong, MT and Shah, NP. Mechanisms of *in-vitro* cholesterol removal by strains of *Lactobacillus acidophilus* and *Lactobacillus casei*. **SFAM Summer Conference**, 12-15 July 2004, Cork, Ireland.
2. Liong, MT and Shah, NP. Galactooligosaccharides and human health. **World Nutra Conference**, 7-10 November 2004, San Francisco, USA.
3. Liong, MT and Shah, NP. Synergistic effects of *Lactobacillus casei* ASCC 292, FOS and maltodextrin for *in-vitro* removal of cholesterol: a response surface approach. **IFT Annual Meeting**, 15-20 July 2005, New Orleans, USA.
4. Liong, MT and Shah, NP. Development of synbiotic products for *in-vitro* removal of cholesterol using response surface methodology. **IFT Annual Meeting**, 15-20 July 2005, New Orleans, USA.
5. Liong, MT and Shah, NP. The mechanisms of *in-vitro* cholesterol removal by probiotics and the effects of synbiotics on serum lipoproteins, intestinal microbiology, intestinal organic acids, and morphology of red blood cells in animal models. Malcolm Bird Award, **AIFST Annual Convention**, 9-12 July 2006, Adelaide, Australia.

Chaired Poster Presentations

1. Liong, MT and Shah, NP. Benefits of *Lactobacillus casei* ATCC 292, fructooligosaccharide and maltodextrin on reduction of serum cholesterol in rats. **AIFST Annual Convention**, 10-13 July 2005, Sydney, Australia.
2. Liong, MT, Dunshea, FR and Shah, NP. Improved serum lipid profiles and morphology of red blood cells in pigs fed a high-cholesterol diet by *Lactobacillus acidophilus* ATCC 4962, fructooligosaccharide, inulin and mannitol. **IFT Annual Meeting**, 24-28 June 2006, Orlando, USA.
3. Liong, MT, Dunshea, FR and Shah, NP. *Lactobacillus acidophilus* ATCC 4962 in the presence of fructooligosaccharide, inulin and mannitol improved serum lipid profiles and morphology of red blood cells in pigs fed a high-cholesterol diet. **AIFST Annual Convention**, 9-12 July 2006, Adelaide, Australia.

Awards and Grants

1. **Studentship Grant** from the Society of Applied Microbiology for the SFAM Summer Conference, 12-15 July 2004, Cork, Ireland.
2. Runner-up for the **Summer Conference Student Oral Prize** from the Society of Applied Microbiology, 15 July 2004, Cork, Ireland.
3. **Outstanding Final Year Research Student Award** from the Faculty of Health, Engineering and Science, Victoria University, 25 May 2006, Melbourne, Australia.
4. **Honorarium for Travel** from SPI Polyols Inc., Denmark for the IFT Annual Conference, 24-28 June 2006, Orlando, USA.
5. Runner-up for the **Malcolm Bird Award** from the Australian Institute of Food Science and Technology, 13 July 2006, Adelaide, Australia.

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VIII. List of Abbreviations

ANOVA = Analysis of variance

ANS = 8-anilino-1-napthalenesulfonic acid

ASRC = Australia starter culture research centre

ATCC = American type culture collection

BSA = Bovine serum albumin

BSH = Bile salt hydrolase

CCD = Central composite design

CE = Cholesteryl ester

CETP = Cholesteryl esters transport protein

CFU = Colony forming unit

C/P = Cholesterol: phospholipids ratio

CSCC = Commonwealth scientific culture collection

DE = Dextrose equivalent

DF = Degree of freedom

DP = Degree of polymerization

DPH = 1,6-diphenyl-1,3,5-hexatriene

FA = fatty acids

FAn = Fluorescence anisotropy

FOS = Fructooligosaccharide

GC = Gas chromatography

GRAS = Generally regarded as safe

HDL = High-density lipoprotein

HDL-C = High-density lipoprotein cholesterol

LDL = Low-density lipoprotein

LDL-C = Low-density lipoprotein cholesterol

MRS agar = de Mann, Rogosa, Sharpe agar

OD = Optical density

RBC = Red blood cell

RSM = Response surface methodology

SCFA = Short-chain fatty acids

TC = Total cholesterol

TG = Triglycerides

TMA-DPH = 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene

VLDL = Very low-density lipoprotein

1.0 Introduction to Thesis

Elevated levels of certain blood lipids have been reported to be the principal cause of cardiovascular disease and the leading cause of death and disabilities in developed countries (Pekkanen *et al*., 1990). Studies and clinical trials have shown a continuous, positive association between cholesterol levels and the risks of coronary heart disease (Stamler *et al*., 2000). Various approaches have been used to alleviate this issue, including the use of probiotics and/or prebiotics. Probiotics have been defined as 'live microorganisms that could confer health benefits on the host when administered in adequate amounts' (Reid *et al.*, 2003), while prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating growth, activity, or both of one or a limited number of bacterial species already resident in the colon (Gibson and Roberfroid, 1995). The combination of both probiotic and prebiotics has been termed synbiotic.

Before any strains of probiotic could be used as a food adjunct, their tolerance on gastrointestinal stresses particularly under acidic and bile conditions have to be assessed. Once administered, probiotics are exposed to the harsh digestive processes for more than 90 minutes before being released from the stomach into the intestine (Berada *et al.* 1991). Acid tolerance is often a crucial factor considering that the pH of the stomach could be as low as 1.5 in fasting subjects (Gibson *et al.*, 2000). In addition, when probiotics pass through the upper intestinal tract, they are subjected to varying bile types and concentrations that may decrease their viability.

Probiotics and prebiotics have received much attention on their proclaimed health benefits which include improvement in lactose intolerance, increase in natural resistance to infectious disease in gastrointestinal tract, suppression of cancer, reduction in serum cholesterol level and improved digestion (Gibson and Roberfroid, 1995). In addition, there are numerous reports on cholesterol removal ability of probiotics and prebiotics in *in-vitro* experiments (Pereira and Gibson, 2002; Tahri *et al.,* 1996), and also the hypocholesterolemic effects in *in-vivo* subjects (Bukowska *et al.*, 1997; Delzenne and Kok, 2001; Schaafsma *et al.*, 1998). Despite these claimed benefits, a decisive outcome failed to be reached due to controversies raised. Several *in-vivo* trials have shown insignificant effect of probiotics and prebiotics on blood lipid properties, such as yoghurt enriched with *L. acidophilus* did not alter serum lipids of healthy humans (De Roos *et al*., 1999), milk product fermented by probiotic did not increase cholesterol excretion in ileostomy subjects (Marteau *et al.*, 1995), ingestion of probiotic supplement had no hypocholesterolemic effect on post-menopausal women (Greany *et al.*, 2004), consumption of fructooligosaccharides did not lower serum cholesterol levels of diabetes type-II patients (Alles *et al*., 1999) while resistant starch did not affect serum lipoproteins in rats (Jenkins *et al*., 1987).

Also, the exact mechanism for cholesterol removal is poorly understood. While *in-vivo* trials imposed variation in subjects that would complicate most assessments on the mechanisms involved, such trials also failed to reach a conclusive outcome. Several possible mechanisms for cholesterol removal by probiotics and prebiotics based on various *in-vitro* studies have been proposed including assimilation of cholesterol by growing-cells (Pereira and Gibson, 2002), binding of cholesterol to cellular surface (Noh *et al*., 1997), incorporation of cholesterol into the cellular membrane (Kimoto *et al*., 2002), deconjugation of bile via bile salt hydrolase (Kurdi *et al.*, 2003), co-precipitation of cholesterol with deconjugated bile, binding action of bile by fibre (Marlett, 1997) and production of short-chain fatty acids by oligosaccharides (Hara *et al.*, 1994). However, some of the mechanisms mentioned above were found to be strain dependent and conditions generated under laboratory conditions would not be practical in the *in-vivo* systems.

The combinations of probiotics in the presence of prebiotics on several aspects of health have also been documented (Gibson and Roberfroid, 1995; Niness, 1999). A study using rats showed improved colon carcinogenesis after consumption of *Bifidobacterium* and oligofructose (Gallaher and Khil, 1999) while the combination of several probiotics including *L. plantarum* and *L. paracasei*, and prebiotics such as inulin and resistant starch reduced post-operative infection (Rayes *et al.*, 2005). However, very little attempt has been made to develop a synbiotic product with a specific need to remove cholesterol in order to exert hypocholesterolemic effect *in-vivo*. Most studies in the past had emphasised on the cholesterol reducing or lowering properties of probiotics or prebiotics individually but not synergistically. The main reasons for this would be the complication raised due to the number of possible probiotic-prebiotic combinations and also the complex evaluation of the synergism from the interaction effects. Although all these would seem unfeasible with the conventional screenings, there is no attempt to develop such cholesterol lowering synbiotic product using statistical approaches. The response-surface approach would be an extremely useful tool for developing, optimizing and estimating interaction effects (Lee and Chen, 1997).

Numerous studies have shown promising results in reducing triglyceride, total- and LDL cholesterol levels after consumption of probiotic and/or prebiotics (Jin *et al.*, 1998), but no attempt has been made to study the effect of synbiotics on the compositions of plasma lipoproteins. This is important as lipoproteins are the major lipid transporters, and alterations in their properties would affect serum lipid profiles in general. The information generated would also provide better understanding on the effect of synbiotics on lipid metabolism. Moreover, very little information is available on the effects of synbiotics on the

characteristics of erythrocytes in hypercholesterolemic subjects. This is also of upmost importance considering that morphological defects of the RBC such as the formation of spur cells have been reported in hypercholesterolemic animals (Cooper *et al.*, 1980).

This study was aimed at achieving two broad objectives. The first aim was to screen and develop three synbiotic products with cholesterol removing properties *in-vitro*. The second aim was to evaluate the cholesterol lowering effects of the developed synbiotics in animal models using rats and pigs. Thus, the specific aims of this project were to:

1. evaluate the acid and bile tolerance of four strains of *Lactobacillus acidophilus*, seven strains of *L. casei* and five strains of *Bifidobacterium*,
2. study the possible mechanisms of cholesterol removal *in-vitro* by *Lactobacillus* and *Bifidobacterium*,
3. screen and select one strain from each of *L. acidophilus, L. casei* and *Bifidobacterium* with cholesterol removal ability that showed good tolerance to acidic and bile conditions for subsequent development of synbiotics,
4. optimize cholesterol removal *in-vitro* by strains of the selected probiotics in the presence of prebiotics,
5. evaluate the growth properties of the probiotics and fermentation patterns of the prebiotics within the optimized regions for maximum removal of cholesterol,
6. evaluate the cholesterol lowering properties of the developed synbiotics and their effects on intestinal microflora and intestinal concentration of organic acids, using rats as an animal model, kept on a high-cholesterol diet,
7. evaluate the effect of the best cholesterol-lowering synbiotic on serum lipoprotein compositions and on RBC morphology and membrane properties in hypercholesterolemic pigs on high- and low-fat diets.

Chapter 2.0 of this thesis deals with the literature review that highlighted the use of probiotics, prebiotics and synbiotics on cholesterol removal, the mechanisms involved, and *in-vivo* studies. Chapter 3.0 emphasises on the evaluation of acid and bile tolerance and several cholesterol removal mechanisms by *Lactobacillus* and *Bifidobacterium*. Chapter 4.0 reports on the bile salt deconjugation ability of these strains via bile salt hydrolase activity, and the possible cholesterol removal via co-precipitation of cholesterol with the deconjugated bile. Chapter 5.0 focuses on the screening and optimization of *L. casei* ASCC 292, *L. acidophilus* ATCC 4962 and *B. infantis* ATCC 17930 in the presence of six prebiotics for maximum removal of cholesterol *in-vitro*. In addition, the growth properties of these organisms and the fermentation patterns of the prebiotics have also been examined. Chapter 6.0 reports the administration of the developed synbiotics in rats kept on a high-

cholesterol diet. The effects of these synbiotics on serum lipid profiles, intestinal microbiology and intestinal production of organic acids have also been evaluated. Chapter 7.0 focuses on further evaluation of the synbiotic containing *L. acidophilus* ATCC 4962, which showed best hypocholesterolemic effect in rats, using hypercholesterolemic pigs on high- and low-fat diets. The effect of this synbiotic on serum lipid profiles, lipoprotein compositions and properties of RBC is also examined. The overall conclusions are included in Chapter 8.0, and the future directions of research are highlighted in Chapter 9.0. All references are listed in Chapter 10.0.

2.0 Literature Review

2.1 Probiotic organisms

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Reid *et al.*, 2003). Over the years, many species of microorganisms have been used. They not only consist of lactic acid producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* and fungi such as *Saccharomyces* and *Aspergillus*. Postulated health advantages associated with probiotics intake are improvement of lactose intolerance, increase in humoral immune responses, biotransformation of isoflavone phytoestrogen to improve post-menopausal symptoms, bioconversion of bioactive peptides for antihypertension, improvement of serum lipid profiles, increase in natural resistance to infectious disease in gastrointestinal tract, suppression of cancer, reduction in serum cholesterol level and improved digestion (Gibson and Roberfroid, 1995).

2.1.1 *Lactobacillus*

Lactobacillus is a Gram-positive facultative bacteria. They are a major part of the lactic acid bacteria group having the ability to convert lactose and other monosaccharides to lactic acid. Intestinal lactic acid bacteria for humans are closely associated with the host's health because they act as an important biodefense in preventing colonization and subsequent proliferation of pathogenic bacteria in the intestine (Goldin and Gorbach, 1992; Naidu *et al.*, 1999). Some species of *Lactobacillus* and *Bifidobacterium* that have been claimed as probiotics include *L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. casei*, *L. fermentum*, *L. plantarum*, *L. reuteri, B. infantis, B. breve, B. animalis, B. adolescentis* and *B. longum*. The gastrointestinal tract of a healthy human is a harsh environment and poses a significant threat to probiotic strains. In addition, low surface tension and immune response also affect the survival of probiotic strains (Gilliland, 1979). *Lactobacillus acidophilus* strains are widely used as probiotic cultures in dairy and pharmaceutical products because this species is one of the most dominant lactobacilli in the human intestine (Sandine, 1979). This may be contributed by their non-iron requirement for growth, extremely high hydrogen peroxide tolerance, ability to operate using homofermentative metabolism and are aerotolerant despite the complete absence of a respiratory chain.

Lactobacillus have several scientifically established and/or clinically proved health effects, such as reduction and prevention of diarrhoea, improvement of the intestinal microbial balance by antimicrobial activity, alleviation of lactose intolerance symptoms, prevention of food allergy, enhancement of immune potency, and antitumourigenic activities (McFarland, 2000; Andersson *et al.*, 2001; Salminen, 2001). Moreover, some studies have

shown that certain strains of *Lactobacillus* possess antioxidative activity (Kaizu *et al.*, 1993; Peuhkuri *et al.*, 1996; Kullisaar *et al.*, 2002). They are able to decrease the risk of accumulation of reactive oxygen species in the host and could potentially be used in probiotic food supplements to reduce oxidative stress. In a previous study (Kullisaar *et al.*, 2002), it was reported that *Lactobacillus fermentum* strain ME-3 (DSM 14241) has high antimicrobial and antioxidant activity. In healthy volunteers, it has been demonstrated that the consumption of fermented milk containing *L. fermentum* ME-3 exhibited antioxidative and antiatherogenic effects (Kullisaar *et al.*, 2003). Since milk fermented with lactobacilli was first demonstrated to exhibit hypocholesterolemic effects in humans, various studies have shown that some lactobacilli exhibit cholesterol lowering ability in human (Anderson and Gilliland, 1999), particularly lowering of total- and LDL cholesterol (Sanders, 2000).

2.1.2 *Bifidobacterium*

Bifidobacterium is anaerobic, rod shaped, Gram positive bacteria that is normal inhabitant of the human colon constituting a predominant part of the anaerobic flora. Previous research on human found that *Bifidobacterium* is one of the most predominant cultures in the human colon, with numbers reaching 10^9-10^{10} cfu/g (Benno *et al*., 1989) and is also the predominant intestinal organisms of breast fed infants (Hudault *et al.*, 1994).

Bifidobacteria are regarded to be beneficial for human health and are widely used in probiotic preparations and foods. Several positive effects related to bifidobacteria include synthesis of vitamins, supplementation in ingestion and absorption, inhibition of putrefactive bacteria and stimulation of the immune system (Mitsuoka, 1992). Bifidobacteria have been associated with the treatment of viral diarrhoea (including rotavirus diarrhoea), prevention of travellers' diarrhoea (Black *et al.*, 1989), modulation of intestinal flora (Marteau *et al*., 1990), modulation of immune response (Link *et al.*, 1994), improvement of constipation and alleviation of atopic dermatitis symptoms in children (Fukushima *et al*., 1998). In addition, consumption of yoghurt containing *Bifidobacterium longum* lowered the frequency of antibiotic-induced gastrointestinal disorders (Colombel *et al*., 1987). Experimental studies in mice fed bifidobacteria have also shown lower numbers of chemically induced tumours in the large bowel (Koo and Rao, 1991).

2.2 Prebiotics

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating growth, activity, or both of one or a limited number of bacterial species already resident in the colon (Gibson and Roberfroid, 1995). To exhibit such effects, a prebiotic must neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract, and must be selective for one or a limited number of potentially beneficial bacteria residing in the colon (Collins and Gibson, 1999). The numbers of probiotic in the human gut tend to decrease with age (Mitsuoka, 1992). Two major strategies have been proposed to maintain a high level of probiotics to sustain beneficial health effects; 1) continuous ingestion of probiotic-containing foods or 2) supplementation of food with prebiotics (Gomes and Malcata, 1999).

2.2.1 Inulin and fructooligosaccharides (FOS)

Most widely researched prebiotics fall in the group of oligosaccharides. Oligosaccharides are sugars consisting of 2 to 20 sugar units. Some occur naturally in foods such as leek, asparagus, chicory, garlic, onion and soybean. There are numerous properties of oligosaccharides which serve the desired attributes in prebiotics. High selectivity and efficiency in metabolism by bifidobacteria and lactobacilli have enabled the supplementation of prebiotic at low dosage. It is reasonable to postulate that the longer the oligosaccharide, the further it may penetrate the distal colonic regions. Oligosaccharides are reported to possess receptor sequences that inhibit adhesion of pathogens to mucosal cells. Binding of the pathogens to specific receptor sites in oligosaccharides (Table 2.1) is postulated to increase host resistance to infection, reducing the likelihood of pathogen establishment and subsequent elaboration of virulence (Gibson *et al.*, 2000).

Inulin and fructooligosaccharides (FOS, a lower molecular weight version) are the most studied oligosaccharides for their prebiotic properties. Inulin-type fructans contain both glucosyl and fructosyl molecules, with number of fructose units varying from 2 to 70 units. Inulin naturally occurs in thousands of different plants with garlic, onion, asparagus, chicory and artichoke being especially a rich source (Gibson *et al.*, 1994). The manufacturing of inulin involves extraction from chicory roots producing an average degree of polymerization (DP) of 10 to 12 with lengths of molecules ranging from 2 to 60 units. Inulin is a polydisperse $\beta(2\rightarrow1)$ fructan with linear fructose polymers and oligomers which are each linked by $\beta(2\rightarrow1)$ bonds, while the glucose molecule normally resides at the end of each fructose chain linked by an $\alpha(2\rightarrow1)$ bond. Such $\beta(2\rightarrow1)$ bonds are unique as these linkages

prevent inulin from being digested like any typical carbohydrate, reduced it caloric value and impose dietary fibre effects (Niness, 1999).

Table 2.1 Examples of oligosaccharide receptor sequences for common gut pathogens (Gibson *et al*., 2000).

Receptor sequence	**Pathogen**
Galα1→4Gal	*E. coli*
Galβ1→4GlcNAcβ1→3Gal	*S. pneumoniae*
GalNAcβ1→4Gal	*S. aureus, K. pneumoniae, H. influenzae*
Sialic acids	*E. coli*
Galα1→3Galβ1→4GlcNAc	*C. difficile*
Fucose	*V. cholerae*
GlcNAc	*E. coli, V. cholerae*
Mannose	*E. coli, Salmonella sp., K. aerogenes*

The IUB-IUPAC Joint Commission on Biochemical Nomenclature and the AOAC defined oligofructose as 'fructose oligosaccharide (FOS) containing 2 to 10 monosaccharide residues linked by glycosidic bonds' (Niness, 1999). Common types of FOS include 'neosugar', which is a mixture of three oligosaccharides of different lengths, such as kestose (GF_2), nystose (GF_3) and β-fructosylnystose (GF_4). Oligofructose derived from chicory contains both fructose chains and fructose chains with terminal glucose units while synthesized oligofructose contains only fructose chains with terminal glucose units. Both types of oligofructose contain β(2→1) bonds between the fructose molecules, and have the same nutritional effects (Roberfroid *et al.*, 1998). FOS is not absorbed or degraded in the upper human gastrointestinal tract. It enters the colon intact and metabolized by the resident microbiota. Similar to inulin, the β configuration in fructose monomers of FOS contributes to resistance towards hydrolysis by human digestive enzymes. Thus, inulin and FOS pass through the mouth, stomach and small intestine without being metabolized. A vast majority of FOS ingested could be recovered in ileostomy fluid (Ellegard *et al*., 1997).

The metabolic transport for FOS by lactic acid bacteria and bifidobacteria was studied by Kaplan and Hutkins (2003) using radiolabeled FOS and *Lactobacillus paracasei*. The uptake and hydrolysis of FOS are induced by sucrose or higher oligosaccharides, but are repressed by products of their hydrolysis. FOS uptake was competitively inhibited in the

presence of excess glucose, fructose or sucrose. However, excess of FOS would not inhibit uptake of radiolabeled glucose, fructose or sucrose, leading to the belief that separate transport systems exist between these simple sugars and FOS, and the FOS uptake system is specific for a narrow range of substrates. The main requirement for the uptake system was hypothesized to involve β-type sugars linked to α-glucose. Uptake systems for FOS in lactobacilli were also found to be affected by substrate size, when the GF_4 moiety of FOS was not consumed during growth even after the GF_2 and GF_3 fractions had been mostly depleted (Kaplan and Hutkins, 2000). Moreover, substrate competition assays revealed that inulin had little affinity for the FOS transport system (Kaplan and Hutkins, 2003). Interestingly, extracts from lactobacilli cells grown in inulin and supernatant from inulin-grown cells were able to hydrolyse FOS. The enzymes involved were extracellular inulinase and intracellular exo-β-fructofuranosidase, that target the α-1,2 bond between fructose and glucose (Kaplan and Hutkins, 2003).

Previous studies indicated that almost all of the inulin and FOS ingested enters the colon and is totally fermented by colonic microflora (Knudsen and Hessov, 1995; Ellegard *et al*., 1997). All these fermentation produced energy as a result of the production of short-chain fatty acids (SCFA) and lactate, and by-products such as bacterial biomass and gasses that are excreted (Niness, 1999). Lactate is the major end-product of the lactate-producing species. An increase in lactate concentrations decrease the luminal pH, and act as a potent antimicrobial substance to putrefactive microorganisms. Swanson *et al.* (2002b) found that FOS increased lactate production in the gut of canine subjects and decreased putrefactive compounds such as indoles and phenols. FOS reportedly increased butyrate concentrations from both *in-vitro* studies (Gibson and Wang, 1994) and *in-vivo* trials using rats (Campbell *et al.*, 1997). This is encouraging because butyrate is the main energy source for colonocytes, and the preferred energy substrate for colonic epithelium (Roediger, 1982). The bifidobacteria fermentation pathway results in 3 moles of acetic acid and 2 moles of lactic acid for every 2 moles of glucose in ideal synthetic medium, yielding a theoretical molar ratio (acetic:lactic) of 1.5 (Scordovi and Trovelli, 1965). FOS was found to enhance the production of acetic acid compared to lactic acid and reached a ratio as high as 1.9, while inulin produced a lower ratio of 1.5 (Shin *et al.,* 2000).

Swanson *et al.* (2002b) found that the ingestion of FOS increased the sheer numbers of aerobes and lactobacilli in the gut of dog subjects. Bifidobacterial numbers increased followed by the decrease in the *Clostridia perfringens* concentrations, a positive indicator of colon health. In a separate study using mixed batch and chemostat culture, inulin and its hydrolysate were found to stimulate the growth of bifidobacteria, which at the end of

incubation period, became predominant (Wang and Gibson, 1993). Dramatic positive shifts in the composition of microflora have been shown through *in-vivo* human studies at doses between 5 and 20 g/day over a 15-day period (Figure 2.1) (Gibson et al., 1995). This is contributed by the possession of a competitive β-fructosidase enzyme that catalysed a cleavage of glycosidic linkages in FOS so that fructose is released for metabolism via the "bifidus" pathway (Imamura *et al.*, 1994). The bifidogenic effects of different forms of inulin and oligofructose are bifidogenic independent of chain length, terminal units of fructose or glucose (Roberfroid *et al.,* 1998). It was postulated that bifidobacteria have an advantage over other intestinal bacteria due to their outer membrane that locates the related site of the enzyme activity. Bifidobacteria were found to have preference for FOS over glucose (Gibson and Wang, 1994). Three-stage gut models found enhanced proliferation of bifidobacteria by FOS in conditions resembling human colon (McBain and Macfarlane, 1997). However, the enzyme kinetics of bifidobacteria is poorly understood (Gibson *et al.*, 2000). Up to now, very little information is available on the uptake of carbohydrate by bifidobacteria, although it appears that the substrate transport systems may be more efficient for dimeric and oligomeric carbohydrates (Shin *et al.*, 2000).

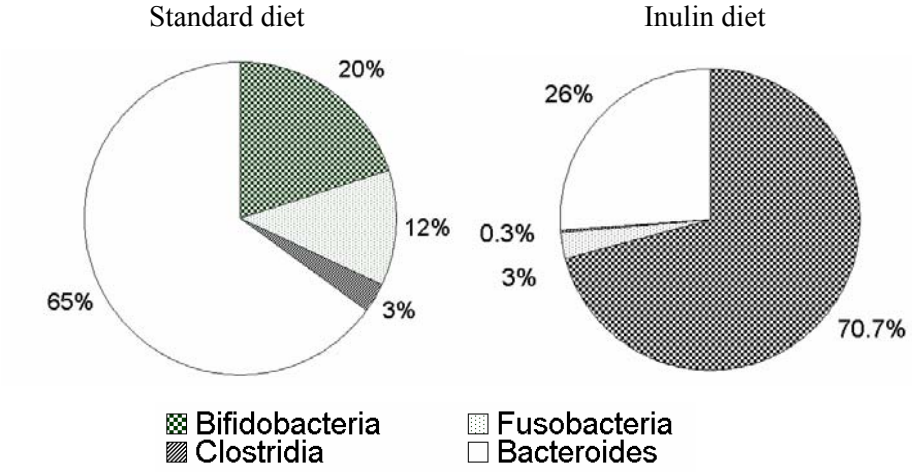


Figure 2.1 Distribution of faecal microflora in human provided a diet with and without inulin (Gibson et al., 1995).

2.2.2 Resistant starch and fibre

Starch that is resistant to the upper gut digestion is metabolized by bifidobacteria and lactobacilli. The name 'resistant starch' was coined to described incomplete digestion *in-vitro* of starch in foods that has been cooked and then cooled (Berry, 1986), but now includes

all starch and starch degradation products that resist small intestinal digestion and enter the large bowel in normal humans (Asp, 1992). There are four main types of resistant starch, namely RS-1 (physically inaccessible starch), RS-2 (resistant granules and high amylose starches), RS-3 (retrograded starches) and RS-4 (chemically modified starches) (Bird *et al.*, 2000). Raw potato starch which contains a large proportion of resistant granules of starch, is considered to be RS 2. Feeding raw potato starch was reported to lead to very active fermentations in the distal parts of the digestive tract (Levrat *et al.*, 1991).

Resistant starch is defined strictly in terms that exclude the small intestine. Small intestine amylolysis can occur at different rates, but only incompletely digested starch can contribute to resistant starch (Truswell, 1995). Resistant starch is not hydrolysed by pancreatic amylases but can be metabolized by bacterially produced enzymes, such as those from saccharolytic clostridia, bacteroides and bifidobacteria (Roberfroid *et al.*, 1998). Some non-digestible properties of resistant starch were also contributed by the presence of non-starch polysaccharides and also the degree of mastication. High-amylose starch has generated much interest as a resistant starch with prebiotic potentials. High-amylose content in corn has been found to lower the small intestinal enzymatic hydrolysis of the starch (Brown *et al.*, 1997). Feeding trials with high-amylose starch in humans have shown loss of starch in the ileum (Muir *et al.*, 1994) and studies in pigs have shown increased starch concentration in the proximal colon (Topping *et al.*, 1997). Faecal volatile fatty acids excretion was higher in humans consuming high amylose starch and this was consistent with enhanced large bowel bacterial fermentation (Noakes *et al*., 1996).

The AOAC produced the analytical definition of dietary fibres as “remnants of plant cells resistant to hydrolysis by the alimentary enzymes of man” (Trowel and Burkitt, 1986). There is a mixed opinion about fibre to be considered as prebiotics as they were reported to be non-specific in stimulatory effect and therefore not considered as a true prebiotic (Gibson *et al*., 2000). However, with increasing interest on the benefits of prebiotic, various sources of fibre have been studied for having prebiotic properties. Epidemiological studies showed that dietary fibre reduced the risk of coronary artery disease (Rimm *et al*., 1996; Ripsin *et al.*, 1992) and lowered total- and LDL cholesterol (Glore *et al*., 1994; Truswell, 1995). Despite this, there have been contradictory outcomes of cholesterol lowering degrees from various dietary fibres. These variations were found to be contributed by small sample sizes, and different dosages of fibre, background diets of trial subjects, type of subjects with various dietary control and body weight changes (Brown *et al*., 1999).

The mechanism by which fibre reduces serum cholesterol remains unclear. Anderson and Tietyen-Clark (1986) suggested that bile acids or cholesterol were bound to soluble fibres during intraluminal formation of micelles. This resulted in decreased cholesterol content in liver cells and that led to the up-regulation of the LDL receptors, thus increased excretion of LDL cholesterol. However, such excretion of bile acids may not be sufficient to account for the observed cholesterol reduction. This led to other mechanisms that proposed that the effect of dietary fibres were contributed by the high viscosity of fibres that slowed absorption of macronutrients and subsequently increased insulin sensitivity (Schneeman, 1987), inhibition of hepatic fatty acid synthesis (Nishina and Freedland, 1990) and changes in intestinal motility (Schneeman and Galleher, 1985).

2.2.3 Polyols

Recently, the usage of polyols or sugar alcohols such as lactitol, sorbitol, mannitol and xylitol as prebiotics has generated much interest mainly contributed by their inexpensive and uncomplicated production. Although polyols have been proven to contribute to the fermentable carbohydrate load in the large gut (Roberfroid *et al.*, 1998), the arguments on the fermentability of sugar alcohols and their ability to reach the large bowel undigested had intensified both *in-vitro* and *in-vivo* studies. Lactitol (a disaccharide alcohol) is not absorbed in the human small intestine and thus arrives at the colon as a potential substrate for microbial fermentation (Kontula *et al.*, 1998; Koutsou *et al.*, 1996). *In-vitro* studies showed that lactitol is too reduced to be metabolized under anaerobic conditions and contributed to decreased population of bacteroides (Probert *et al.*, 2004). *In-vitro* experiment using ruminal-based inoculum showed that sorbitol acted as a substrate that supported the growth of luminal bacteria which competitively inhibited growth of *E. coli*, suggesting the potential of sorbitol as a prebiotic (De Vaux *et al*., 2002). Using pig and human cecal digesta, sorbitol was also found to be slowly fermented and stimulated the production of butyrate and propionate (Kiriyama *et al.*, 1992; Mortensen *et al.*, 1988). This has been a desirable attribute as rapid fermentation often causes rapid growth of Gram-positive cocci, such as Streptococci in the bowel (Dawson and Allison, 1988). Despite all the reported positive health benefits, there is very little information on the effect of sugar alcohols on serum cholesterol levels (Ellwood et al., 1999), whether used individually or in combinations with probiotic organisms.

2.3 Concept of synbiotic

Another possibility of gut microflora management is the use of synbiotics, where probiotics and prebiotics are used in combination. The combination of suitable probiotics and prebiotics enhances survival and activity of the organism, for example a FOS in conjunction with a *Bifidobacterium* strain or lactitol in conjunction with *Lactobacillus* (Gibson and Roberfroid, 1995). The combination of prebiotic and probiotic has synergistic effects because in addition to promoting growth of existing strains of beneficial bacteria in the colon, synbiotics also act to improve the survival, implantation and growth of newly added probiotic strains. The synbiotic concept has been widely used by European dairy drink and yoghurt manufacturers such as Aktifit (Emmi, Switzerland), Proghurt (Ja Naturlich Naturprodukte, Austria), Vifit (Belgium, UK) and Fysiq (Netherlands) (Niness, 1999).

The combination of *Bifidobacterium* and oligofructose was reported to synergistically improve colon carcinogenesis in rats compared to when both were given individually (Gallaher and Khil, 1999). Another study reported that a synbiotic containing *Pediococcus pentoseceus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei*, and *L. plantarum* with four fermentable fibres namely β-glucan, inulin, pectin, and resistant starch reduced the occurrence of post-operation infections from 48% to 13% in 66 liver transplant patients (Rayes *et al.*, 2005). Most of the claims on benefits of different synbiotics are on general health (Gibson and Roberfroid, 1995). There have yet been any clinical trials on suitable combinations of synbiotics that specifically target reduction of serum cholesterol level in animals and humans.

2.4 Survivability of probiotics under acidic and bile conditions

Probiotic bacteria used as food adjuncts are commonly delivered in a food system and begin their journey to the lower intestinal tract via the mouth. The dominant food vehicles for probiotics remain to be yoghurts and fermented milks, both of which provide a relatively low-pH environment in which the probiotic bacteria must survive. In addition, probiotic bacteria should be resistant to the enzymes in the oral cavity such as lysozyme (Fuller, 1992) and resist the gastric acidity, bile toxicity and stress conditions in the colon (Gibson *et al*., 2000). The time from entrance to release from the stomach was reported to be approximately 90 min (Berada *et al.,* 1991). However, further digestive processes have longer residence times and thus, there is a need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine, which contain bile. Cellular stress begins in the stomach, which has pH as low as 1.5 (Lankaputhra and Shah, 1995) and the upper intestinal tract where bile is secreted into the gut. The concentration of bile in the human

gastrointestinal system is variable and is difficult to predict at any given moment (Lankaputhra and Shah, 1995). Thus, strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile acids, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits.

Hence, acid and bile tolerances are one of the first properties screened when selecting probiotic strains. Simple *in-vitro* tests have been applied to lactic acid bacteria and *Bifidobacterium* strains used in the dairy industry prior to use as probiotics. The results of these tests very often predicted the ability of the strains to survive in acidic and bile environments. These *in-vitro* tests for selection of acid- and bile-tolerant strains can readily be applied to ensure the quality of probiotic cultures during manufacture, storage, and shelf life of the product (Tuomola *et al.*, 2001).

However, the quantitative extrapolation to probiotic performance *in-vivo* has been difficult, due to intraspecies variation between potential probiotic strains. In addition, *in-vivo* experiments would also involve environmental factors that affect culture growth phase and induce stress causing changes in culture performance (Lee and Salminen, 1995). Thus, *in-vitro* experiments have often been used and efforts have been made to mimic the *in-vivo* systems in the best possible ways. Human gastric juice was used in *in-vitro* studies to better evaluate the survivability of strains through the stomach (Dunne *et al.*, 2001). To minimize variations, Draser *et al.* (1969) adjusted the human gastric juice from healthy subjects prior to use, as the pH of the stomach is known to fluctuate, with pH as low as 1.5 from fasting individuals. The authors reported that most *Lactobacillus* strains showed the ability to transit the human stomach and reaching the intestinal environment to function effectively there. *Bifidobacterium*, however, proved less acid resistant to human gastric juice than lactobacilli.

Bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form (Hoffman et al., 1983). These acids then undergo extensive chemical modifications in the colon as a result of microbial activity (Hylemon and Glass, 1983). Both conjugated and deconjugated bile acids exhibit *in-vitro* antibacterial activity inhibiting the growth of *Escherichia coli*, *Klebsiella* and *Enterococcus* (Stewart *et al*., 1986). However, Gram-positive bacteria are found to be more sensitive to these acids than gram-negative bacteria (Floch *et al.*, 1972). Also, bile sensitivity is found to be dependent of bile origins; porcine bile was reported to have more inhibition effects on *Lactobacillus* and *Bifidobacterium* strains than bovine bile. Dunne *et al.* (2001) found that most of the human derived *Lactobacillus* and *Bifidobacterium* had the ability to tolerate the physiological concentrations of human bile.

2.5 Mechanisms of cholesterol removal by probiotics and prebiotics

2.5.1 Deconjugation of bile salts

Bile, composed mainly of bile salts, is produced by liver cells, and secreted into the duodenum via the bile duct. Bile salts are glycine and taurine conjugates of bile acids, and act as natural ionic detergents. In the intestine, bile salts play an important role in emulsifying lipids, which enable intra-luminal lipolysis and absorption of lipolytic products by enterocytes. Cholic acid, one of the most common free bile acids in the intestine, is produced mostly by the deconjugation of bile salts (Kurdi et al., 2003).

The major route of cholesterol excretion from humans and other mammals is through faeces. Cholesterol is the precursor of primary bile salts that are formed in the liver and stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo and Gilliland, 1999). The secretion of conjugated bile salts into the small intestine helps absorption of hydrophobic vitamins and other dietary fat soluble compounds. Deconjugated bile salts are more hydrophobic than conjugated bile salts, resulting in lower absorption in the intestinal lumen and are lost in faeces. At the physiological pH of the intestinal lumen, deconjugated bile salts can be transported through the epithelium and into the blood stream of the host, or precipitated. Thus, in a steady state situation, deconjugation of bile salts can reduce serum cholesterol levels by increasing the formation of new bile salts that are needed to replace those that have escaped the enterohepatic circulation (Reynier et al., 1981).

Some probiotics such as *Lactobacillus acidophilus* was found to excrete bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), the enzyme that catalyses the hydrolysis of glycine and/or taurine conjugated bile salts into amino acid residues and free bile salts (bile acids) (Corzo and Gilliland, 1999). A study on the formation of deconjugation products reported that cholate, chenodeoxycholate and deoxycholate were found on the agar plugs containing typical colonies on plates containing taurocholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid (Ahn et al., 2003). Most bile salts found in faeces are of deconjugated forms and consist almost entirely of deoxycholate and lithocholate (Korpela et al., 1988). Strains of *L. acidophilus* that could deconjugate bile salt *in-vitro* contributed to higher faecal excretion of deoxycholate in *in-vivo* human trials, which led to the suggestion that the increase of deoxycholate was contributed by deconjugation of glycine or taurine by *L. acidophilus* and the transformation of cholate into deoxycholate through 7α-dehydroxylation of other intestinal bacteria (Ahn et al., 2003).

This section has been published: *Liong, MT and Shah, NP. 2005. Roles of probiotics and prebiotics on cholesterol: the hypothesized mechanisms.* ***Nutrafoods*** 4: 45-57.

Bile salt hydrolases are active on both glycine and taurine conjugated bile salts. However, in experiments resembling human intestinal pH of 6.5 and glycocholate to taurocholate ratio of 2:3, glycine conjugated bile salt was found to be more efficiently deconjugated by strains of *L. acidophilus* from both human and porcine origins than taurine conjugated bile salt (Corzo and Gilliland, 1999). It was postulated that this contributed to the solubility of both conjugated bile salts under acidic conditions. At the normal pH of the upper intestinal tract (5.50-6.50), about 50 percent of free bile salts and a small amount of glycine-conjugated bile salts were found to be protonated (nonionized), while no protonation occurred in taurine-conjugated bile salt (Carey and Cahalane, 1988). This can be explained by the pK_a values of taurine and glycine conjugated bile salts, and of deconjugated bile salts, which are at pH 1.9, 3.9 and 5.0, respectively. Thus, at acidic pH, deconjugated bile salts are protonated and precipitated, while taurine-conjugated bile salts remain ionized in solution, and glycine-conjugated bile salts are partially precipitated without hydrolysis (Dashkevics and Feighner, 1989). It was previously reported that the removal of cholesterol from a medium was contributed by the disruption of destabilized cholesterol micelles as a result of bile salt deconjugation, followed by the precipitation of cholesterol with the free bile salts as pH decreased (Klaver and Van der Meer, 1993). However, cholesterol was also reported to be removed *in-vitro* by *L. acidophilus* strains when the pH was maintained at 6.0 (Noh et al., 1997). It was hypothesized that cholesterol removal was not solely contributed by bile salt deconjugation, co-precipitation of cholesterol with deconjugated bile salt or destabilization of cholesterol micelles (Brashears et al., 1998). Nevertheless, low pH and strong deconjugation activities were reported to be important factors for the precipitation of soluble cholesterol *in-vitro* (Ahn et al., 2003). The regulation of BSH activity by pH is still unclear although BSH activities were shown to be higher in lower pH values. Various strains of the same bacterial species also exhibited different BSH activity under similar pH levels (Corzo and Gilliland, 1999; Lunden and Salvage, 1990).

Considering that sodium glycocholate predominates in the human intestine, it was postulated that strains that prefer to deconjugate sodium glycocholate may have better potential to lower serum cholesterol concentrations if the deconjugation mechanism is important in decreasing serum cholesterol (Brashears et al., 1998). In addition, it was proposed that higher substrate specificity was obtained toward glycine conjugates than taurine conjugates. It has been found that the enzyme hydrolysed cholyglycine with the Michaelis-Menten kinetics and there was competitive inhibition by cholytaurine, as if both conjugated bile salts were hydrolysed at a single site. The products of hydrolysis, taurine and cholic acid, were found to inhibit the hydrolysis of cholyglycine (Dean et al., 2002). In contrast, using crude bovine bile, another study reported that the enzyme completely

hydrolysed all the conjugated bile salts without interference by other bile components (cholesterol, phospholipids) or reaction products (taurine, glycine, cholic acid) (De Smet et al., 1995). Until now, the molecular fate of the amino acid released by the deconjugation by the bacterial cell and the benefit the organism may derive from such activity is unclear. A mechanistic approach using isolated and purified BSH from *B. longum* SBT 2928 found that BSH was an intracellular enzyme and hydrolysis of bile salts makes the amino acid nitrogen atoms of the released amino acids available for cells (Tanaka et al., 2000).

Studies have suggested that the distribution of BSH activity in *Bifidobacterium* and *Lactobacillus* correlated with the habitat of a genus, species or even strains, as shown in Table 2.2. Most of the probiotic organisms that originated from human/mammalian intestines and faeces exhibited BSH activity. However, it must also be noted that not all strains isolated from the intestine or faeces have BSH activity, suggesting that bacteria without this enzyme could survive through bile acids environment (Tanaka et al., 1999). Deconjugation of bile salts also exhibit host specificity. In mammalian host, the exact location of deconjugation is dependent on the host species. In mice, *Lactobacillus* flora is present in the small intestine where bile salt deconjugation starts (Tannock et al., 1994), while in humans, a significant flora starts only at the end of the ileum and fully developed in the large intestines, indicating that bile salt deconjugation activities begin at the end of the ileum and active in the large bowel (Marteau et al., 1995). Thus, if the deconjugation of bile is an essential mechanism for lowering cholesterol levels, it would be an important consideration that cultures used for human *in-vivo* trials are selected from suitable origins.

There have also been debatable arguments stating that growth of selected strains of *L. acidophilus* in laboratory media containing cholesterol and bile resulted in much of the cholesterol being removed along with the deconjugated bile. Cholesterol might coprecipitate with deconjugated bile salts which were observed at pH values below 5.5, contributed by bacterial fermentation and formation of short chain fatty acid. The amount of soluble cholesterol in medium containing taurocholic acid (TCA) and glycocholic acid (GCA) decreased during growth of *L. acidophilus*. Precipitation of soluble cholesterol was greater in culture broth containing dihydroxy-conjugated bile salt (taurochenodeoxycholic acid) compared to trihydroxy-conjugated bile salt, which was suggested to be contributed by lower solubility from the former in aqueous environment (Hofmann and Roda, 1984). Taurodeoxycholate was found to be the most effective bile salt in the precipitation of [^{3}H] cholesterol (74%) followed by glycodeoxycholate (62%) and glycocholate (42%) (Taranto et al., 1997).

Table 2.2 Influence of the habitat of a genus, species or even strains of bifidobacteria and lactobacilli on the distribution of bile salt hydrolase (BSH) activity.

Probiotic organisms	BSH activity[a]	Source of microorganism	Reference
Bifidobacterium adolescentis	+	Human faeces	Tanaka et al., 1999
B. animalis	+	Human faeces	Tanaka et al., 1999
B. breve	+	Human faeces	Tanaka et al., 1999
B. coryneforme	-	Human faeces; honey bee gut	Tanaka et al., 1999; Wood and Holzapfel, 1995
B. infantis	+	Human faeces	Tanaka et al., 2000
B. longum	+	Human faeces	Tanaka et al., 2000
Bifidobacterium sp.	+	Intestines of mammals and insects; Human faeces	Grill et al., 2000; Wood and Holzapfel, 1995
Lactobacillus acidophilus	+	Faeces, mammalian intestines	Corzo and Gilliland, 1999; Wood and Holzapfel, 1995
L. buchneri	-	Human intestine	Moser and Salvage, 2001
L. casei	+	Human faeces	Brashears et al., 1998
L. fermentum	+ (-)	Infant faeces (fermented beets)	Moser and Salvage, 2001
L. gasseri	+	Human faeces; mammalian intestines	Tanaka et al., 1999; Wood and Holzapfel, 1995
L. helveticus	+	Human faeces	Tanaka et al., 1999
L. johnsonii	-	Mammalian intestines	Wood and Holzapfel, 1995
L. paracasei subsp. *paracasei*	+	Raw milk	Moser and Salvage, 2001
L. plantarum	-	Human faeces	De Smet et al., 1995
L. rhamnosus	+	Human faeces; bowel drain, yoghurt	Moser and Salvage, 2001; Tanaka et al., 1999

[a] + : Exhibited bile salt deconjugation activities; - : Without bile salt deconjugation capability.

When cholesterol precipitation was studied in medium using resting cells of *Bifidobacterium* species, oxgall and pH lower than 5.4, cholesterol that was removed from the medium reportedly redissolved in washing buffer and no cholesterol was found in the cell extracts, suggesting that the precipitation of cholesterol could be a transient phenomenon (Tahri et al., 1995). However, this proposed mechanism has generated much debate, especially when higher amount of cholesterol was removed when higher concentration of bile salts (0.4% w/v) was used. This is quite impractical as it is unlikely to occur *in-vivo* since bile acid content in the small intestine is below that level (Pereira and Gibson, 2002). Furthermore, only a minimal amount of cholesterol was co-precipitated with deconjugated bile and it occurred at pH less than 5. These did not appear to be a major factor in controlling serum cholesterol because only negligible amount of cholesterol was removed and the pH of the intestine is unlikely to be lower than 6.0 (Brashears et al., 1998).

2.5.2 Incorporation of cholesterol into cellular membrane

Another proposed mechanism for cholesterol reduction suggested that *L. acidophilus* incorporates some of the cholesterol removed from media into the cellular membrane during growth (Noh et al., 1997). Cholesterol incorporated into or adhere to the bacterial cells would more likely be less available for absorption from the intestine into the blood. An interesting attribute of this mechanism involves the correlation between incorporation of cholesterol and pH of the growth medium. Cell membranes from culture grown without pH control was found to contain significantly more cholesterol compared with cultures grown under controlled pH of 6.0. Cell membranes from culture without pH control were reportedly higher in cholesterol concentration as compared to the whole cells grown under same conditions, while there was no significant difference of cholesterol concentration between cell membranes and whole cells grown at controlled pH 6.0. However, the amount of cholesterol removed in the membrane fraction of *L. acidophilus* did not account for the total amounts removed by the culture. The authors suggested that some cholesterol might loosely associate with the cells and do not get incorporated in the membrane resulting in loss during the isolation procedure.

Conducting assays of the membrane fractions that were isolated from cells of the bacteria grown in the presence of cholesterol, it was reported that 20 percent of the cholesterol that was removed by the culture was recovered with the cellular membrane. Cells grown in the presence of cholesterol were found to be more resistant towards lysis by sonication, with only 85 percent cells lysis as compared to more than 90 percent in absence of cholesterol, which indicated differences in cellular envelop (Dambekodi and Gilliland,

1998). This was supported by a previous study that reported cells of *L. acidophilus* ATCC 43121 grown in the presence of cholesterol micelles showed greater resistance to lysis by sonication than did those grown in control broth (Noh et al., 1997). The authors postulated that incorporation of cholesterol into the cells of *L. acidophilus* may have altered the cellular membrane or wall of the lactobacilli and increased tensile strength of the membranes. The fact that cholesterol changed cells membrane properties was supported by cells of *L. acidophilus* that were grown in cholesterol did not all stain Gram positive, but those grown without cholesterol micelles did.

Based on another approach and using *Lactococcus lactis* in *in-vitro* studies, it was found that there was a difference in fatty acid distribution pattern for cells grown with and without cholesterol. Cells grown in the presence of cholesterol contained significantly higher amount of 18:1, 18:2 and total unsaturated fatty acids, but lower amount of 16:0, 18:0 and total saturated fatty acids compared to those grown in the absence of cholesterol (Kimoto et al., 2002). The authors suggested that the fatty acid composition of the cells was altered as a result of cholesterol being incorporated into the cellular membrane after its removal from the media. Although the changes in membrane fatty acids composition does not necessarily manifest direct incorporation of cholesterol into the cellular envelop, it does provide evidence that cholesterol removed from the medium was somehow associated with the changes in the membrane. Whether the changes were due to cellular resistance or cholesterol removal capabilities, more information is needed to elucidate this phenomenon when controversially, other researchers reported no incorporation of cholesterol of measurable amount was obtained from membrane of *L. casei* strains grown in broth containing cholesterol (Brashears et al., 1998).

2.5.3 Assimilation of cholesterol by probiotics

A study have shown that there appeared to be no relationship between the amounts of cholesterol removed *in-vitro* and the degree of bile salt deconjugation, which led to another hypothesis that cholesterol removal may be contributed by assimilation of cholesterol (Dambekodi and Gilliland, 1998). In order to be able to assimilate cholesterol, probiotics have to be viable and growing. The relationship between frozen and refrigerated cells and cholesterol assimilation abilities were studied using *L. acidophilus* strains ATCC 43121 and NCFM-L during frozen storage at -196 °C in liquid nitrogen (Piston and Gilliland, 1994). No significant difference in bile tolerance and cholesterol assimilation capabilities were found during the 28 days frozen storage period. However, a significant decrease in viability and cholesterol assimilation were observed for NCFM-L during

refrigerated storage of nonfermented milk, as compared to strain ATCC 43121 which exhibited greater viability and subsequently better cholesterol assimilation abilities. It was postulated that the decrease in activity during refrigerated storage caused a decrease in activity *in-vivo* and suggested that cholesterol assimilation activity is growth dependent of the bacteria. This supported a recent study reporting that the cholesterol assimilation ability of the bacteria is highly dependent on their growth and may reflect the growth stage of the inoculum used (Pereira and Gibson, 2002). Although controversial, several factors affecting growth such as pH, bile, fatty acids and origin of probiotics appeared to affect their cholesterol assimilation capabilities.

Cellular stresses begin in the stomach, with low pH in the stomach and bile in the upper intestine. In order to exhibit cholesterol assimilation capability *in-vivo*, probiotics will have to maintain survivability in both acid and bile environments. *In-vitro* experiments showed that strains that did not grow well in medium containing bile salts were unable to assimilate cholesterol to the extent as those that grew well in the presence of bile salts, with uptake of cholesterol was particularly higher in the medium containing higher concentration of bile salts (Pereira and Gibson, 2002). This has been supported by various studies that showed strains of bacteria have greater tendency to remove cholesterol from culture medium in the presence of bile salts (Gilliland et al., 1985; Rasic et al., 1992). Using various concentration of sodium taurocholate, assimilation of cholesterol increased with increasing concentration of the bile salt. More cholesterol was assimilated in the medium containing 0.006 M taurocholate than from those containing any other level (Dambekodi and Gilliland, 1998). Dairy calves were fed strains of *L. acidophilus* of different bile tolerance. The calves supplemented with bile tolerant strains had more lactobacilli in their intestinal tract compared with those given strains which were less bile tolerant. The more bile tolerant strain was also found to develop better in the upper small intestines than lesser bile tolerant strain. However, controversy arises when certain bile tolerant strains of *L. acidophilus* and *B. longum* did not exhibit greatest ability to assimilate cholesterol, while strains that possessed minimal bile tolerant abilities assimilated more cholesterol (Dambekodi and Gilliland, 1998; Gilliland et al., 1985).

Previous studies showed that cholesterol assimilation was influenced by host specificity. The assimilation of cholesterol was compared using 12 commercial cultures containing *L. acidophilus* originated from the human intestine with *L. acidophilus* ATCC 43121 which originated from pigs (Gilliland and Walker, 1990). None of the commercial cultures were favourably compared to those porcine counterparts. Screening of 123 *L. acidophilus* strains from human volunteers found only eight cultures of *L. acidophilus* that

assimilated as much cholesterol as ATCC 43121, none that exhibited greater cholesterol assimilation (Buck and Gilliland, 1994). Apparently the porcine originated strain performed better *in-vitro* than the human isolated ones. More studies are needed to support evidence of such a correlation.

2.5.4 Binding of cholesterol to cells

The mechanism of cholesterol removal was studied from a different angle when non-growing lactococci cells reportedly removed cholesterol *in-vitro* (Kimoto et al., 2002). The authors found that both heat-killed cells and resting cells removed cholesterol although the amount of cholesterol removed was less compared to growing cells, leading to the hypothesis that cholesterol not only may be removed by living cells during growth but also via binding of cholesterol to dead cells. Less cholesterol per milligram of protein was also detected in the membrane fraction than the whole cell of *B. longum*, indicating that the cholesterol was not closely associated with the membrane, but merely attached to the cell surface (Dambekodi and Gilliland, 1998). A large amount of cholesterol was found retained by growing bacterial cells. More than 40 percent of the cholesterol was extracted by sonication from cells of *Bifidobacterium breve* ATCC 15700. The absorbed cholesterol could not be detached even after several washings indicated that the binding between cholesterol and growing cell was intense (Tahri et al., 1995).

2.5.5 Effects of prebiotics

Lactic acid bacteria have been reported to produce exocellular polysaccharides (EPS) by either excreting into medium as free EPS or adhering to the cells as capsular EPS. Some EPS classified as prebiotics could contribute to human health and positively affect gut microflora (Ruas-Madiedo et al., 2002). Consumption of milk fermented with an EPS producing lactic acid bacteria reduced serum cholesterol level in rats as compared to milk fermented with a non-EPS producing strain (Nakajima et al., 1992), suggesting that the EPS contributed to the reduced absorption of cholesterol or bile acids. Due to the resistance of EPS to digestive enzymes and its water holding capacity, it was hypothesized that the bacterial polymer has similar manner as plant-based polysaccharides or dietary fibre. *In-vitro* experiments using strains of *Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus* found that strains that produced greatest amount of EPS bound the most cholic acid from medium (Pigeon et al., 2002). Since that cultures tested did not bind conjugated bile acid (glycocholic acid), it was suggested that the consumption of such fermented product could increase secretion of free bile acids from the body leading to subsequent

cholesterol reduction. Up to 45 mg of cholic acid could be removed by a serving of yoghurt (8 oz) made by *L. delbrueckii* spp. *bulgaricus* strain LB 10442, while up to 26 mg of cholic acid was reportedly removed by strains of streptococci. In manufacturing practice, strains of starter cultures that are able to bind free bile acid could be used to produce yoghurt containing probiotic bacteria that are able to deconjugate bile salts.

Consumption of soluble fibre has been shown to lower LDL cholesterol levels through a series of processes that alter cholesterol metabolism. The mechanism of action is thought to involve increasing faecal bile acid excretion and interference with faecal bile acids readsorption (Marlett, 1997). The effect of fibre was also suggested to be contributed by its binding and dilution actions on bile acids and lowering of intestinal pH, which inhibits conversion of primary bile acids to secondary bile acids, thus resulting in a reduction in absorption of fat and cholesterol (McIntyre et al., 1993).

A substantial increase in short chain fatty acids (SCFA) was observed when rat were fed transgalactooligosaccharide at 5% (w/v) for seven weeks (Morishita and Konishi, 1994). Using human subjects and supplementation with soybean oligosaccharides at 3-6 g/day for 2 weeks, another study found similar results, with significant increase in organic acids especially lactate (Hara et al., 1994). It has been postulated that SCFA, especially propionic acid, inhibited cholesterol synthesis in the liver, by suppressing a rise in the serum concentration of free fatty acids (Nishina and Freedland, 1990). Propionic acid production by bacteria has been associated with bacterial fermentation of fibre. It was hypothesized that propionic acid increased HDL concentration in human trials by affecting the peroxisome proliferator-activated receptor α, a factor stimulating the expression of genes responsible for the synthesis of apolipoproteins, which form the structure of the HDL molecule (Schoojans et al., 1996). Recent studies in rats showed for the first time that resistant rice starch in the diet increased serum concentrations of propionate, leading to a reduction in the concentration of total and LDL cholesterol (Cheng and Lai, 2000). This suggests that the potential effect of fructooligosaccharide (FOS) is based on the hypothetical effects of propionate produced during fermentation on glucose and lipid metabolism. Acetate, on the other hand, has dissimilar effects with propionate on glucose and lipid metabolism (Delzenne and Kok, 2001). Acetate was reported as a lipogenic substrate and act as a precursor for cholesterol synthesis. Another study also reported the possibility of acetate to decrease concentration of glucose and increase the concentration of cholesterol, while propionate increased glucose production and decreased cholesterol concentration (Kok et al., 1996). This is because cholesterol, like other long-chain fatty acids, is made from acetyl-CoA, which is formed from three condensed acetate units. Acetyl-CoA is later converted into the intermediate,

mevalonate and subsequently to cholesterol in a series of more than 20 reactions (Figure 2.2) (Nelson and Cox, 2000). High ratio of propionate to acetate after supplementation of FOS decreased total serum cholesterol concentration (Wang and Gibson, 1993). The combination of *L. fermentum* KC5b with galactooligosaccharide had also significantly decreased acetate production by 9 to 27%, while propionate increased by 50 to 90% (Pereira et al., 2003). However, an *in-vivo* study involving patients with type-II diabetes reported that FOS did not have important effects on lipid concentrations (Alles et al., 1999). In a randomized, single-blind, crossover design, the authors found that FOS did not significantly affect fasting concentrations of serum total-, HDL- and LDL cholesterol, serum triacylglycerols and serum free fatty acids levels.

Again, the hypothesis regarding the effect of prebiotics and SCFA has been a controversial one. SCFA reportedly reduced the accumulation of cholic acid, the deconjugated bile acid form. Using various mixture of SCFA which correspond to the concentration in the ascending colon, cholic acid accumulation by *Bifidobacterium breve* was reduced by at least 50 percent. The presence of SCFA at the physiological concentrations found in the colon appears to exert a severe environmental stress on the bifidobacterial cells (Kurdi et al., 2003). The authors speculated that since SCFA are weak acids that can reduce the internal pH of bacterial cells, such property may contribute to the decreased cholic acid accumulation. If such hypothesis is true, SCFA may contribute to an increase in free deconjugated bile acids in the enterohepatic circulation which subsequently decreases the synthesis of bile acids from blood cholesterol. This may alter cholesterol lowering effects of probiotics *in-vivo*.

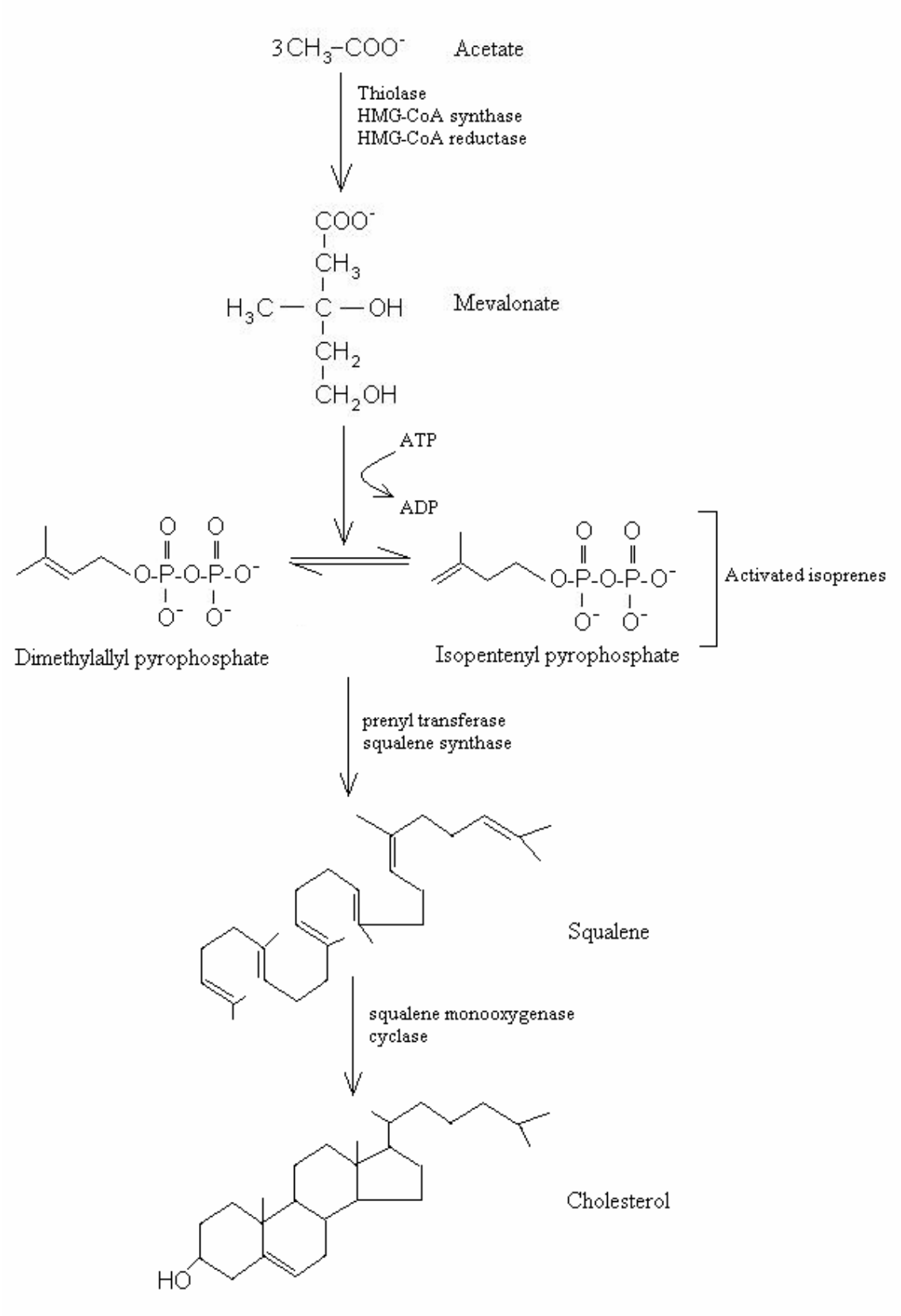


Figure 2.2 Biosynthesis of cholesterol from acetate. Adapted and simplified from Nelson and Cox (2000).

2.6 Safety of probiotics from translocational properties

Most probiotics are marked as foodstuffs or drug, and strains of probiotic have achieved the generally regarded as safe (GRAS) status. However, in recent years, many species of the genera *Lactobacillus, Leuconostoc, Pediococcus, Enterococcus* and *Bifidobacterium* have been isolated from infected lesions such as bacterial endocarditis and blood stream infections (Gasser, 1994). Adams and Marteau (1995) reported that with the exception of enterococci, the overall risk of infection due to lactic acid bacteria is very low. Bacterial translocation is a phenomenon caused by diminished intestinal barrier, resulting in passage of bacteria to mesenteric lymph nodes and then to other organs. This may result in multiple organ failure (Van Eldere *et al.*, 1988). Translocation from the intestine is difficult to induce in healthy animals. However, translocation may be induced by changes of the gut environment due to antibiotic treatment or administration of an immunosuppressive agent (Ishibashi and Yamazaki, 2001).

Most studies of bacterial translocation in healthy subjects reported positive safety. The safety of administration of novel probiotic, *Propionibacterium jensenii* 702 was assessed in healthy rat model by Huang *et al.* (2003). The authors reported that even at high dose of 10^{10} CFU/rat/day, no viable cells of *P. jensenii* 702 was recovered from blood, mesenteric lymph nodes, liver and spleen of rats. A detail probiotic translocation assessment was conducted by Zhou *et al.* (2000b), who administered lactobacilli and bifidobacteria in healthy mice at a high dose of 10^{12} CFU/kg body weight/day. *L. rhamnosus, L. acidophilus* and *B. lactis* were administered for 4 consecutive weeks. DNA fingerprinting did not identify any recovery of viable bacteria from blood and tissue samples, and no adverse effect on haematology, blood chemistry and gut mucosal histology parameter was observed. This is supported by another study that showed no translocation of lactic acid bacteria even at an administration level of 10^{11} CFU/mouse/day. This oral intake of probiotic supplement was equivalent to 50 g/kg/day per mouse while the acceptable daily intake value is 35 g/day for a 70-kg person, suggesting that the strains studied were safe for human consumption (Zhou *et al.*, 2000a).

Recent studies on bacterial translocation assessment using unhealthy subjects provided information that most probiotic strains used were safe and reduced the level of translocation of pathogenic bacteria to other organs and tissues. Pavan *et al*. (2003) evaluated the safety of lactic acid bacteria used in treatment of inflammatory bowel disease using mouse models. Bacterial translocation of *L. plantarum* NCIMB8826 was not found in both healthy mice and mice suffering from colitis even at a daily administration of 10^9 CFU viable cells. Moreover, the translocation of endogenous microflora to the mesenteric lymph

nodes and spleen was reduced. Chiva *et al.* (2002) reported similar positive effects with *Lactobacillus johnsonii* La1 using rats with experimental cirrhosis. The administration of *L. johnsonii* La1 at a high dose of 10^9 CFU/day also did not exhibit translocation in the blood systems. In addition, translocation of intestinal enterococci and enterobacteria in cirrhotic rats was lowered. *Lactobacillus rhamnosus* HN001 exhibited immune protection against *E. coli* infection in mice. Administration of *L. rhamnosus* at a dose of 3×10^8 CFU/g for a week reduced the severity of *E. coli* infection and translocation of pathogens to spleen, liver and blood, compared to mice that were fed a placebo (Shu and Gill, 2002). Romond *et al.* (1997) found that the administration of *B. longum* in mice led to the disappearance of *E. coli* from kidney, liver, spleen and lung, contributed by the alteration of lipopolysaccharide in *E. coli* by *B. longum*.

However, reports suggest that various strains of probiotic affect bacterial translocation in different manners. Using liver injury in rat models, Adawi *et al.* (2001) found that *L. acidophilus, L. rhamnosus* and *L. plantarum* decreased bacterial translocation but *B. animalis* was found to increase bacterial translocation to the mesenteric lymph nodes. These led to the postulation that the administration of different lactobacilli and bifidobacteria strains in acute injury models has different effects on bacterial translocation and possible hepatocellular damage. Bacteria of the indigenous microflora are not normally found in extraintestinal sites, such as the mesenteric lymph nodes, spleen, liver or blood of pathogen-free mice. Indigenous bacteria that would translocate across the mucosal epithelium are killed by the host immune defences. However, lactobacilli or enterococci were the most commonly identified strains in mesenteric lymph nodes of healthy pathogen-free mice that contained translocating bacteria (Berg and Garlington, 1979). Indigenous *L. acidophilus* has been found to translocate to the mesenteric lymph nodes of antibiotic-decontaminated pathogen-free mice. Mice injected with immunosuppressive agents exhibited increased translocation of *L. acidophilus* to the mesenteric lymph nodes, spleen or liver (Berg, 1983). Lactobacilli and enterococci were most likely to translocate because they normally colonize the gastrointestinal tract at high population levels. On the other hand, obligate anaerobes populate the gastrointestinal tract at highest levels but are rarely found to translocate spontaneously in pathogen-free mice. *L. acidophilus* does not cause severe disease even when it did translocate from the gastrointestinal tract.

Prebiotic such as the FOS stimulate the protective gut microflora, resulting in an increased production of organic acids that inhibited acid-sensitive pathogens. However, rapid fermentation of FOS that leads to high concentrations of organic acids may impair the barrier function. Bruggencate *et al.* (2003) found that the administration of FOS for 2 weeks

increased cytotoxicity of faecal water and faecal mucin excretion in rats, indicating mucosal irritation. FOS was found to enhance translocation of salmonella, increased their numbers in mucosa and impaired resistance to infection by this organism in rats. Although the effect of prebiotics on translocation of probiotics has not been documented, it is of upmost importance that such possibility is carefully examined so that their negative consequences will not outweigh their benefits.

2.7 *In-vivo* effects of probiotics and prebiotics

2.7.1 Hypocholesterolemic benefits

Because *in-vitro* studies have shown that bacteria can remove cholesterol from culture media, much attention has been given to the cholesterol-lowering potential of probiotics using animal models and human subjects. The cholesterol-lowering potential of *L. acidophilus* has been most widely studied. Using a human trial with 23 subjects receiving tablets containing 3 x 10^7 CFU *L. acidophilus* (ATCC 4962) and *Lactobacillus delb.* ssp. *bulgaricus* (ATCC 33409) daily for 16 wk, Lin *et al.* (1989) reported that serum cholesterol in the control group remained stable at 4.9 mmol/L while serum cholesterol in the experimental group decreased from 5.7 to 5.4 mmol/L. Another study performed in India showed that consumption of buffalo milk fermented with a specific strain of *L. acidophilus* reduced serum cholesterol by 12–20% after one month (Khedkar *et al.*, 1993). A placebo-controlled crossover study with 30 volunteers found a 0.23-mmol/L reduced serum cholesterol concentration after consumption of yoghurt enriched with a specific strain of *L. acidophilus* (Schaafsma *et al.*, 1998). *Lactobacillus plantarum* 299 from the food product Pro-Viva has been reported to affect cholesterol levels in humans with moderately elevated serum cholesterol. Using 30 healthy men in a randomized-placebo design, Bukowska *et al.* (1997) found significant decrease in total cholesterol, LDL cholesterol and fibrinogen levels in groups taking 200 mL/day of Pro-Viva for 6 weeks, suggesting its potential for patients with moderately elevated cholesterol to prevent cardiovascular disease.

Very few studies have shown the potential of hypocholesterolemia effects of bifidobacteria. Rasic *et al.* (1992) found that the consumption of *Bifidobacterium bifidum* that assimilated cholesterol in *in-vitro* experiments, reduced concentration of serum cholesterol in human subjects with elevated serum cholesterol. In another study, Xiao *et al*. (2003) found that milk fermented by *Bifidobacterium longum* BL1 not only reduced serum total cholesterol, LDL cholesterol and triglycerides in rats, but also in hypercholesterolemia patients and healthy adults. The human study compromising 32 human subjects showed that serum total cholesterol was reduced by approximately half of those in the control group,

whom were given milk fermented only with yoghurt starter cultures *Streptococcus thermophilus* and *L. delb* ssp. *bulgaricus*. Subjects with moderate hypercholesterolemia (serum total cholesterol > 240 mg/dl) also showed a significant decrease in serum total cholesterol, indicating the potential of *B. longum* BL1 in lipid improvement.

Until now, numerous studies designed to evaluate the potential reduction of serum cholesterol levels by the consumption of probiotics or of certain cultured dairy products as carriers for probiotics have given variable data (Taylor and Williams, 1998). In most of these studies a decrease in serum cholesterol was only observed during the consumption of very high doses of products, while more 'normal' doses of the probiotic product failed to deliver such a conclusion. Such contradictory results obtained could be related to experimental design, lack of statistical power, use of inadequate sample sizes, failure to control nutrient intake and energy expenditure during the experiments and variations in the baseline levels of blood lipids (Pereira and Gibson, 2002). Dietary studies using random double-blind placebo procedures and higher ranges of human subjects have also reached the same conclusion (Table 2.3). Some showed the cholesterol-lowering effect of various fermented dairy carriers, while others failed to demonstrate a significant effect on cholesterol or lipoprotein levels by dietary supplementation of these products. Differences in the type and quantity of the fermented milk product, age and sex distribution, starting plasma cholesterol levels of the subjects studied and length of study period made direct comparisons difficult.

In addition, despite all the claimed hypocholesterolemic effects of probiotics, not all investigations support this outcome. Reports also suggest no changes in lipid profiles of healthy human subjects after consumption of probiotic food products. De Roos *et al.* (1999) found that yoghurt enriched with *L. acidophilus* had no effect on blood lipids in healthy men and women with normal to borderline serum cholesterol levels, indicating the probiotic product did not exhibit medically relevant changes in healthy humans. Similarly, Jahreis *et al*. (2002) reported that sausages incorporated with *L. paracasei* did not influence blood lipids profiles in healthy subjects. Another study also found that the consumption of a probiotic milk product did not increase cholesterol excretion in ileostomy subjects (Marteau *et al.*, 1995), while Greany *et al.* (2004) reported that probiotic consumption did not enhance the cholesterol-lowering effect in postmenopausal women. Norin et al. (1991) found that in spite of being established in high numbers (more than 10^8 cells/g of cecum and colon contents), *L. acidophilus* A10 and *B. bifidum* B11 were not able to mediate any alterations in the conversion of cholesterol to coprostanol in germfree mice. All these controversies have led to the inconclusive hypocholesterolemic effects of probiotics.

Table 2.3 Evaluation of the cholesterol-lowering properties of fermented dairy products supplemented with probiotics

Product type Dose (vol/day) Intake period Organism	No. of subjects Subject types Age (years)	Study design	Cholesterol levels	Reference
FLFM 11 3 weeks *L. acidophilus* L1	9 Ileostomy patients 29-67	Crossover, 2 weeks washout	= TC = LDL-C	Andersson *et al.* (2001)
FM 200 mL 6 weeks NA	58 (all males) Normolipidemic 44	Randomised, DB, placebo-controlled	- 6% TC -10% LDL-C	Agerbaek *et al.* (1995)
FM 200 mL 6 months NA	87 Normolipidemic 44	Randomised, DB, placebo-controlled	= TC = LDL-C	Richelsen *et al.* (1996)
FM 200 mL 3 months NA	78 males, 76 females Hyperlipidemic	Multicentered, double-blind, placebo controlled	= TC = LDL-C	Sessions *et al.* (1998)
FM 200 g 8 weeks *S. thermophilus, E. faecium*	11 males, 21 females Hyperlipidemic 36-65	Prospective, randomised, double-blind, crossover, placebo controlled	- 5.3% TC - 6.15% LDL-C = HDL-C = TG	Bertolami *et al.* (1999)
LA yoghurt 500 mL 6 weeks	78 Normolipidemic	Randomised, placebo controlled, paralled	= TC = LDL-C = HDL-C	De Roos *et al.* (1999)
LA Yoghurt 300g 52 days	29 (all females) Normal and hyperlipidemic	Randomised, double blind, placebo controlled	= TC -50% LDL-C	Schaarmann *et al.* (2001)

FLFM, fermented low fat milk; FM, fermented milk; LA, *Lactobacillus acidophilus*; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; =, no change; -, reduced.

Prebiotics such as nondigestable but fermentable dietary carbohydrates and fibre have also been reported to regulate lipemia and tryglyceridemia in both humans and animals. This has attracted considerable attention, mainly because unlike probiotics, prebiotic substances by definition are not subject to problems associated with their viability. Such substances also have greater possibilities for incorporation into a wide range of common foodstuffs. Oligofructose has been the most studied non-starch polysaccharide for their cholesterol reducing properties (Table 2.4). However, the mechanism of their serum lowering effect remains to be elucidated. Oligofructose was found to decrease triacylglycerol when given to rats. The hypothesis on triacylglycerol-lowering action of oligofructose was due to reduction of fatty acid synthesis in the liver (Delzenne and Kok, 2001). Using hepatocytes isolated from rats given oligofructose, another study reported a slightly lower capacity to esterify [14C]palmitate into triacylglycerol but a 40% lower capacity to synthesize triacylglycerol from [14C]acetate compared to control rats, indicating a decrease in the hepatic synthesis of triacylglycerol (Kok *et al.*, 1996). These led to the postulation that oligofructose decreased de novo lipogenesis in the liver and subsequently reduced secretion of VLDL-triglyceride, via reduction of the activity of all lipogenic enzymes. In combination with the low activity of fatty acid synthase, the enzyme that controls lipogenesis, administration of oligofructose appeared to modify lipogenic enzyme gene expression (Delzenne and Kok, 2001).

Similar to probiotics, the effects of prebiotics on serum cholesterol are also a subject of controversy, with studies indicating no hypocholesterolemic effects (Table 2.4). The consumption of FOS was found to have zero net effect on lipid concentrations in serum of patients with type II diabetes (Alles *et al*., 1999), while resistant starch has shown cholesterol-lowering properties in rats but did not affect plasma cholesterol in humans (Jenkins *et al.*, 1987). Using arabic gum and pectin, Davidson *et al.* (1998) also reported insignificant hypocholesterolemic effect in hypercholesterolemic male and female subjects. Again, all these controversies have led to the indecisive hypocholesterolemic effect of prebiotics.

2.7.2 Modification of gut microflora

The role of probiotics to modify gut microflora is well documented and several mechanisms have been proposed. Probiotic bacteria produce a variety of substances that are inhibitory to both Gram-positive and Gram-negative bacteria, organic acids, hydrogen peroxide and bacteriocins. These compounds may reduce not only the number of viable cells but may also affect bacterial metabolism or toxin production (Rolfe, 2000).

Table 2.4 Summary of human dietary studies on the effect of oligosaccharides on lipid metabolism

Oligosaccharides Dose (vol/day) Intake period (day)	No. of subjects Subject types Age (years)	Study design	Cholesterol levels	Reference
FOS 8 14	18 males, 10 females NIDD 47 ± 7	Parallel, double blind, placebo controlled	- 8% TC - 10% LDL-C = TG	Yamashita *et al.* (1984)
Inulin 9 28	12 males Normolipidemic 23 ± 0.5	Sequential, placebo controlled	- 5% TC - 27% TG	Canzi *et al.* (1995)
Inulin, FOS 17 3	10 Ileostomy	Double blind, crossover, 4 days washout	= TC, LDL-C, HDL-C	Ellegard *et al.* (1997)
Inulin 14 28	64 females Normolipidemic	Randomised, double blind, crossover	= TC, LDL-C, HDL-C, TG < LDL:HDL ratio	Pedersen *et al.* (1997)
Inulin 10 56	54 Moderate to hyperlipidemic 35-70	Double blind, randomised, crossover, 6 weeks washout	- 8.7% TC -14.4% LDL-C = HDL-C, TG	Davidson *et al.* (1998)
Inulin 20 21	12 males Hypercholesterolemic 27-49	Randomised, double blind, crossover, no washout	= TC, LDL-C, HDL-C - 14% TG	Causey *et al.* (2000)

NIDD, non-insulin dependent diabetes; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; =, no change; -, reduced.

Lactic acid bacteria especially *Lactobacillus* produce a number of antimicrobial substances, such as organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like substances. Bacteriocins or bacteriocin-like substances are peptides or proteins, which exhibit inhibitory activity against sensitive strains of bacteria (Jack *et al.*, 1995). Antimicrobial peptides have been found to be widely distributed in microorganisms as well as some insects and rodents (Montville and Kaiser, 1993). Numerous bacteriocins from lactic acid bacteria such as the *L. acidophilus* 30SC against other organisms have been extensively studied (Table 2.5). Some bacteriocins such as acidocin, lactacin, acidophilin, acidolin and lactocidine that are produced by strains of *L. acidophilus* have been purified and characterized (Oh *et al.*, 2000). Another report showed that a colonisation resistance mechanism against *Salmonella typhimurium* in mice inoculated with a mixture of faeces from breast fed infants containing *B. bifidum* was related to lowering of the pH level and the presence of short chain fatty acids in the caecal contents (Hentges *et al*., 1995). Such antimicrobial properties of *Bifidobacterium* provided protection to breast fed infants against gut infection (Saavedra *et al.*, 1994). Another strain of *Bifidobacterium* was also found to inhibit the growth of pathogens by developing a broad spectrum of antimicrobial compounds, unrelated to acid production (Gibson and Wang, 1994).

Competitive inhibition for bacterial adhesion sites on intestinal epithelial surfaces is another mechanism of action for probiotics (Goldin and Gorbach, 1992; Kleeman and Klaenhammer 1982). Consequently, some probiotic strains have been chosen for their ability to adhere to epithelial cells. Selected strains of *Bifidobacterium* isolated from adult human stools were found to inhibit binding of human enteropathogens onto cultured enterocyte-like cells (Bernet *et al.*, 1993).

There is evidence that suggested stimulation of specific and non-specific immunity may be another mechanism by which probiotics can protect against intestinal disease (Fukushima *et al.*, 1998; Malin *et al.*, 1996). Peroral administration of *Lactobacillus* sp. GG during acute rotavirus diarrhoea is associated with an enhanced immune response to rotavirus (Kaila *et al.*, 1992). This may account for the shortened course of diarrhoea seen in treated patients. The underlying mechanisms of immune stimulation are not well understood, but specific cell wall components or cell layers may act as adjuvants and increase humoral immune responses (Rolfe, 2000). This may also explain the reason that strains of *Bifidobacterium* increased the resistance of rats to salmonella infection (Bovee-Oudenhoven *et al.*, 1996), while *B. breve* YIT4064 enhanced antigen specific antibody directed against rotavirus, protecting pups that receive milk against the rotavirus challenge (Yasui *et al.*, 1995).

Table 2.5 Antimicrobial spectrum of crude bacteriocin from *Lactobacillus acidophilus* 30SC against Gram-positive and Gram-negative bacteria.

Bacteria	Inhibition	Reference
Bacillus cereus ATCC 11778	+	ATCC
Bacillus subtilis 1A650	+	Roncero (1983)
B. subtilis 1A651	+	Roncero (1983)
Lactobacillus acidophilus ATCC 43121	-	ATCC
Lactobacillus casei Y2	+	Oh *et al.* (2000)
Lactobacillus delbruekii subsp. *lactis* ATCC 4797	+	ATCC
Lactobacillus fermentum ATCC 11931	+	ATCC
Lactobacillus helveticus 1213	-	Oh *et al.* (2000)
Lactobacillus plantarum	+	Oh *et al.* (2000)
Lactococcus lactis ATCC 1145	-	ATCC
Lactococcus ssp. CU216	-	Oh *et al.* (2000)
Leuconostoc ssp. K2	+	Oh *et al.* (2000)
Listeria innocua	-	Oh *et al.* (2000)
Listeria ivanovii	+	Oh *et al.* (2000)
Listeria monocytogenes	-	Oh *et al.* (2000)
Staphylococcus aureus	+	Oh *et al.* (2000)
Streptococcus faecalis	-	Oh *et al.* (2000)
Acinetobacter baumani	-	Oh *et al.* (2000)
Escherichia coli O157 ATCC 43889	-	ATCC
E. coli O157 ATCC 43893	-	ATCC
E. coli O157 ATCC 43894	-	ATCC
E. coli O157 ATCC 43895	-	ATCC
Klebsiella pneumoniae	-	Oh *et al.* (2000)
Salmonella typhimurium	-	Oh *et al.* (2000)
Yersinia enterocolitica	-	Oh *et al.* (2000)

+, Inhibited by crude bacteriocin; -, not inhibited. ATCC, American Type Culture Collection.

Competition for nutrients has been proposed as a mechanism for probiotics. Probiotics may utilize nutrients otherwise consumed by pathogenic microorganisms (Rolfe, 2000). However, very little *in-vivo* evidence has been generated on this mechanism. Probiotics have also been reported to inhibit colonization of pathogens by degradation of toxin receptor in the host. *Saccharomyces cerevisiae* ssp. *boulardii* was believed to protect animals against *Clostridium difficile* intestinal disease via degradation of the toxin receptor on the intestinal mucosa (Castagliuolo *et al*., 1999; Pothoulakis *et al.*, 1993).

An attractive alternative to gut microflora management is the use of prebiotics. Studies on feeding of lactosucrose (1.5 g of lactosucrose/d) to 8 healthy dogs for 2 wk resulted in a desirable change to the gut flora as determined by faecal microbiological analysis. A 0.5-log increase in bifidobacteria was seen together with a 1.6-log decrease in clostridia levels. Decreases were also seen in toxin levels and faecal odour (Terada *et al.*, 1992a). Lactosucrose was also fed to 8 healthy cats at a level of 750 mg/d for 2 wk (Terada *et al.*, 1992b). This resulted in an increase in the incidence of recovery of bifidobacteria and a significant 0.9-log increase in lactobacilli numbers. A substantial decrease of 0.4 log was seen with levels of clostridia and Enterobacteriaceae. Similarly, 20 adults dogs given FOS at 4 g/d showed increase counts of *Bifidobacterium* (0.58 log) and *Lactobacillus* (0.86 log) with a decrease in clostridia by 0.11 log (Swanson *et al.*, 2002b). A study on cats (Sparkes *et al.*, 1998) given a diet that contained 0.75% FOS for 12 wk showed an increase in *Lactobacillus* accompanied by a decrease in clostridia (1.47 log) and *Escherichia coli* (0.52 log).

The exact mechanisms involved in modification of gut microflora by prebiotics remained to be elucidated. The production of short-chain fatty acids (SCFA) from fermentation of prebiotics coupled by decreased in luminal pH levels have been reported to alter intestinal bacteria population (Swanson *et al.*, 2002b). Prebiotics have been shown to be a source of SCFAs both *in-vitro* and *in-vivo*, although no particular distinguishing feature of the pattern of SCFA production has emerged as yet. It has been previously found that starch consistently produces relatively more butyrate whereas oligofructose and inulin are the lowest producers. Arabinogalactan and polydextrose yield relatively more propionate, and oligofructose yields predominantly acetate (Cummings, 1995). Despite these *in-vitro* findings, the *in-vivo* study of SCFAs is more difficult and relies mostly on determination of the concentrations of these acids in faeces. Because of the limitations of this approach, it is not surprising that this mechanism remains controversial. In addition, previous human studies have reported that neither inulin nor oligofructose at doses of between 4 and 40 g/d produced any significant change in the concentration or molar ratios of faecal SCFA (Gibson *et al.*, 1995).

2.8 Response surface methodology (RSM) as a useful approach in product development

RSM is a group of important statistical and mathematical techniques for developing, improving and optimizing various processes, and for enhancing existing product designs. The experiments are designed to allow estimation of interaction and even quadratic effects, and therefore give the pattern of the response surface investigated. RSM designs are aimed at finding improved or optimal process settings, troubleshoot process problems and weak points, and at making a product or process more robust against external and non-controllable influences (ESH, 2006a). It has been successfully utilized to optimize composition of fermentation medium, enzyme hydrolysis conditions, food preservative parameters and fermentation processes (Lee and Chen, 1997).

2.8.1 Statistical versus conventional approach in *in-vitro* studies

Conventional methods in optimization processes have produced erroneous results caused by misinterpretations of interactions between parameters studied and huge variation between parameters. These have misled the determination of true optimum region and other experimental regions were mistakenly identified as the optimum points (Figure 2.3). Crosses and circles represent experimental points, which indicate experimental runs. Points represented by crosses were initially determined followed by points represented by circles, while the optimum region was obtained from the combination of both. It is clearly indicated that the true optimum region was failed to be detected (Selber *et al*., 2000).

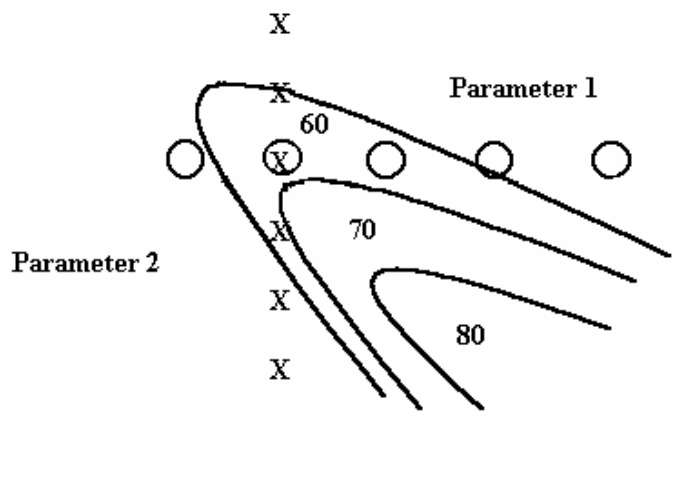


Figure 2.3 Variations from experimental parameters as determined by conventional optimization approach (Selber et al., 2000).

RSM was utilized to determine functions or mathematical equations that predict response values from a set of conditions within the experimental region. In chemical reactions, Y yield (response variable) is influenced by variables X_1, X_2,…, X_k (k is number of factors). If all variables are quantified, response variable Y may be defined as:

$$Y = f(X_1, X_2, \ldots, X_k)$$ (Montgomery, 1996).

The utilization of the above equation for first or second order equations will depend on the relationship between response and independent variables used. First order equations are utilized when responses are obtained from:

$$Y = \beta_0 X_0 + \beta_1 X_1 + \beta_2 X_2 + E$$

with E represents error (Wanasundara and Shahidi, 1996).

It is adequate for response surface models to contain only main effects and interaction effects when experimental results do not indicate quadratic effects in response obtained or when constrains exist within design matrix with practical maximum or minimum experimental limits. Quadratic or cubic models are needed when responses indicate curvature and "lack-of-fit" analyses confirm lower order models are inadequate:

$$Y = \beta_0 X_0 + \beta_1 X_1 + \beta_2 X_2 +$$ (linear effect)

$$\beta_{11} X_1^2 + \beta_{22} X_2^2 +$$ (quadratic effect)

$$\beta_{12} X_1 X_2 + E$$ (interaction effect) (Embuscado *et al*., 1994).

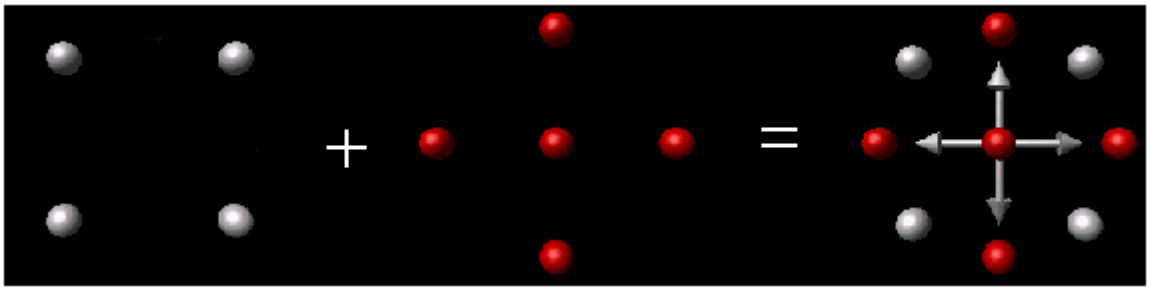
2.8.2 Sequencing in RSM

Response surfaces are often influenced by various variables. The primary purpose of screening experiments is to select or screen out the few important main effects from the many less important ones. Factorial designs are used for screening in order to select significant variables (Myers and Montgomery, 1995), to determine the magnitude and direction of changes in responses contributed by changes in variables, and to determine changes that occurred from combinations of variables as compared to changes from single variables (Montgomery, 1996).

2.8.2.1 Full factorial design

Compared to conventional optimization processes that utilize a factor at a time, full factorial designs enable manipulation of multiple variables within shorter time frame, determination of factors with best influence towards responses, and provide better illustration of interactions between various factors studied (El-Helow and El-Ahawany, 1999).

Factorial design class 2^k (two levels, k factors) is the fundamental of full factorial designs with utmost importance in screenings to determine system variables or initial response surface experiments of important processes, to achieve first order response surface models and subsequently to produce factor effect estimates for steepest ascent formation, and as building blocks to produce other response surface designs (Figure 2.4).



(A) (B) (C)

Figure 2.4: The combinations of a 2^2 design (A) with an axial design (B), to generate a central composite design (CCD; C) (Montgomery, 1996).

General cubic design of 2^3 (Figure 2.5) and Table 2.6 illustrate an experimental standard sequencing (non random). Column-i (X_i) was generated from repetition of 2^{i-1} times for –1, followed by repetition of 2^{i-1} times for +1, and so on. When all factors are scaled with value +1 for the highest value and –1 for the lowest value, the design matrix will have a column that is orthogonally paired, and all columns will have a sum of zero. Orthogonality is an important characteristic that demolishes correlation effects between main factor estimation effects and interaction effects. This enables full factorial design to estimate all higher-level interaction effects (Ragonese *et al*., 2000).

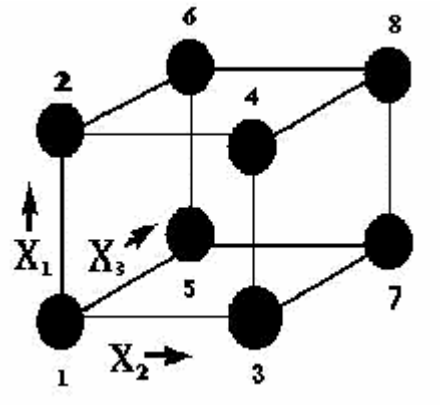


Figure 2.5 Cubic figure for a 2^3 design. Arrows indicate direction of increment for individual factors. Numbers indicate experimental standard order (ESH, 2006b).

Table 2.6 Full factorial design of 2^3 experimental standard order (ESH, 2006b)

Experimental runs	Factor X_1	Factor X_2	Factor X_3	Response, Y
1	-1	-1	-1	Y1
2	+1	-1	-1	Y2
3	-1	+1	-1	Y3
4	+1	+1	-1	Y4
5	-1	-1	+1	Y5
6	+1	-1	+1	Y6
7	-1	+1	+1	Y7
8	+1	+1	+1	Y8

Full factorial designs are found to be capable of accommodating any number of variables studied, but may involve higher experimental runs. When the number of factors is five or greater, a full factorial design requires a large number of runs and is not very efficient. Thus, fractional factorial designs are more appropriately used (Houston *et al.*, 2001).

2.8.2.2 Fractional factorial design

Fractional factorial designs are derived from full factorial designs. It is capable of identifying important variables and determining interaction effects between variables, using less number of experimental runs as compared to full factorial designs, and without loss of information on main variable effects and their interactions (Li *et al*., 2002). Properly chosen fractional factorial designs for 2-level experiments have the desirable properties of being both balanced and orthogonal. A balance design has the same number of observations for all treatment combinations. An experimental design is orthogonal if the effects of any factor balance out across the effects of the other factors. Two vectors of the same length are orthogonal if the sum of the products of their corresponding elements equals to zero (ESH, 2006a).

Total magnitudes of main effects are higher than the total magnitude of two-factor interaction effects, which subsequently are higher than three-factor interaction effects. At a certain level, higher-factor interaction effects decrease and are negligible, thus, fractional factorial designs could be utilized to estimate main effects and in certain cases, interaction effects of higher-factors (Ragonese *et al*., 2000). Experiments with two levels and three factors will require eight runs ($2^3 = 8$) which can be reduced to four runs (2^{3-1}) using fractional factorial designs, which produce three degree of freedom for main effects (Montgomery, 1996).

2.8.2.3 Steepest ascent

Steepest ascent is a selective procedure for approaching the optimum region. A steepest ascent path refers to the vector generated by first order regression, based on chosen parameters and initial screening experiments (Figure 2.6). The fundamentals for a new design are formed from the highest value within the steepest ascent path. Vectors are scaled to enable determination of extreme points. Thus, steepest ascent is the initial step in optimization procedures (Selber *et al*., 2000).

2.8.2.4 Optimization experiments

Two-level factorial designs are adequate to estimate interaction effect and first order effect but fail to detect second order effect. In a central composite design (CCD), axial points detect pure quadratic effect in a model within experimental region. Factorial and middle

points are used to fulfil first order models via lack-of-fit tests and to subsequently determine second order effect. The integration of axial points with factorial and middle points will produce CCD that fits second order models (Ghosh, 1996).

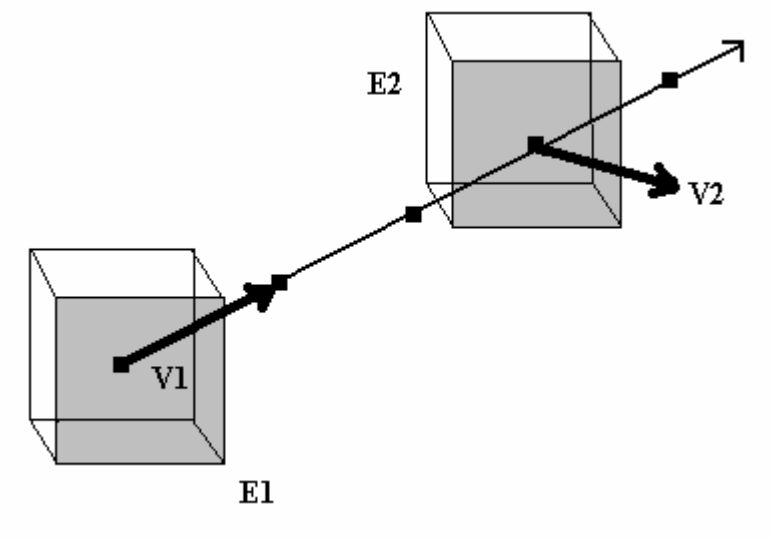


Figure 2.6 A steepest ascent path. Points in dark squares represent experimental sequences. E1 and E2 refer to the initial and subsequent experimental groups. V1 and V2 refer to the vectors generated that approach the steepest ascent path (Selber et al., 2000).

Optimization purposes that include linear, quadratic and interaction effects, second order effects are often taken into consideration and achieved with the application of CCD. Although various types of CCD are utilized, the circumscribed CCDs are most commonly practiced. Circumscribed-CCDs (Figure 2.7) are the original form of the central composite design. The star points are at α-distance from the center, and are based on the properties desired for the design and the number of factors in the design. The star points establish new extremes for the low and high settings for all factors. These designs have circular, spherical, or hyperspherical symmetry. In circumscribed CCD, the design points describe a circle *circumscribed* about the factorial square. For three factors, the design points describe a sphere around the factorial cube. Such designs are also rotatable when $\alpha = 1.414$ that produces circular geometry designs. A design is rotatable if the variance of the predicted response at any point X depends only on the distance of X from the design center point and is not a function of the direction the point lies from the center. A design with this property can be rotated around its center point without changing the prediction variance at X. Before a study begins, little or no knowledge may exist about the region that contains the optimum

response. Therefore, the experimental design matrix should not bias an investigation in any direction (ESH, 2006c). Rotatability is a desirable property not present in 3-level factorial designs used for response surface designs such as the quadratic model designs. In rotatable designs, points with the same distance from middle points will have similar estimated error. Rotatability of designs that are centered at middle points does not interfere with the produced contour of variance (Araujo and Brereton, 1996).

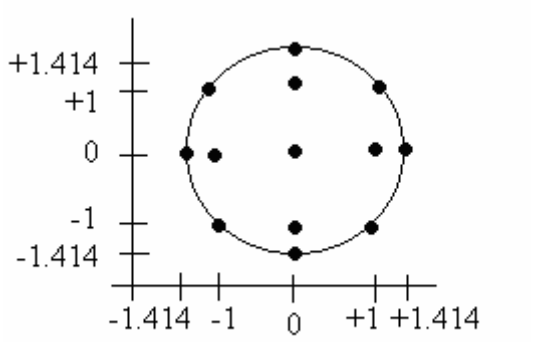


Figure 2.7: Circumscribed CCD with α = 1.414 that produces the rotatability geometry (ESH, 2006c).

Circumscribed CCDs provide high quality predictions over the entire design space, but require factor settings outside the range of the factors in the factorial part. With α = 1.414, experimental region are expanded to include chosen factorial values. However, this requires five levels for each factor as compared to only three levels in simple CCDs. Thus, circumscribed CCDs are suitable if estimated error may be reduced significantly and outweigh the saddle experimental operations at five levels. When the possibility of running such a design is recognized, factor spacings can be reduced to ensure that $\pm\alpha$ for each coded factor corresponds to feasible reasonable levels (ESH, 2006c).

3.0 Acid and bile tolerance, and cholesterol removal ability of *Lactobacillus* and *Bifidobacterium*

3.1 Acid and Bile Tolerance, and Cholesterol Removal Ability of *Lactobacillus acidophilus* and *L. casei* Strains

3.1.1 INTRODUCTION

Studies have shown that elevated serum cholesterol was associated with risks of coronary heart disease. For each 1 mmol higher than normal cholesterol level, the risk of coronary heart disease was approximately 35 percent greater, while coronary death was 45 percent higher. Small reduction in serum cholesterol of 1 per cent was also found to reduce risk of coronary heart disease by 2 to 3 percent (Manson et al., 1992). Consumption of dairy products containing probiotics has been proposed to lower serum cholesterol (Greenwald, 1991).

Probiotics are defined as 'live microbial supplement that beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1992). In order to provide health benefits, probiotics must overcome physical and chemical barriers such as acid and the presence of bile in the gastrointestinal tract (Gibson et al., 2000). Milk fermented with lactobacilli was first demonstrated to exhibit hypocholesterolemic effects in humans as early as 1963 (Shaper et al., 1963; Mann, 1974). Various studies have shown that some lactobacilli could lower total cholesterol and low-density-lipoprotein (LDL) cholesterol (Anderson and Gilliland, 1999; Sanders, 2000).

The exact mechanisms of serum cholesterol reduction by probiotic bacteria are unclear. Some strains of *Lactobacillus acidophilus* were found to secrete bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), which catalyses the hydrolysis of glycine and/or taurine conjugated bile salts into amino acid residues and free bile salts (bile acids) (Corzo and Gilliland, 1999). Free bile salts are less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen. Deconjugation of bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation (Reynier et al., 1981). Another mechanism for cholesterol reduction was proposed by Noh et al. (1997), who suggested that *L. acidophilus* incorporates some of the cholesterol removed from the medium into their cellular membrane during growth. Cholesterol incorporated into or adhered to the bacterial cells would be less available for absorption from the intestine into the blood. Cholesterol incorporated into the cells of *L. acidophilus* was postulated to alter the cellular membrane or cell wall of the organism. This was supported by Gram-staining of *L. acidophilus*, which showed only a proportion of cells that were grown in the presence of cholesterol stained Gram-positive, while all those grown without cholesterol did not stain Gram-positive.

This section has been published: *Liong, MT and Shah, NP. 2005. Acid and bile tolerance and cholesterol removal ability of lactobacilli strains.* ***J Dairy Sci*** *88: 55-66.*

However, this was debated as experiments using membrane isolated from strains of *L. casei* grown in broth containing cholesterol revealed no incorporation of cholesterol of measurable amount (Brashears et al., 1998).

Dambekodi and Gilliland (1998) have shown no relationship between the amount of cholesterol removal *in-vitro* and the degree of bile salt deconjugation, which led to another hypothesis that cholesterol removal may be related to assimilation of cholesterol. *In-vitro* experiments showed that strains of lactobacilli that were able to assimilate cholesterol were also able to reduce cholesterol *in-vivo*. Cholesterol assimilation was associated with the presence of bile salts and cholesterol removal from the medium increased with increasing concentration of bile salt (Rasic et al., 1992; Tahri et al., 1996). However, bile tolerant strains of *L. acidophilus* did not exhibit greatest ability to assimilate cholesterol, while strains that possessed minimal bile tolerant abilities actually assimilated more cholesterol (Dambekodi and Gilliland, 1998). Cholesterol assimilation by strains of *L. acidophilus* during refrigerated storage of non-fermented milk suggested that cholesterol uptake was associated with bacterial growth and their viability (Piston and Gilliland, 1994). Similarly, Pereira and Gibson (2002) suggested that the cholesterol assimilation ability of the bacteria was growth dependent.

The aims of this study were to investigate the probiotic properties (acid and bile tolerance) of eleven lactobacilli strains, and their cholesterol removal abilities in order to understand the possible mechanisms of cholesterol removal.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Bacteria

Seven strains of *Lactobacillus casei* were obtained from the Victoria University Culture Collection (Werribee, Australia). *L. casei* CSCC 2607 was originally obtained from the Commonwealth Scientific and Industrial Organization (CSIRO) (Highett, Australia), while *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 279, *L. casei* ASCC 290, *L. casei* ASCC 292 and *L. casei* ATCC 15820 were originally obtained from the Australia Starter Culture Research Centre (ASRC) (Werribee, Australia). Four strains of *Lactobacillus acidophilus* were obtained from ASCC. All strains are of human origin. Stock cultures were stored in 40% glycerol at –80 °C. The organisms were subcultured three times prior to use in sterile de Mann, Rogosa, Sharpe (MRS) broth using 1% inoculum and 20 h incubation at 37 °C.

3.1.2.2 Acid tolerance

Acid tolerance of the cultures was studied by incubating the organisms in MRS broth supplemented with 0.30% oxgall, the pH was adjusted to 2.0 with HCl and incubated at 37 °C for 2 h. Each of the 11 strains of *L. acidophilus* and *L. casei* was subcultured at least three times prior to experimental use, followed by centrifugation after the final subculture, inoculation (10% vol/vol) into the broth, and growth monitoring by using the plate count method. Bacilli generally divide in one plane, but can produce chains of cells by the failure to separate completely. Thus, fermentation broth containing probiotic cultures were sonicated for 5 s to disrupt clumps of lactobacilli (Bermudez et al., 2001) before serial dilutions were performed. Subsequent serial dilution blanks were vortexed for 30 s individually. One millilitre sample was taken every 30 min for 2 h, and 10-fold serial dilutions were made using peptone water diluent. MRS agar was used for plating and the plates were incubated anaerobically at 37 °C for 24 h in an anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD., U.S.A.) with a Gas Generating Kit® (Oxoid, Ltd.). Acid tolerance was determined by comparing the final plate count after 2 h with the initial plate count at 0 h. The experiments were repeated twice.

3.1.2.3 Bile tolerance

Three types of different bile were used, namely oxgall, cholic acid and taurocholic acid to study bile tolerance of the organisms. The bile tolerance was studied according to the method of Gilliland and Walker (1990). Briefly, MRS broth containing 0.30% (wt/vol) of oxgall, cholic acid or taurocholic acid was inoculated with each strain, and incubated at 37 °C. MRS broth without bile salt was used as a control. According to the material safety data sheet (MSDS) and Merck solubility index, cholic acid has a solubility of 0.28 g/L in water at 15°C, while taurocholic acid was completely soluble in water. Bacterial growth was monitored by measuring absorbance with a spectrophotometer (Pharmacia Novaspec II, Cambridge, England) at 620 nm at hourly intervals for 7 to 8 h. The absorbance values obtained were plotted against the incubation time, and bile tolerance of each strain was based on the time required for the absorbance value to increase by 0.3 unit. pH values of all the fermentation broths at time = 0 were measured, and another measurement was taken after the absorbance increased by 0.3 unit. All the experiments were replicated twice.

3.1.2.4 Cholesterol removal

Freshly prepared MRS broth was supplemented with 0.30% oxgall as a bile salt. Water soluble cholesterol (polyoxyethanyl-cholesteryl sebacate) was filter sterilized and

added to the broth at a final concentration of 70-100 μg/mL, inoculated with each strain at 1% level and incubated anaerobically at 37 °C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a modified colourimetric method as described by Rudel and Morris (1973). One millilitre of the aliquot was added with 1 mL of KOH (33% wt/vol) and 2 mL of absolute ethanol, vortexed for 1 min, followed by heating at 37 °C for 15 min. After cooling, 2 mL of distilled water and 3 mL of hexane were added and vortexed for 1 min. One millilitre of the hexane layer was transferred into a glass tube and evaporated under nitrogen. The residue was immediately dissolved in 2 mL of o-phthalaldehyde reagent. After complete mixing, 0.5 mL concentrated sulphuric acid was added and the mixture was vortexed for 1 min. Absorbance was read at 550 nm (Pharmacia Novaspec II, Cambridge, England) after 10 min. All experiments were replicated twice.

3.1.2.5 Cholesterol removal by dead- and resting cells

Freshly prepared MRS broth containing 0.30% oxgall was inoculated with each strain of lactobacilli and incubated at 37 °C for 20 h. Cells were harvested after the incubation period by centrifuging at 10000 x g (Microspin 24, Sorvall Instruments, Melbourne, Australia) at 4 °C for 10 min. The cell pellet was washed twice with sterile distilled water. For preparation of heat-killed cells, the cell pellet was suspended in 10 mL sterile distilled water and autoclaved for 15 min at 121 °C. The heat-killed cells were suspended into MRS broth containing 0.30 % oxgall and water-soluble cholesterol. For preparation of resting cells, the cell pellet was suspended in 10 mL sterile 0.05 M phosphate buffer (pH 6.8) containing oxgall and water-soluble cholesterol (Kimoto et al., 2002). All strains were incubated at 37 °C for 20 h. The spent broth was assayed for cholesterol content as mentioned above. Cholesterol assimilation by growing-, resting- and dead-cells was expressed in dry weight to obtain uniformity in all treatments. The following equation was used:

Cholesterol assimilation = $(C_1 – C_2)/(W_2-W_1)$;

where C_1 and C_2 were the amount of cholesterol present in the fermentation broths at time = 0 and time = 20 h, respectively, and W_1 and W_2 were the dry weight of the individual culture at time = 0 and time = 20 h, respectively, for all treatment studied. The experiments were repeated twice.

3.1.2.6 Cellular fatty acids composition

Cellular lipids were extracted using a modified method of Murga et al. (2000). The cells pellet was mixed with 1 mL of methanol: chloroform (2: 1, v: v) and vortexed at room temperature for 2 min. Aliquots were left to stand for 24 h at 4 °C and centrifuged (10000 x g, 10 min, 4 °C). The supernatant was collected and the cell pellet was washed twice with 1 mL of methanol: chloroform: distilled water (2: 1: 0.8). All three supernatants were pooled and vortexed for 1 min. To this, 1 mL each of distilled water and chloroform were added, followed by vortexing for 2 min. The mixture was allowed to separate and the chloroform layer (bottom) was collected and evaporated. The lipid residue was dissolved in 0.5 mL of hexane and converted into fatty acid methyl esters with the addition of 100 µl methanolic KOH (2 M). HCl (2 M) was added until methyl orange indicator changed to pink and the mixture was allowed to settle. The organic layer (10 µl) was collected and injected into a gas chromatograph (Variance Star 3400 CX, Walnut Creek, CA, USA), equipped with a flame ionization detector. A stainless steel column (30 m x 0.25 mm) packed with 70% cyanopropyl polysilphenylene siloxane was employed. The oven temperature was held at 100 °C after sample injection and increased to 225 °C with a rate of 5 °C /min. The injector and detector temperatures were 260 °C and 280 °C, respectively. Helium was used as the carrier gas (3 mL/min), split ratio 1: 100 and the injection volume was 1 µl. The concentration of fatty acid methyl esters in samples was determined using standards (Sigma Chemical Co., St. Louis, MO, USA). All experiments were repeated twice.

3.1.2.7 Statistical analysis

Data analysis was carried out with SPSS Inc. software (version 10.0). One-way analysis of variance was used to study significant difference between means, with significance level at $\alpha = 0.05$. Tukey's-test was used to perform multiple comparisons between means. All data are presented as mean ± standard error of means. The acid tolerance experiment and cellular fatty acid profiles were independently replicated two times (n = 2), with two measurements per replicate. The mean of the repeated measurements yielded the value for each replicate. The bile tolerance and cholesterol assimilation experiments were also independently replicated two times (n = 2), with three measurements per replicate. The mean of the repeated measurements yielded the value for each replicate.

3.1.3 RESULTS

3.1.3.1 Acid tolerance of lactobacilli

The effect of acid on the viability of lactobacilli is shown in Table 3.1. All strains showed tolerance to pH 2.0 for 2 h despite variations in the degree of viability. *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 were the most acid tolerant strains, with more than 10^7 total CFU/mL after incubation for 2 h at pH 2.0, while *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 279, *L. casei* ATCC 15820 and *L. casei* CSCC 2607 were the most acid sensitive strains with only 10^4 total CFU/mL after 2 h of incubation. In general, total CFU for the strains of *L. acidophilus* reduced at a higher level as compared to those of *L. casei* for the first hour of incubation; the former decreased by 2.41 to 2.79 log cycles, while the latter only decreased by 1.58 to 2.26 log cycles. However, strains of *L. acidophilus* showed greater acid tolerance over the entire 2 h incubation period, and their counts decreased by 2.66 to 4.38 log cycles, compared to 3.16 to 6.20 log cycles for *L. casei*. For acid sensitive strains, the viability decreased slowly for the first hour of incubation, followed by a rapid decline at the end of the 2 h incubation period. *L. casei* CSCC 2607, being the most acid sensitive strain, showed a total reduction of 6.31 log cycles over the 2 h incubation period, with 4.20 log cycle reduction mainly in the second hour of incubation.

3.1.3.2 Bile tolerance of lactobacilli

The effects of oxgall, cholic acid and taurocholic acid on the growth of lactobacilli are presented in Table 3.2. Growth of lactobacilli in MRS broth without bile source was used as a control. Cholic acid was used as the deconjugated bile, taurocholic acid as the conjugated bile, while oxgall contained both conjugated and deconjugated bile. Due to the addition of bile acids to the media, the effect of pH was monitored. Generally, media containing cholic acid had lower pH values compared to the other media. However, the reduction in pH values due to the production of acid by these lactic acid bacteria, were more likely to be strain dependent. pH of the media decreased highest from strains *L. casei* ASCC 290 and CSCC 2607 incubated in media supplemented with oxgall. The average reduction was highest from media supplemented with oxgall (0.81), despite the fact that media with cholic acid had the lowest initial pH values, while the lowest pH reduction was from media supplemented with taurocholic acid (0.11). These indicated that the initial pH of media with different bile sources had minimal influence towards subsequent growth and bile tolerance of the cultures, and pH-related inhibitory actions of bile salts. Most strains of *L. acidophilus* showed better growth in MRS broth without bile as compared to those of *L. casei*. *L. acidophilus* ATCC 4356 showed similar growth in the presence of bile salt (oxgall and

cholic acid) and without bile, while the growth of *L. casei* ASCC 290 was inhibited in the presence of oxgall compared to the control. All strains showed faster growth in MRS broth containing cholic acid, while slower growth was observed in presence of taurocholic acid. *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 290, *L. casei* ATCC 15820 and *L. acidophilus* ATCC 4357 were most bile tolerant in the presence of cholic acid, while *L. acidophilus* ATCC 4356 and *L. casei* CSCC 2607 were the least tolerant. Most strains of *L. casei* showed better tolerance to cholic acid as compared to those of *L. acidophilus*. In the presence of taurocholic acid, strains of *L. acidophilus* showed overall greater tolerance compared to those of *L. casei*. However, when comparing control and taurocholic acid on the average, there was a 1.37 hour difference with *L. acidophilus* (principally due to strain 4356), while it was only 1.06 hour with *L. casei*. Thus, we postulate that the slow growth of *L. casei* in the presence of taurocholic acid may have also been influenced by its slower growth in MRS than inhibition effects by conjugated bile. *L. acidophilus* ATCC 33200, *L. acidophilus* ATCC 4357, *L. acidophilus* ATCC 4962 and *L. casei* ASCC 1521 were most tolerant towards taurocholic acid, while *L. casei* ASCC 279 was the least tolerant strain ($P < 0.05$). *L. casei* and *L. acidophilus* showed various levels of tolerance to oxgall; *L. casei* ASCC 1520, *L. casei* ASCC 292, *L. casei* ATCC 15820 and *L. acidophilus* ATCC 33200 showed better tolerance than *L. casei* ASCC 1521, *L. casei* ASCC 279 and *L. casei* ASCC 290.

3.1.3.3 Cholesterol assimilation by lactobacilli

Levels of cholesterol assimilation during 20 h of growth of the strains are shown in Table 3.3. Cholesterol removal varied among strains ($P < 0.05$) and ranged from 3.76 to 34.69 μg/mL. In general, both *L. acidophilus* and *L. casei* assimilated more cholesterol in the presence of cholic acid compared to the control. In broth containing cholic acid, overall cholesterol removal was observed to be higher for strains of *L. casei* compared to those of *L. acidophilus*. *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 292, *L. casei* ATCC 15820 and *L. acidophilus* ATCC 4962 assimilated more than 25 μg/mL cholesterol in the presence of cholic acid, and were better than *L. acidophilus* ATCC 4356, *L. acidophilus* ATCC 4357 and *L. casei* CSCC 2607, which assimilated less than 15 μg/mL cholesterol. In broth containing taurocholic acid, lowest cholesterol assimilation was observed for most strains of lactobacilli studied compared to the control. Strains of *L. acidophilus* ATCC 33200 and *L. casei* ASCC 1521 removed highest level of cholesterol with more than 20 μg/mL, however, the level was lower than other bile salts studied ($P < 0.05$). Overall cholesterol assimilation by strains of *L. acidophilus* was significantly higher than those of *L. casei* in the

presence of taurocholic acid. Cholesterol assimilation in broth containing oxgall varied from 12.03 to 32.25 μg/mL. Most strains were able to remove higher level of cholesterol compared to the control, except for strains of *L. acidophilus* ATCC 4357, *L. casei* ASCC 279 and *L. casei* ASCC 290. Cholesterol assimilation may be influenced by different biomass levels which were not identical in the various treatments. Thus, growth and cholesterol assimilation patterns were further studied.

3.1.3.4 Growth and cholesterol assimilation patterns

Growth of all lactobacilli studied in the presence or absence of, and the cholesterol assimilation patterns are illustrated in Figures 3.1 and 3.2. Oxgall was chosen instead of cholic acid because it represents closely the actual human gut model system, although the latter produced higher growth and cholesterol assimilation. Most strains exhibited higher growth in the presence of cholesterol with the exception of strain *L. acidophilus* ATCC 4962 (Figure 3.1D), ASCC *L. casei* 1520 (Figure 3.2A) and *L. casei* ASCC 1521 (Figure 3.2B), which showed better growth in the absence of cholesterol. Most strains had higher growth for the first 9 to 15 h in the medium containing no cholesterol followed by slower growth until the end of the 24 h incubation period. In the presence of cholesterol, most strains showed gradual but slower initial growth for the first 12 to 18 h, followed by a rapid growth thereafter. Cholesterol assimilation patterns varied with strains studied. Cholesterol assimilation patterns and growth curves indicated that cholesterol removal was growth associated.

3.1.3.5 Cholesterol removal by growing-, dead- and resting cells

We wanted to find out whether non-growing cells could remove cholesterol. The amount of cholesterol removed by growing-, resting- and dead-cells is illustrated in Figure 3.3. Heat-killed- and resting-cells showed a small degree of cholesterol removal, ranging from 0.79 to 3.82 mg/g dry weight, compared to 4.53 to 16.03 mg/g dry weight for growing cells. Although there was no significant difference between cholesterol removal by resting and dead cells, most strains exhibited higher cholesterol removal when cells were resting as suspended in phosphate buffer (pH 6.8), compared to heat-killed cells, except for *L. casei* ASCC 1520, which removed more cholesterol when cells were killed than resting form. *L. casei* ASCC 292, *L. casei* ATCC 15820 and *L. casei* ASCC 1520 assimilated more cholesterol during growth, but not during resting and in dead form. On the other hand, *L. acidophilus* ATCC 4357 and *L. casei* ASCC 1521 assimilated less cholesterol during growth but showed relatively higher cholesterol assimilation when cells were resting and dead. *L.*

casei ASCC 279 and *L. casei* ASCC 290 assimilated lowest level of cholesterol during growth.

3.1.3.6 Effect of cholesterol on cellular fatty acid composition

Effect of cholesterol on cellular fatty acid compositions is presented in Tables 3.4 and 3.5. Cellular fatty acid patterns varied between strains grown with or without cholesterol. Strains grown in the absence of cholesterol had higher percentages of oleic and linoleic acids compared to those grown in the presence of cholesterol. Cellular fractions of cells grown in the presence of cholesterol showed relatively higher percentage of hexadecanoic and octadecanoic acids compared to cells grown in medium without cholesterol. All strains grown in the medium with or without cholesterol showed higher percentage of total saturated fatty acids than unsaturated fatty acids. Total saturated fatty acids were higher in cellular fractions of cells grown in the presence of cholesterol, while total unsaturated fatty acids were lower. However, ATCC *L. acidophilus* 4356 and *L. acidophilus* 4357 showed lower total level of saturated fatty acids per gram dry weight of cells when grown in the medium with cholesterol compared to that without cholesterol, while strains *L. casei* ATCC 4962, *L. casei* 15820, *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 290, *L. casei* ASCC 292 and *L. casei* CSCC 2607 showed higher cellular total unsaturated fatty acids when cells were grown in the medium with cholesterol compared to that without cholesterol. Most strains showed higher level of total fatty acids when cells were grown in the presence of cholesterol compared to those in its absence.

3.1.4 DISCUSSION

High level of serum cholesterol has been associated with risks of coronary heart disease. The use of probiotic bacteria in reducing serum cholesterol levels has attracted much attention. Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant in order to survive in the human gastrointestinal tract. The time from entrance to release from the stomach has been estimated to be approximately 90 min, with further digestive processes requiring longer residence time (Berada et al., 1991). Stresses to organisms begin in the stomach, with pH between 1.5 and to 3.0, and in the upper intestine that contains bile (Lankaputhra and Shah, 1995; Corzo and Gilliland, 1999). Survival at pH 3.0 for 2 h and in bile concentration of 1000 mg/L is considered optimum for acid and bile tolerance for probiotic strains (Usman and Hosono, 1999). Strains of lactobacilli used in this study showed varying levels of viability at pH 2.0 after 2 h incubation. *L. acidophilus* ATCC 4357, *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 survived best

under the acidic conditions, while viability of strains ASCC 1520, ASCC 1521, ASCC 279, ATCC 15820 and CSCC 2607 was greatly reduced. In general, *L. acidophilus* strains survived better under acidic conditions than *L. casei*.

Growth observed in the presence of different bile sources suggested that conjugated bile, taurocholic acid was more inhibitory towards strains of lactobacilli as compared to deconjugated bile (cholic acid) and oxgall. The resistance of the lactobacilli towards deconjugated bile may be due to the fact that conjugated bile salts have greater solubility and detergent activity, and may, therefore be more toxic than their deconjugated counterpart. This was supported by the fact that the cholic acid added to the fermentation broths was far less soluble than taurocholic acid based on the solubility index. Taurocholic acid was not accumulated by *Lactobacillus salivarus* JCM 1044 due to its hydrophilicity (Kurdi et al., 2000). Such preference was also supported by previous reports (Kurdi et al., 2000; Yokota et al., 2000) that showed *Lactobacillus* species actively accumulated cholic acid in an ATP-dependent manner, or when they were energized by glucose. However, results from our study contradicted with previous studies that found deconjugated bile salts to have greater tendency to damage the cell membrane due to their hydrophobic nature. Free bile acids and deconjugated bile acids have the ability to disaggregate the ordered structure of biological membrane and cytoplasmic membrane, respectively. Deconjugated sodium taurocholate was reported to have such properties and therefore, have higher inhibitory effects against bacterial cells (Tannock et al., 1997). Similarly, according to Yokota et al. (2000), the degree of sensitivity of *Lactococcus lactis* was consistent with the degree of hydrophobicity of the compounds, the highest being for deconjugated bile followed by conjugated one. Our results suggest that under high concentration of conjugated bile, strains of *L. acidophilus* are likely to survive best, while in presence of deconjugated one, *L. casei* would survive best. Growth in the presence of oxgall showed that *L. casei* ASCC 1520, *L. casei* ASCC 290 and *L. casei* ATCC 15820 survived best, while *L. casei* ASCC 1521, *L. casei* ASCC 279 and *L. casei* ASCC 290 would be least bile tolerant.

Cholesterol assimilation in the presence of different bile source showed good relationship (R^2 = 0.71 to 0.89) with bile tolerance of the strains studied (Figure 3.4). Strains showing greater tolerance towards deconjugated bile exhibited overall higher cholesterol assimilation in the presence of cholic acid, while those showing greater inhibition by taurocholic acid produced lower cholesterol assimilation. Also, most strains exhibited better growth in the presence of cholesterol, indicating that cholesterol stimulated their growth. Furthermore, regression analyses suggested that cholesterol removal was closely associated with the bacterial growth. Cholesterol assimilation by growing cells was significantly higher

than resting and dead counterparts, however, there was no significant difference ($P < 0.05$) in the level of cholesterol removal by resting and dead cells. The capability of strains to remove cholesterol in dead and resting stage indicated that cholesterol might also be removed via binding to cells. Higher cholesterol removal by growing cells indicated that the degree of bound cholesterol might be dependent on the growth of cells. The physiological pH in the intestinal tract of humans is usually neutral to alkaline (Kimoto et al., 2002). Although cholesterol assimilation occurred mainly with growing cells, results on cholesterol removal from media at pH 6.8 by heat-killed cells indicated the potential of non-viable cells to reduce cholesterol concentration in the gastrointestinal system.

To further examine the possibility of cholesterol incorporation into the membrane fraction of cells, changes in cellular lipid profiles were quantified using a gas chromatograph. Methanolic HCl does not methylate free fatty acids, thus methylated fatty acids were components of the membrane phospholipids. The lipids in Gram-positive bacteria are found predominantly in the membrane (Kimoto et al., 2002), and fatty acids in bacteria are primarily the precursors of cellular phospholipids (Li and Cronan, 1993). Thus, cholesterol removed *in-vitro* could be incorporated into the cellular membrane, and this may alter the fatty acid profiles, especially hexadecanoic, octadecanoic, total saturated and unsaturated acids. Such alteration of the cellular envelop was supported by Noh et al. (1997) who found that cells of *L. acidophilus* ATCC 43121 grown in the presence of cholesterol micelles showed greater resistance to lysis by sonication than those grown without any cholesterol. Kimoto et al. (2002) found that there was a difference in the fatty acid distribution pattern for *Lactococcus lactis* cells grown with or without cholesterol, suggesting that the fatty acids composition of the cells were altered as a result of cholesterol being incorporated into the cellular membrane after its removal from the media. Our results showed that total fatty acids obtained from most cells grown in the presence of cholesterol were higher than those grown in the absence, with the exception of strains *L. acidophilus* ATCC 4356 and *L. acidophilus* ATCC 4357. We postulate that the increment in total fatty acids was contributed by cholesterol incorporation into the membrane and not by cellular synthesis, because lactic acid bacteria growing under high level of lipid were previously reported to lose the ability to synthesize fatty acids (Kiatpapan et al., 2001).

3.1.5 CONCLUSIONS

All strains of lactobacilli studied survived the acidic condition and bile concentration to a variable extent. Similarly, all strains have varying capabilities to remove cholesterol *in-vitro*. Three possible mechanisms for removal of cholesterol from media by lactobacilli are

proposed: assimilation of cholesterol during growth, incorporation of cholesterol into the cellular membrane of cells, and binding of cholesterol to cell surface. *L. casei* ASCC 292 and *L. acidophilus* 4962 strains showed good acid and bile tolerance, and highest cholesterol removal from media, indicating that these strains may be promising candidates for use as a dietary adjunct to lower serum cholesterol *in-vivo*.

Table 3.1. Effect of pH 2.0 on viability of lactobacilli

Strains	Viable count (log CFU/mL) [1]				
	0 min	30 min	60 min	90 min	120 min
L. acidophilus ATCC 33200	10.05 ± 0.64[a]	9.41 ± 0.27[a]	7.64 ± 0.59[a]	6.55 ± 0.18[bc]	5.67 ± 0.24[bc]
L. acidophilus ATCC 4356	10.13 ± 0.58[a]	8.27 ± 0.75[a]	7.60 ± 0.03[a]	6.73 ± 0.32[b]	5.77 ± 0.17[bc]
L. acidophilus ATCC 4357	10.18 ± 0.64[a]	8.59 ± 0.62[a]	7.67 ± 0.03[a]	6.26 ± 0.04[bc]	6.15 ± 0.71[b]
L. acidophilus ATCC 4962	10.21 ± 0.09[a]	8.11 ± 0.33[a]	7.42 ± 0.13[a]	6.27 ± 0.10[bc]	7.55 ± 0.20[a]
L. casei ASCC 1520	10.41 ± 0.86[a]	9.41 ± 0.27[a]	8.29 ± 0.06[a]	6.49 ± 0.38[bc]	4.51 ± 0.14[c]
L. casei ASCC 1521	10.56 ± 0.60[a]	9.10 ± 0.63[a]	8.30 ± 0.02[a]	6.37 ± 0.08[bc]	4.70 ± 0.08[bc]
L. casei ASCC 279	10.40 ± 0.23[a]	9.50 ± 0.51[a]	8.36 ± 0.31[a]	6.39 ± 0.09[bc]	4.20 ± 0.81[c]
L. casei ASCC 290	10.65 ± 0.31[a]	10.20 ± 0.88[a]	9.07 ± 0.86[a]	8.68 ± 0.21[a]	7.00 ± 0.71[ab]
L. casei ASCC 292	10.62 ± 0.21[a]	9.91 ± 0.50[a]	8.70 ± 0.73[a]	8.44 ± 0.35[a]	7.46 ± 0.13[a]
L. casei ATCC 15820	10.28 ± 0.15[a]	9.16 ± 0.55[a]	8.61 ± 0.06[a]	5.73 ± 0.48[c]	4.43 ± 0.27[c]
L. casei CSCC 2607	10.36 ± 0.18[a]	9.06 ± 0.98[a]	8.25 ± 0.09[a]	7.43 ± 0.08[ab]	4.05 ± 0.97[c]

[abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

[1]Results are expressed as mean ± standard error of means; each data point is the average of two repeated measurements from two independently replicated experiments, n = 2.

Table 3.2. Bile tolerance of lactobacilli in different bile media

Strains	Growth media[1,2,3]											
	MRS broth			MRS broth + 0.3% oxgall			MRS broth + 0.3% cholic acid			MRS broth + 0.3% taurocholic acid		
	h	pH		h	pH		h	pH		h	pH	
		T1	T2		T1	T2		T1	T2		T1	T2
L. acidophilus ATCC 33200	$3.53 \pm 0.13^{b,AB}$	6.09	5.95	$3.29 \pm 0.18^{ab,AB}$	6.13	5.98	$3.03 \pm 0.14^{bc,A}$	5.84	5.61	$4.03 \pm 0.19^{a,B}$	6.05	5.89
L. acidophilus ATCC 4356	$2.80 \pm 0.20^{a,A}$	6.10	5.60	$3.86 \pm 0.13^{b,A}$	6.11	5.98	$3.78 \pm 0.19^{c,A}$	5.78	5.38	$5.40 \pm 0.29^{b,B}$	6.09	6.00
L. acidophilus ATCC 4357	$3.04 \pm 0.21^{a,AB}$	6.08	5.80	$3.92 \pm 0.40^{b,AB}$	6.11	5.57	$2.65 \pm 0.25^{b,A}$	5.88	5.53	$4.40 \pm 0.26^{ab,B}$	6.05	6.01
L. acidophilus ATCC 4962	$3.73 \pm 0.32^{bc,AB}$	6.06	5.91	$3.83 \pm 0.11^{b,AB}$	6.12	5.92	$2.89 \pm 0.16^{b,A}$	5.89	5.70	$4.74 \pm 0.39^{ab,B}$	6.08	6.02
L. casei ASCC 1520	$3.53 \pm 0.24^{b,AB}$	6.09	5.71	$2.36 \pm 0.13^{a,AB}$	6.11	4.80	$1.78 \pm 0.19^{a,A}$	5.87	5.34	$5.00 \pm 0.33^{b,C}$	6.05	6.00
L. casei ASCC 1521	$3.98 \pm 0.32^{bc,B}$	6.10	5.68	$4.03 \pm 0.12^{bc,B}$	6.10	4.73	$2.20 \pm 0.31^{a,A}$	5.91	5.77	$4.37 \pm 0.18^{ab,B}$	6.09	5.97
L. casei ASCC 279	$5.30 \pm 0.13^{d,BC}$	6.07	5.93	$4.24 \pm 0.28^{bc,AB}$	6.12	5.98	$3.19 \pm 0.35^{bc,A}$	5.86	5.70	$6.21 \pm 0.21^{c,C}$	6.08	6.00
L. casei ASCC 290	$3.93 \pm 0.19^{bc,AB}$	6.09	5.69	$4.36 \pm 0.15^{c,B}$	6.10	4.53	$3.14 \pm 0.23^{bc,A}$	5.88	5.68	$5.82 \pm 0.14^{bc,C}$	6.08	5.81
L. casei ASCC 292	$3.95 \pm 0.13^{bc,C}$	6.09	5.80	$3.17 \pm 0.12^{ab,B}$	6.10	5.99	$2.04 \pm 0.16^{a,A}$	5.90	5.45	$5.52 \pm 0.12^{bc,D}$	6.07	6.01
L. casei ATCC 15820	$4.62 \pm 0.41^{cd,B}$	6.08	5.61	$3.21 \pm 0.28^{ab,AB}$	6.09	4.25	$2.22 \pm 0.36^{a,A}$	5.84	5.53	$5.19 \pm 0.33^{b,B}$	6.06	5.95
L. casei CSCC 2607	$5.01 \pm 0.25^{cd,AB}$	6.06	5.71	$3.88 \pm 0.19^{b,A}$	6.12	4.52	$3.82 \pm 0.33^{c,A}$	5.80	5.47	$5.64 \pm 0.39^{bc,B}$	6.09	5.97

[abcd]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

[ABCD]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

[1]Results are expressed as mean ± standard error of means; each data point is the average of three repeated measurements from two independently replicated experiments, n = 2; [2]Time (h) required to increase 0.3 absorbance units at 620 nm in each medium; [3]pH at time = 0 (T1) and at time after absorbance increased 0.3 units (T2).

Table 3.3. Cholesterol assimilation by lactobacilli in different bile media[1]

Strains	MRS broth	MRS broth + 0.3% oxgall	MRS broth + 0.3% cholic acid	MRS broth + 0.3% taurocholic acid
	Cholesterol assimilated (μg/mL)	Cholesterol assimilated (μg/mL)	Cholesterol assimilated (μg/mL)	Cholesterol assimilated (μg/mL)
L. acidophilus ATCC 33200	$21.61 \pm 0.19^{a,BC}$	$22.09 \pm 0.21^{cd,AB}$	$23.80 \pm 0.52^{c,A}$	$20.80 \pm 0.25^{a,C}$
L. acidophilus ATCC 4356	$20.52 \pm 0.46^{ab,A}$	$21.78 \pm 0.23^{d,A}$	$14.01 \pm 0.14^{d,C}$	$9.07 \pm 0.30^{e,D}$
L. acidophilus ATCC 4357	$21.30 \pm 0.26^{a,A}$	$19.65 \pm 0.23^{e,B}$	$14.32 \pm 0.13^{d,D}$	$16.88 \pm 0.31^{b,C}$
L. acidophilus ATCC 4962	$19.22 \pm 0.47^{bc,C}$	$23.40 \pm 0.39^{cd,B}$	$27.60 \pm 0.25^{b,A}$	$14.69 \pm 0.25^{c,D}$
L. casei ASCC 1520	$20.68 \pm 0.21^{ab,C}$	$32.25 \pm 0.42^{a,B}$	$34.69 \pm 0.45^{a,A}$	$8.86 \pm 0.27^{ef,D}$
L. casei ASCC 1521	$18.02 \pm 0.44^{cd,C}$	$18.71 \pm 0.40^{e,BC}$	$29.64 \pm 0.35^{b,A}$	$20.32 \pm 0.31^{a,B}$
L. casei ASCC 279	$13.07 \pm 0.25^{e,B}$	$12.03 \pm 0.58^{f,B}$	$20.57 \pm 0.55^{d,A}$	$3.76 \pm 0.24^{g,C}$
L. casei ASCC 290	$18.75 \pm 0.46^{bc,B}$	$12.66 \pm 0.42^{f,C}$	$23.02 \pm 0.64^{c,A}$	$6.93 \pm 0.26^{f,D}$
L. casei ASCC 292	$16.67 \pm 0.17^{d,B}$	$29.33 \pm 0.16^{b,A}$	$34.53 \pm 0.35^{a,A}$	$12.14 \pm 0.17^{d,B}$
L. casei ATCC 15820	$16.04 \pm 0.44^{d,C}$	$23.97 \pm 0.37^{c,B}$	$28.80 \pm 0.22^{b,A}$	$11.93 \pm 0.85^{d,D}$
L. casei CSCC 2607	$10.00 \pm 0.44^{f,B}$	$20.38 \pm 0.31^{de,A}$	$10.94 \pm 0.32^{e,B}$	$7.82 \pm 0.35^{ef,C}$

^{abcdef}Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

^{ABCD}Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

[1] Results are expressed as mean ± standard error of means; each data point is the average of three repeated measurements from two independently replicated experiments, n = 2.

.**Table 3.4.** Fatty acid composition of *L. acidophilus* strains grown in medium with or without cholesterol[1]

Fatty acid	Medium	ATCC 33200		ATCC 4356		ATCC 4357		ATCC 4962	
		%[2]	mg/g[3]	%	mg/g	%	mg/g	%	mg/g
10:0	Cholesterol	9.39	1.11	9.88	1.14	7.99	0.75	10.94	1.35
	Without cholesterol	7.16	0.59	7.11	1.02	6.78	1.56	7.99	0.39
12:0	Cholesterol	10.66	1.11	11.42	1.16	9.83	0.81	11.18	1.21
	Without cholesterol	7.58	0.55	8.47	1.07	8.32	1.69	9.52	0.41
14:0	Cholesterol	11.61	1.10	12.53	1.16	10.45	0.79	12.19	1.21
	Without cholesterol	8.24	0.54	9.41	1.09	9.41	1.74	10.05	0.39
16:0	Cholesterol	20.12	1.80	18.39	1.60	20.13	1.43	18.66	1.74
	Without cholesterol	15.71	0.98	18.33	1.99	18.18	3.17	17.38	0.64
16:1	Cholesterol	7.20	0.67	8.06	0.72	8.61	0.63	7.80	0.75
	Without cholesterol	6.01	0.39	5.96	0.67	6.30	1.14	6.94	0.26
18:0	Cholesterol	14.59	1.25	13.97	1.16	15.07	1.02	14.08	1.25
	Without cholesterol	11.86	0.70	12.98	1.34	12.37	2.06	14.30	0.50
18:1	Cholesterol	10.12	0.87	8.70	0.73	9.55	0.65	8.61	0.77
	Without cholesterol	18.98	1.13	16.66	1.74	18.67	3.13	14.36	0.51
18:2	Cholesterol	8.49	0.69	8.74	0.69	9.51	0.62	8.43	0.72
	Without cholesterol	17.93	1.02	14.74	1.46	13.48	2.14	11.98	0.50
others	Cholesterol	7.81	1.12	8.31	1.16	8.86	1.01	8.11	1.22
	Without cholesterol	6.53	0.65	6.35	1.11	6.49	1.82	7.49	0.44
SFA[4]	Cholesterol	66.38	6.37	66.19	6.21	63.47	4.79	67.05	6.76
	Without cholesterol	50.55	3.36	56.30	6.51	55.06	10.22	59.24	2.32
UFA[4]	Cholesterol	25.81	2.23	25.50	2.14	27.67	1.90	24.84	2.24
	Without cholesterol	42.92	2.54	37.35	3.86	38.46	6.41	33.27	1.17
Total FA[4]	Cholesterol		9.72		9.51		7.70		10.22
	Without cholesterol		6.55		11.48		18.45		3.93

[1]Results are expressed as mean ± standard error of means; each data point is the average of two repeated measurements from two independently replicated experiments, n = 2; [2]% indicates percentage of total fatty acid content; [3]mg/g indicates milligrams of fatty acids per gram (dry weight) of cells; [4]Total saturated fatty acids; Total unsaturated fatty acids; Total saturated, unsaturated and other fatty acids.

Table 3.5. Fatty acid composition of *L. casei* strains grown in medium with or without cholesterol[1]

Fatty acid	Medium	ASCC 1520		ASCC 1521		ASCC 279		ASCC 290		ASCC 292		ATCC 15820		CSCC 2607	
		%[2]	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g
10:0	Cholesterol	10.08	2.37	9.08	2.08	8.01	1.14	5.87	0.79	8.83	1.32	11.05	2.22	9.99	2.34
	Without cholesterol	7.12	0.49	9.97	0.32	8.22	1.11	5.82	0.37	4.85	0.73	9.44	0.25	7.62	0.55
12:0	Cholesterol	11.60	2.40	10.88	2.20	8.51	1.07	9.12	1.08	10.18	1.34	11.16	1.98	11.39	2.35
	Without cholesterol	8.71	0.53	6.42	0.18	9.86	1.17	7.34	0.41	6.31	0.83	8.79	0.21	7.72	0.49
14:0	Cholesterol	12.74	2.41	12.13	2.24	10.49	1.20	9.43	1.02	10.87	1.31	12.15	1.97	12.37	2.33
	Without cholesterol	10.01	0.56	7.78	0.20	11.33	1.23	7.79	0.39	7.16	0.86	9.78	0.21	8.36	0.49
16:0	Cholesterol	18.79	3.34	18.52	3.22	14.86	1.60	21.04	2.14	22.65	2.56	18.27	2.78	18.47	3.27
	Without cholesterol	15.43	0.81	19.30	0.46	15.40	1.57	11.39	0.54	21.75	2.47	18.30	0.37	16.34	0.89
16:1	Cholesterol	8.27	1.52	7.89	1.42	9.62	1.07	7.98	0.84	6.77	0.79	7.78	1.22	8.00	1.47
	Without cholesterol	5.35	0.29	8.40	0.21	7.92	0.84	6.60	0.32	5.69	0.67	7.75	0.16	6.50	0.37
18:0	Cholesterol	14.15	2.40	13.91	2.31	16.94	1.74	16.08	1.56	15.16	1.64	13.78	2.00	13.97	2.36
	Without cholesterol	13.23	0.66	10.65	0.24	12.04	1.17	14.23	0.64	8.71	0.94	11.94	0.23	12.59	0.66
18:1	Cholesterol	6.75	1.15	10.95	1.83	10.89	1.13	11.48	1.12	10.22	1.11	8.60	1.26	8.75	1.49
	Without cholesterol	17.55	0.89	14.31	0.33	14.63	1.44	21.77	0.99	28.80	3.14	14.08	0.27	21.81	1.15
18:2	Cholesterol	9.03	1.46	8.53	1.35	10.58	1.04	10.52	0.98	7.86	0.81	9.19	1.28	8.71	1.41
	Without cholesterol	14.75	0.71	14.30	0.31	12.13	1.13	19.23	0.83	12.17	1.26	11.51	0.21	14.07	0.70
others	Cholesterol	8.59	2.46	8.10	2.26	10.10	1.75	8.82	1.44	7.47	1.36	8.02	1.96	8.34	2.38
	Without cholesterol	7.86	0.66	8.88	0.34	8.47	1.39	5.84	0.45	4.56	0.83	8.41	0.27	4.97	0.44
SFA[3]	Cholesterol	67.36	12.92	64.52	12.04	58.82	6.76	61.54	6.56	67.68	5.84	66.41	10.95	66.19	12.65
	Without cholesterol	54.50	3.06	54.12	1.40	56.85	6.26	46.57	2.35	48.78	8.17	58.25	1.26	52.64	3.08
UFA[3]	Cholesterol	24.05	4.14	27.38	4.60	31.09	3.24	29.97	2.93	24.85	5.07	25.58	3.76	25.47	4.36
	Without cholesterol	37.65	1.88	37.01	0.85	34.68	3.41	47.59	2.15	46.66	2.72	33.35	0.64	42.39	2.22
Total FA[3]	Cholesterol		19.52		18.90		11.75		10.93		12.27		16.67		19.39
	Without cholesterol		5.60		2.59		11.06		4.95		11.72		2.17		5.74

[1]Results are expressed as mean ± standard error of means; each data point is the average of two repeated measurements from two independently replicated experiments, n = 2; [2]% indicates percentage of total fatty acid content; mg/g indicates milligrams of fatty acids per gram (dry weight) of cells; [3]Total saturated fatty acids; Total unsaturated fatty acids; Total saturated, unsaturated and other fatty acids.

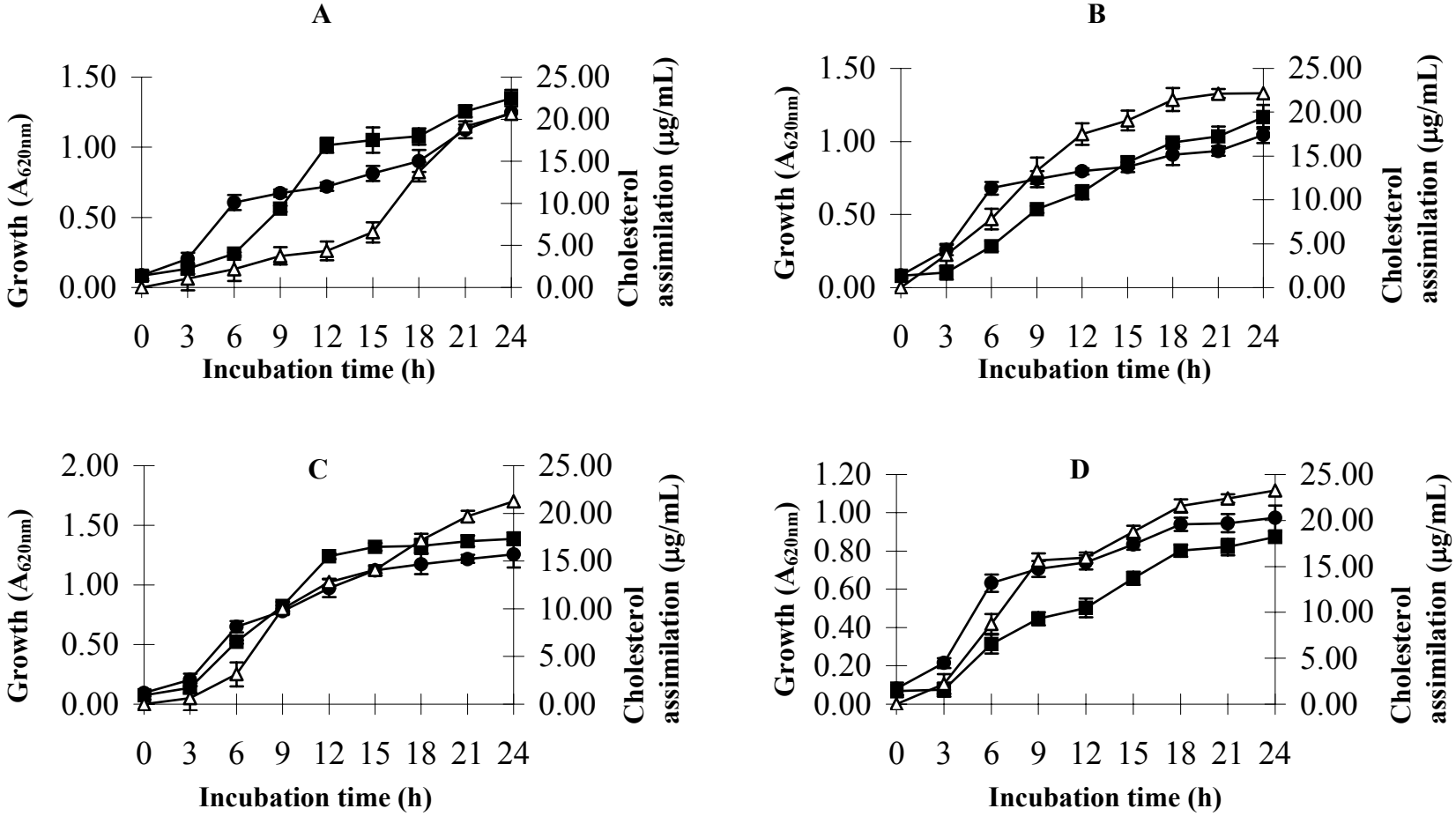


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Figure 3.1. Growth profiles in medium containing cholesterol (■), without cholesterol (●), and cholesterol assimilation patterns (△) for *L. acidophilus* ATCC 33200 (A), *L. acidophilus* ATCC 4356 (B), *L. acidophilus* ATCC 4357 (C) and *L. acidophilus* ATCC 4962 (D). Error bars represent standard error of means; n = 2.

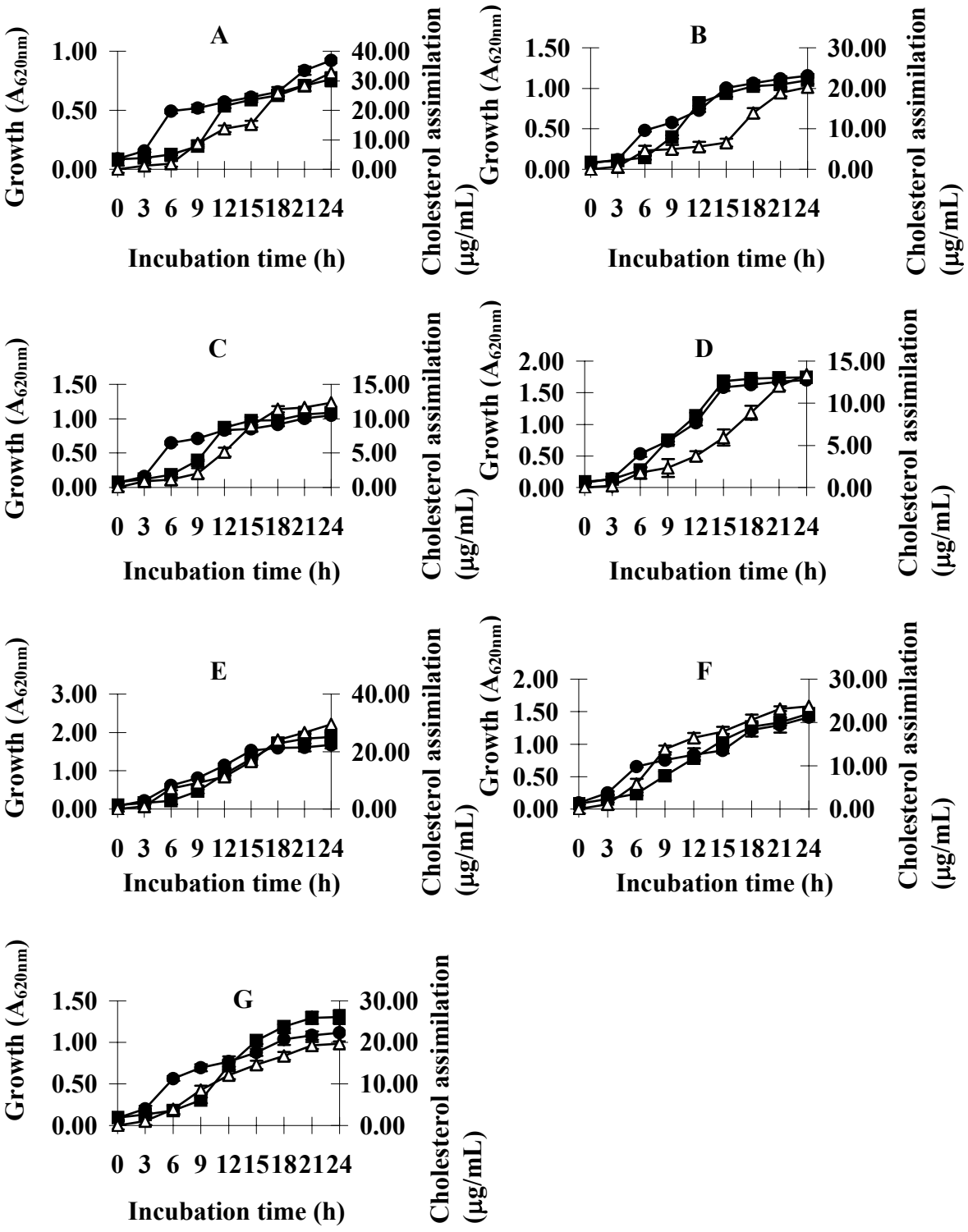


Figure 3.2. Growth profiles in medium containing cholesterol (■), without cholesterol (●), and cholesterol assimilation patterns (△) for *L. casei* ASCC 1520 (A), *L. casei* ASCC 1521 (B), *L. casei* ASCC 279 (C), *L. casei* ASCC 290 (D), *L. casei* ASCC 292 (E), *L. casei* ATCC 15820 (F) and *L. casei* CSCC 2607 (G). Error bars represent standard error of means; each data point is the average of three repeated measurements from two independently replicated experiments, n = 2.

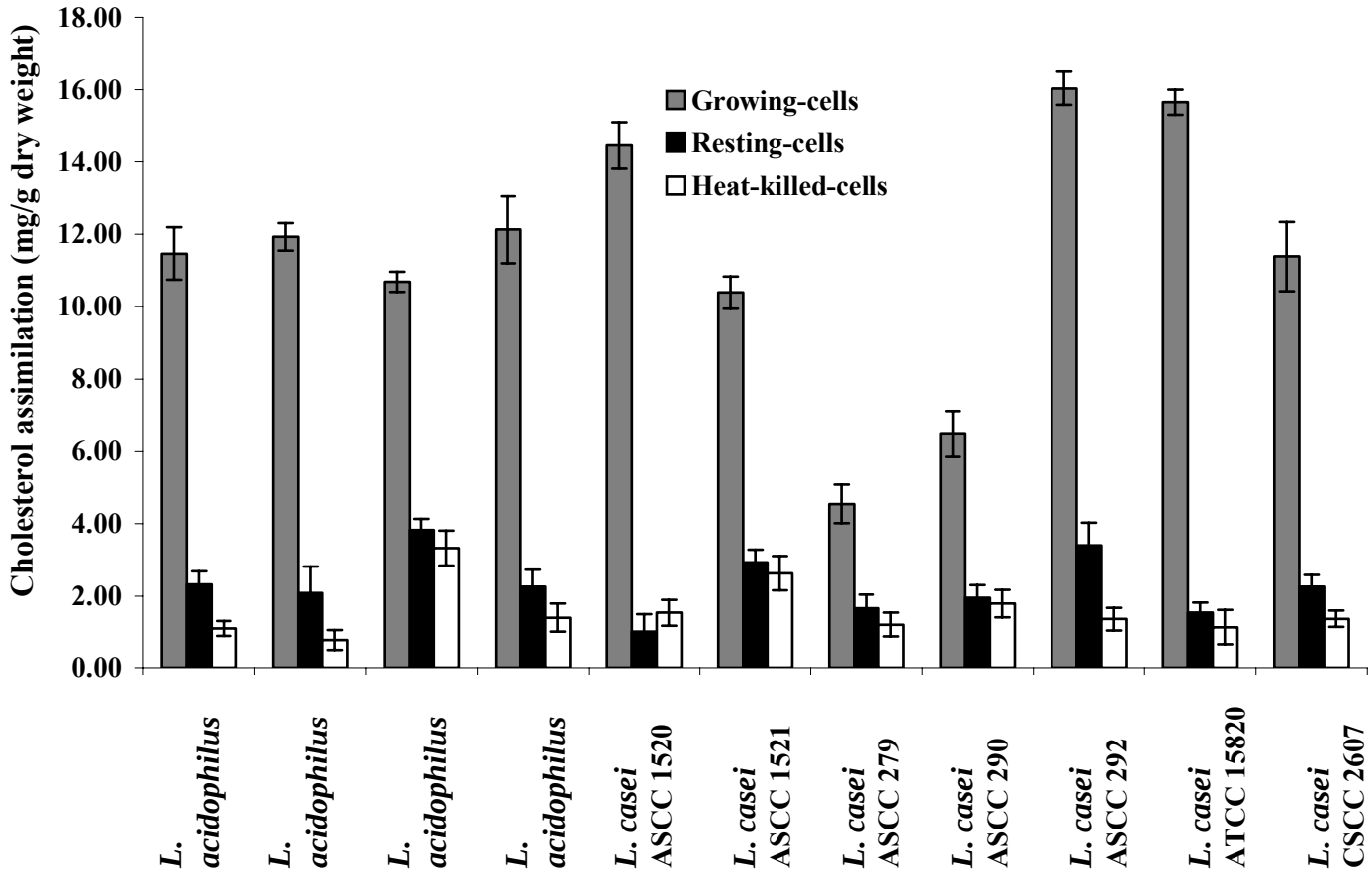


Figure 3.3. Cholesterol removal by growing-, heat-killed- and resting-cells of *L. acidophilus* ATCC 33200, *L. acidophilus* ATCC 4356, *L. acidophilus* ATCC 4357, *L. acidophilus* ATCC 4962, *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 279, *L. casei* ASCC 290, *L. casei* ASCC 292, *L. casei* ATCC 15820 and *L. casei* CSCC 2607. Cholesterol assimilation = $(C_1 - C_2)/(W_2-W_1)$, where C_1 and C_2 were the amount of cholesterol present in the fermentation broths at time = 0 and time = 20 h respectively, and W_1 and W_2 were the dry weight of the individual culture at time = 0 and time = 20 h respectively, for all treatment studied. Error bars represent standard error of means; n = 2.

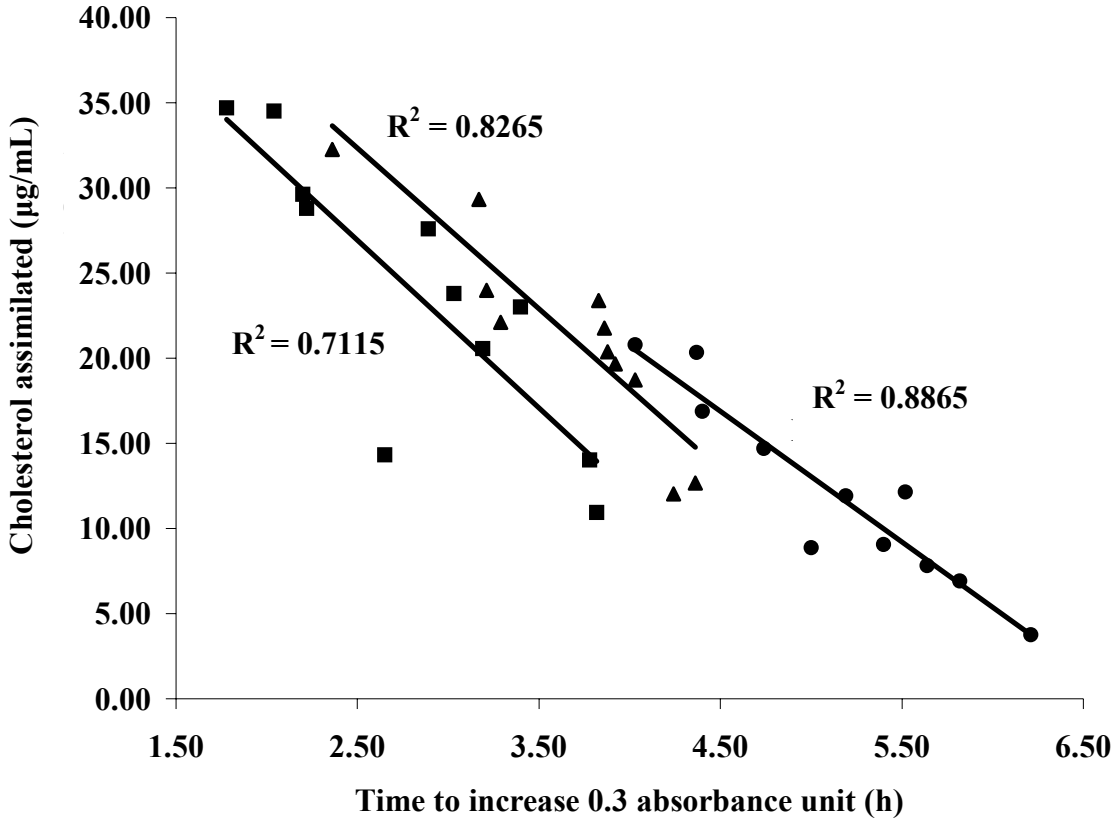


Figure 3.4. Regression analyses of cholesterol assimilation and bile tolerance in MRS media containing 0.30 % oxgall (▲), 0.3 % cholic acid (■) and 0.3% taurocholic acid (●).

3.2 Acid and Bile Tolerance and the Cholesterol Removal Ability of Bifidobacteria Strains

3.2.1 INTRODUCTION

Bifidobacteria are Gram-positive, nonmotile, non-sporeforming and anaerobic organisms that are mainly present in the colon of the intestinal tract, and constitute a major part of natural microflora of the human intestine. When present in sufficient numbers, bifidobacteria create a healthy equilibrium between beneficial and harmful microorganisms in the human gut (Shah and Lankaputhra, 2002). In order to exert such positive influence, bifidobacteria must be resistant to bile salts present in the lower intestine, gastric pH of 1.0 to 4.0, enzymes present in the intestine (lysozyme) and toxic metabolites produced during digestion (Hoier, 1992). It was previously reported that a rapid decline in numbers of bifidobacteria was observed in acidic environment such as yoghurt (Shah and Jelen, 1990). Growth of *Bifidobacterium* spp. was retarded below pH 5.0 and the organisms died in less than 3 h in pH below 2.5 (Shah and Lankaputhra, 2002). Gram-positive bacteria reportedly possess a myriad of acid resistance systems to survive in an acidic environment via use of proton pumps, changes in cell membrane, alteration of metabolism and production of alkali (Cotter and Hill, 2003). Previous studies showed that bile tolerance by bifidobacteria was dependent on strains and types of bile, with resistant levels ranging from bile concentrations of 0.125% to 2.0%. It was considered that the critical concentration of bile salts to screen for resistant strains for human use is 0.3% (Lian et al., 2003; Margolles et al., 2003).

Probiotics are defined as 'live microbial supplement that beneficially affects the host by improving its intestinal microbial balance' (Fuller 1992). Consumption of fermented milk made using bifidobacteria has increased due to their beneficial effects including lowering of serum cholesterol level (Hughes et al., 1991; Rasic et al., 1992). *Lactobacillus reuteri* that actively assimilated cholesterol in the laboratory medium was found capable to function *in-vivo* to exert hypercholesterolemic effect in mice (Taranto et al., 1998). Rats fed with non-fermented milk containing *Lactobacillus gasseri* reportedly showed reduced serum total cholesterol, low-density-lipoprotein cholesterol (LDL) and bile acids, and increased high-density lipoprotein (HDL) (Usman and Hosono, 2000). However, only a few reports showing such effects are found for bifidobacteria (Dambekodi and Gilliland, 1998; Tahri et al, 1995; Tahri et al., 1996).

This section has been published: *Liong, MT and Shah, NP. 2005. Acid and bile tolerance and the cholesterol removal ability of bifidobacteria strains.* ***Biosci Microflora*** *24: 1-10.*

From several *in-vitro* studies, a number of mechanisms have been proposed for the cholesterol lowering action of probiotic bacteria. *In-vitro* experiments also showed that cholesterol assimilation was influenced by the presence of bile salts. Strains that did not grow well in a medium containing bile salts assimilated a small amount of cholesterol as compared to those that grew well in the presence of bile salts. Uptake of cholesterol was particularly higher in a medium containing higher concentration of bile salts (Pereira and Gibson, 2002). When newborn dairy calves was fed with strains of *L. acidophilus* that had different bile tolerance, the calves supplemented with bile tolerant strains had more lactobacilli in their intestinal tract compared with those fed with strains which were less bile tolerant (Gilliland et al., 1984). It has been demonstrated that the ability of some strains to take up cholesterol was growth associated because resting cells did not exhibit interactions with cholesterol (Tahri et al., 1995).

Growth of selected lactobacilli strains in laboratory media containing cholesterol resulted in much of the cholesterol being removed along with the cells, hypothesising that cholesterol removal may be attributed to binding to the bacterial cell wall or incorporating into bacterial cellular membrane (Noh et al., 1997; Usman and Hosono, 2000). It has been postulated that cholesterol was not closely associated with the membrane, but merely attached to the cell surface (Dambekodi and Gilliland, 1998). The fact that cholesterol may be incorporated into cellular membrane was supported by changes in membrane properties of *L. acidophilus* that were grown in the presence of cholesterol. Cells that were grown in the presence of cholesterol micelles showed greater resistance to lysis by sonication than did cells grown in control broth (Noh et al., 1997). However, it was also reported that strains of *L. casei* grown in broth containing cholesterol revealed no measurable amount of cholesterol incorporation (Brashears et al., 1998).

The aims of this study were to investigate the acid and bile tolerance properties of several strains of bifidobacteria, and possible mechanisms of cholesterol removal by these organisms.

3.2.2 MATERIALS AND METHODS

3.2.2.1 *Bacteria*

Bifidobacterium longum BB536, *B. infantis* 1912 and *B. longum* 1941 were obtained from the Victoria University Culture Collection (Werribee, Australia), while *B. breve* ATCC 15698 and *B. infantis* ATCC 17930 were obtained from the Australia Starter Culture Research Centre (ASRC) (Werribee, Australia). *B. longum* BB536 (*B. longum* CSCC 5550)

was originally obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), while *B. infantis* 1912 (*B. infantis* CSCC 1912) and *B. longum* 1941 (*B. longum* CSCC 1941) were both originally obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO) (Highett, Victoria, Australia). Working cultures were prepared as described in section 3.1.2.1. de Mann, Rogosa, Sharpe (MRS) broth, supplemented with filter-sterilized L-cysteine HCl (LC) solution (5% w/v) was used for *Bifidobacterium.*

3.2.2.2 *Acid tolerance*

The survivability of *Bifidobacterium* under acidic conditions was determined as described in section 3.1.2.2.

3.2.2.3 *Bile tolerance*

The survivability of *Bifidobacterium* under different bile conditions was determined as described in section 3.1.2.3.

3.2.2.4 *Cholesterol removal*

Removal of cholesterol was determined spectrophotometrically as described in section 3.1.2.4.

3.2.2.5 *Cholesterol removal by growing-, dead- and resting cells*

The removal of cholesterol by growing-, dead- and resting cells were determined using cells grown in growth medium, cells that were heat killed and cells that were suspended in non-growing medium as described in section 3.1.2.5.

3.2.2.6 *Cellular fatty acid composition*

The fatty acid composition of cellular membrane was quantified using gas chromatography as described in section 3.1.2.6.

3.2.2.7 *Statistical analysis*

Data were analysed statistically as described in section 3.1.2.7.

3.2.3 RESULTS AND DISCUSSION

All tests were performed using MRS broth as the suspending medium although most likely these organisms in dairy products such as yoghurt, milk or cheese. This is to eliminate interferences that may occur with other compounds that are present in the media mentioned, and mainly emphasis on the sole effects of the treatment studied. The viability of bifidobacteria during 2 h of incubation at pH 2.0 is shown in Table 3.6. All strains showed varying levels of acid tolerance, with *B. longum* BB536 and *B. infantis* 17930 being the most acid tolerant strains, retaining 66.10% and 30.22% of initial count, respectively after the 2 h incubation period. No viable count of *B. breve* 15698 and *B. longum* 1941 was observed after the first hour of the incubation period, while no growth was observed for *B. infantis* 1912 after 2 h of incubation at pH 2.0. *B. breve* ATCC 15698 and *B. longum* 1941 showed greatest decrease in viability of 6.09 and 4.74 log cycles, respectively after 1 h incubation, compared to the other species of bifidobacteria studied.

Previous studies have shown that serum cholesterol level has been associated with risks of coronary heart disease. Small reduction in serum cholesterol by 1 per cent was found to reduce risk of coronary heart disease by 2 to 3 percent (Manson et al., 1992). When probiotic organisms are administered *in-vivo*, they are exposed to the acid and bile in the stomach and the intestinal tract (Kimoto et al., 2002). Cellular stresses begin in the stomach, with pH as low as 1.5, and in the upper intestine that contains bile (Corzo and Gilliland, 1999; Lankaputhra and Shah, 1995). Bile salts are synthesized from cholesterol in the liver, stored in the gall bladder, and released in the small intestine after ingestion of a fatty meal. Bile salts are critical to microorganisms since their cell membranes are composed of lipids and fatty acids (Erkkila and Petaja, 2000). There are several mechanisms for the removal of cholesterol from media by bifidobacteria, including assimilation by cells during growth (Tahri et al., 1996). In order to remove cholesterol, survivals at pH 3.0 for 2 h and in bile environment containing 1000 mg/L of bile acids are considered important for probiotic organisms (Usman and Hosono, 1999). Our results indicated that all strains showed varying degree of survival at pH 2.0 for 1 h. However, only *B. longum* BB536 and *B. infantis* 17930 could survive under such condition for 2 h. All organisms tolerated 0.30% oxgall, which was 3 times higher than the suggested level (Usman and Hosono, 1999) for probiotic organisms. Our results suggested that all species of bifidobacteria could survive bile concentrations normally encountered in the human gastrointestinal tract, with *B. infantis* 17930 and *B. breve* 15698 surviving the best.

The effects of different bile on the growth of bifidobacteria are presented in Table 3.7. Taurocholic acid was used as the conjugated bile, cholic acid as the deconjugated bile,

while oxgall contained both conjugated and deconjugated bile salts. Growth of bifidobacteria in MRS-LC media without bile source was used as a control, to study the effects of different bile forms. Due to the addition of bile acids to the media, the effect of pH was monitored. Generally, media containing cholic acid had lower pH values compared to the other media. However, the reduction in pH values due to the production of acid by bifidobacteria, were more likely to be strain dependent. pH of the media decreased highest from strains *B. breve* ATCC 15698 and *B. infantis* ATCC 17930 incubated in MRS media without bile. The average reduction was highest from MRS media without bile supplementation (2.38), despite the fact that media with cholic acid had the lowest initial pH values, while the lowest pH reduction was from media supplemented with taurocholic acid (0.57). These indicated that the initial pH of media with different bile sources had minimal influence towards subsequent growth and bile tolerance of the cultures, and pH-related inhibitory actions of bile salts. All strains showed greatest tolerance to cholic acid, while the bacterial growth was slowest in the presence of taurocholic acid. However, no significant difference was observed between bacterial growth in the medium containing taurocholic acid and the control, indicating that the inhibitory effect may be minimal. *B. infantis* 17930 grew best in the medium without bile and subsequently showed highest tolerance to both cholic acid and oxgall, while *B. infantis* 1912 showed slowest growth in the absence of bile and was greatly inhibited by cholic acid and taurocholic acid.

Based on our findings of bile tolerance in the presence of different bile, taurocholic acid appeared to possess inhibitory effect towards the growth of all species compared to cholic acid, although the inhibition was minimal. Although the reason is unclear, it appears that bifidobacteria had better tolerance to cholic acid as compared to taurocholic acid. It was previously reported that conjugated bile salts have greater solubility and detergent activity, and may, therefore be more toxic than its deconjugated counterpart. This was supported by the fact that the cholic acid added to the fermentation broths was far less soluble than taurocholic acid based on the solubility index. Bifidobacteria were found to hydrolyse bile salts with bile salt hydrolase, which decreases the solubility of bile salts, and thus weakening their detergent effect in order to protect against the toxicity of bile acids (Grill et al., 2000).

Cholesterol assimilation by bifidobacteria during 20 h of growth is shown in Table 3.8. Cholesterol removal varied significantly among species ($P < 0.05$) and ranged from 4.17 to 27.14 μg/mL. All species showed higher cholesterol assimilation in the medium containing cholic acid compared to that containing taurocholic acid. Cholesterol assimilation was higher in the presence of cholic acid compared to the control for all species except *B. longum* BB536, while cholesterol assimilation was lowest in the presence of taurocholic acid

for all species. *B. breve* 15698 and *B. infantis* ATCC 17930 assimilated highest level of cholesterol in the presence of oxgall compared to the other bile sources, while *B. longum* BB536 showed higher cholesterol assimilation in the absence of bile.

Cholesterol assimilation patterns under different bile sources indicated that assimilation might be influenced by bile tolerance for all bifidobacteria studied. Overall greater tolerance in the media containing cholic acid compared to taurocholic acid also yielded higher cholesterol assimilation in the presence of deconjugated bile than conjugated bile. Higher cholesterol assimilation was observed in the presence of bile for all bifidobacteria, with the exception of *B. longum* BB536 that assimilated higher amount of cholesterol in the medium without bile. Previous *in-vitro* experiments showed that strains that did not grow well in the medium containing bile salts were unable to assimilate cholesterol (Pereira and Gibson, 2002). This was also supported by various studies that showed strains of bacteria have greater tendency to remove cholesterol from medium in the presence of bile salts (Corzo and Gilliland, 1999; Tahri et al., 1996).

Growth of all bifidobacteria studied in the presence and absence of cholesterol with oxgall as the bile source, and the cholesterol assimilation patterns are illustrated in Figure 3.5. Oxgall was chosen instead of cholic acid because it represents closely the actual human gut model system, and all strains showed good growth and cholesterol assimilation in its presence. All species showed better growth in the medium containing cholesterol compared to that without cholesterol, except *B. infantis* 1912 that grew better in the absence of cholesterol. In the medium containing no cholesterol, *B. infantis* 1912 and *B. longum* 1941 showed higher growth increment for the first 12 h of incubation, followed by slower growth thereafter. In medium containing cholesterol, most species showed higher initial growth for the first 15 h, followed by slower growth until the end of the 24 h incubation period. Cholesterol assimilation patterns showed that cholesterol was increasingly assimilated throughout the entire 24 h incubation period and was growth associated.

Our findings indicated that cholesterol removal and growth are closely related. Cholesterol assimilation in the presence of different bile source showed good relationship (R^2 = 0.77 to 0.94) with bile tolerance of the strains studied (Figure 3.6). Strains showing greater tolerance towards deconjugated bile exhibited overall higher cholesterol assimilation in the presence of cholic acid, while those showing greater inhibition by taurocholic acid produced lower cholesterol assimilation. Also, most strains exhibited better growth in the presence of cholesterol, indicating that cholesterol stimulated their growth. Better growth was obtained when cells were grown in the presence of cholesterol than without cholesterol.

Furthermore, regression analyses suggested that cholesterol removal was closely associated with the bacterial growth.

We determined the degree of cholesterol removal by resting- and dead- cells. The amount of cholesterol removed by growing-, resting- and dead-cells are illustrated in Figure 3.7. Heat-killed- and resting-cells showed a small amount of cholesterol removal, ranging from 1.11 to 3.35 mg/g dry weight, compared to growing cells which ranged from 4.66 to 10.80 mg/g dry weight. However, most species appear to remove more cholesterol when cells were resting compared to those heat-killed, except *B. infantis* 1912 that removed more cholesterol when cells were dead. With the exception of *B. infantis* 1912, all other species of bifidobacteria that showed higher cholesterol removal in growing cells, also removed relatively more cholesterol in resting and dead forms. *B. infantis* 17930 removed highest cholesterol when cells were growing, resting or dead, while *B. longum* 1941 removed lowest cholesterol regardless of whether cells were growing, resting or dead.

Cholesterol removal was higher when cells were grown in the growth medium than those that were heat-killed or suspended in phosphate buffer. However, both dead- and resting-cells removed a small amount of cholesterol. The capability of strains to remove cholesterol even in dead and resting stage indicated that cholesterol might also be removed via binding to cells. The pH of the intestinal tract of humans is usually neutral to alkaline (Kimoto et al., 2002). Our results showed that cholesterol was bound to heat-killed cells suspended in phosphate buffer at pH 6.8, thus suggesting that cholesterol in the intestine could be removed independently of whether cells are live. Higher cholesterol removal by growing cells indicated that the degree of bound cholesterol might be dependent on growth stage of cells. We postulate that cholesterol binding to growing cells could be stronger compared to dead- and resting-cells. It was reported that growing bacterial cells could retain a significant amount of cholesterol and postulated that cholesterol removal *in-vitro* might be dependent on cell growth (Tahri et al., 1995). More than 40 percent of cholesterol was extracted from cells of *Bifidobacterium breve* ATCC 15700 and the bound cholesterol could not be detached even after several washings, indicating that the binding of cholesterol to cell surface was strong.

The quantification of cellular fatty acid composition for *Bifidobacterium* spp. grown in the presence and absence of cholesterol is shown in Table 3.9. There was a difference in fatty acid distribution pattern for cells grown with or without cholesterol. Cells grown in the media without cholesterol showed high levels of hexadecanoic, oleic and linoleic acids, but lower levels of shorter chain fatty acids. In addition, *B. breve* 15698 showed high level of

octadecanoic acid as well. The total amount of saturated fatty acids was higher than the total unsaturated fatty acids. In the presence of cholesterol, most species contained more hexadecanoic and octadecanoic acids compared to organisms grown in the medium without cholesterol. *B. longum* BB536 contained high content of oleic acid when cells were grown in the medium containing cholesterol, while *B. infantis* 1912, 17930 and *B. breve* 15698 contained high myristic acid. All organisms also exhibited higher level of total saturated fatty acids compared to total unsaturated fatty acids. However, organisms grown in the presence of cholesterol had more total saturated fatty acids and lower unsaturated fatty acids levels. All organisms contained more total fatty acids when they were grown in the media containing cholesterol compared to those without cholesterol.

Changes in cellular lipid profiles were quantified using a gas chromatograph and cellular fatty acids were methylated with methanolic HCl. Methanolic HCl does not methylate free fatty acids, thus methylated fatty acids were supposedly components of the membrane phospholipids. The lipids of Gram-positive bacteria are found mainly in the membrane and the fatty acids in bacteria are primarily the precursors of cellular phospholipids (Magnuson et al., 1993). In this study, alterations of fatty acid profiles were found especially with tetradecanoic, hexadecanoic, octadecanoic, total saturated and unsaturated acids. Using lactococci, it was postulated that the changes in fatty acid profiles of cells grown in the presence of cholesterol were a result of cholesterol incorporation into the cellular membrane (Kimoto et al., 2002). Such alteration in the cellular envelop due to cholesterol incorporation has been reported previously, with an estimated of 10 to 15% of the assimilated cholesterol was recovered in the cellular membrane fraction (Noh et al., 1997). Cells grown in the presence of cholesterol were found to be more resistant to lysis by sonication compared to cells grown in absence of cholesterol (Dambekodi and Gilliland, 1998). Cholesterol incorporated into the cell membranes of mycoplasmas reportedly protected the cells from lysis by increasing the tensile strength of the membranes (Razin et al., 1980). Our results showed that total fatty acids obtained from cells grown in the presence of cholesterol were higher than in the absence of cholesterol. We postulate that the increased total fatty acids content was attributed to cholesterol incorporation into the membrane and not by cellular synthesis, because Gram-positive bacteria living under high lipid concentrations were previously reported to lose the ability to synthesize lipids or fatty acids (Kiatpapan et al., 2001). Furthermore, changes in bacterial cellular lipid composition was mainly related to environmental stresses such as salt, acid, oxidative or thermal fluctuations (Guerzoni et al., 2001), or attributed by changes in composition of fermentation medium such as the addition of Tween 80, which reportedly increased oleic acid concentration in membranes of lactic acid bacteria (Johnsson et al., 1995). All strains were grown in similar

incubation conditions, with the only difference being the presence or absence of cholesterol in the medium.

This study showed that *B. longum* BB536 and *B. infantis* 17930 were more acid tolerant than *B. breve* 15698, *B. longum* 1941 and *B. infantis* 1912. All *Bifidobacterium* spp. were able to tolerate bile concentration, generally encountered in the gastrointestinal tract. All strains had varying capabilities to remove cholesterol *in-vitro*. Our results provide experimental support for three possible mechanisms for the removal of cholesterol from media by *Bifidobacterium* spp.: assimilation during growth, incorporation into the cellular membrane of cells, and binding to the cell surface. Among the 5 species of bifidobacteria studied, *B. infantis* 17930 showed highest cholesterol removal from the media, good tolerance to acid and bile concentration, hence it may be a promising candidate to lower serum cholesterol *in-vivo*.

Table 3.6. Effect of pH 2.0 on viability of bifidobacteria[1]

Strains	Viable cell count (log CFU/mL)				
	0 min	30 min	60 min	90 min	120 min
B. longum BB536	9.94 ± 0.78a	8.56 ± 0.56a	7.23 ± 0.59a	7.05 ± 0.38a	6.57 ± 0.21a
B. breve ATCC 15698	10.59 ± 0.60a	6.67±0.63b	4.50 ± 0.07b	ND[2]	ND
B. infantis 1912	9.67 ± 0.23a	7.45 ± 0.35a	6.51±0.36ab	4.66 ± 0.36b	ND
B. infantis ATCC 17930	10.49 ± 0.23a	7.23 ± 0.08a	6.95 ± 0.19a	5.64±0.15b	3.17±0.03b
B. longum 1941	10.36 ± 0.24a	7.32 ± 0.23a	5.62 ± 0.60ab	ND	ND

[1]Results are expressed as mean ± standard error of means; n = 2.

[2]ND, no CFU detected with zero dilution.

[ab]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

Table 3.7. Bile tolerance of bifidobacteria in different bile media

Strains	Growth media[1]											
	MRS broth			MRS broth + 0.3% oxgall			MRS broth + 0.3% cholic acid			MRS broth + 0.3% taurocholic acid		
	h[2]	pH		h	pH		h	pH		h	pH	
		T1	T2		T1	T2		T1	T2		T1	T2
B. longum BB536	5.80 ± 0.30 bc, B	6.09	3.65	4.61 ± 0.40 b, AB	6.13	4.21	3.70 ± 0.16 b, A	5.84	4.70	6.67 ± 0.11 b, C	6.05	5.30
B. breve ATCC 15698	4.80 ± 0.21 b, B	6.10	3.63	3.89 ± 0.40 a, AB	6.11	4.31	3.27 ± 0.20 ab, A	5.78	5.71	4.39 ± 0.19 a, AB	6.09	5.79
B. infantis 1912	5.96 ± 0.12 c, B	6.08	3.65	4.31 ± 0.17 ab, A	6.11	4.29	3.74 ± 0.25 b, A	5.88	4.33	6.75 ± 0.14 b, B	6.05	5.29
B. infantis ATCC 17930	3.66 ± 0.18 a, AB	6.06	3.60	3.30 ± 0.30 a, A	6.12	4.24	2.66 ± 0.15 a, A	5.89	5.72	4.87 ± 0.34 a, B	6.08	5.81
B. longum 1941	5.71 ± 0.10 bc, C	6.09	3.97	4.86 ± 0.11 b, B	6.11	4.78	3.66 ± 0.13 b, A	5.87	4.68	6.21 ± 0.18 b, C	6.05	5.30

[1]Results are expressed as mean ± standard error of means; n = 2.

[2]Time (h) required to increase 0.3 absorbance units at 620 nm in each medium.

[abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

[ABC]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 3.8. Cholesterol assimilation by bifidobacteria in different bile media[1]

Strains	Cholesterol assimilated (μg/mL)			
	MRS broth	MRS broth + 0.3% oxgall	MRS broth + 0.3% cholic acid	MRS broth + 0.3% taurocholic acid
B. longum BB536	15.05 ± 0.38 b, A	14.80 ± 0.29 b, A	11.56 ± 0.25 c, B	4.54 ± 0.20 cd, C
B. breve ATCC 15698	15.83 ± 0.20 b, B	19.75 ± 0.35 a, A	15.99 ± 0.42 b, B	12.87 ± 0.17 b, C
B. infantis 1912	9.95 ± 0.33 d, C	16.47 ± 0.26 b, A	13.39 ± 0.18 bc, B	4.17 ± 0.29 d, D
B. infantis ATCC 17930	19.53 ± 0.66 a, B	21.16 ± 0.53 a, B	27.14 ± 0.39 a, A	18.65 ± 0.20 a, B
B. longum 1941	12.55 ± 0.31 c, B	11.93 ± 0.34 c, B	17.88 ± 0.45 b, A	5.42 ± 0.17 c, C

[1]Results are expressed as mean ± standard error of means; n = 2.

[abcd]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

[ABCD]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 3.9. Fatty acid composition of bifidobacteria grown in medium with or without cholesterol[1]

Fatty acid	Medium	*B. longum* BB536		*B. breve* ATCC 15698		*B. infantis* 1912		*B. infantis* ATCC 17930		*B. longum* 1941	
		%[2]	mg/g[2]	%	mg/g	%	mg/g	%	mg/g	%	mg/g
10:0	Cholesterol	7.73	1.32	9.13	1.11	9.34	1.38	9.62	1.63	9.84	3.10
	Without cholesterol	6.57	0.97	9.77	0.88	7.49	0.85	6.62	0.32	6.17	0.46
12:0	Cholesterol	8.13	1.23	10.45	1.12	10.66	1.37	11.05	1.65	9.85	2.73
	Without cholesterol	7.61	0.98	10.26	0.82	8.85	0.89	9.95	0.43	9.48	0.62
14:0	Cholesterol	9.02	1.24	11.54	1.13	12.00	1.42	12.27	1.67	8.65	2.19
	Without cholesterol	8.20	0.97	11.21	0.81	9.37	0.86	8.65	0.34	10.78	0.64
16:0	Cholesterol	20.23	2.62	18.61	1.71	18.40	2.05	18.36	2.35	17.24	4.11
	Without cholesterol	18.15	2.02	12.42	0.85	18.28	1.57	18.02	0.67	16.01	0.89
16:1	Cholesterol	6.82	0.91	7.37	0.70	7.91	0.91	8.01	1.06	7.96	1.96
	Without cholesterol	6.01	0.69	7.79	0.55	7.71	0.68	7.46	0.28	9.60	0.55
18:0	Cholesterol	16.33	2.02	14.19	1.24	13.91	1.48	14.14	1.73	16.56	3.76
	Without cholesterol	11.86	1.26	16.56	1.08	11.36	0.93	12.25	0.43	11.81	0.63
18:1	Cholesterol	19.15	2.39	10.70	0.95	10.90	1.17	9.41	1.16	10.75	2.46
	Without cholesterol	20.91	2.24	12.47	0.82	16.09	1.33	16.27	0.58	16.74	0.90
18:2	Cholesterol	6.49	0.77	10.06	0.84	8.65	0.88	8.79	1.03	10.37	2.25
	Without cholesterol	14.70	1.49	10.95	0.68	11.93	0.94	12.30	0.41	14.66	0.75
others	Cholesterol	6.10	1.27	7.96	1.18	8.24	1.48	8.35	1.72	8.78	3.36
	Without cholesterol	5.98	1.07	8.57	0.94	8.93	1.24	8.48	0.50	4.76	0.43
SFA[3]	Cholesterol	61.43	8.44	63.91	6.31	64.31	7.71	65.44	9.02	62.14	15.88
	Without cholesterol	52.40	6.20	60.22	4.44	55.34	5.10	55.49	2.19	54.25	3.23
UFA[3]	Cholesterol	32.46	4.07	28.13	2.49	27.45	2.96	26.21	3.24	29.08	6.67
	Without cholesterol	41.62	4.42	31.21	2.04	35.73	2.95	36.04	1.28	40.99	2.20
Total FA[3]	Cholesterol		13.78		9.98		12.15		13.98		25.91
	Without cholesterol		11.69		7.42		9.29		3.97		5.86

[1]Results are expressed as mean ± standard error of means; n = 2; [2]% indicates percentage of total fatty acid content; mg/g indicates milligrams of fatty acids per gram (dry weight) of cells; [3]Total saturated fatty acids, Total unsaturated fatty acids, Total saturated, unsaturated and other fatty acids.

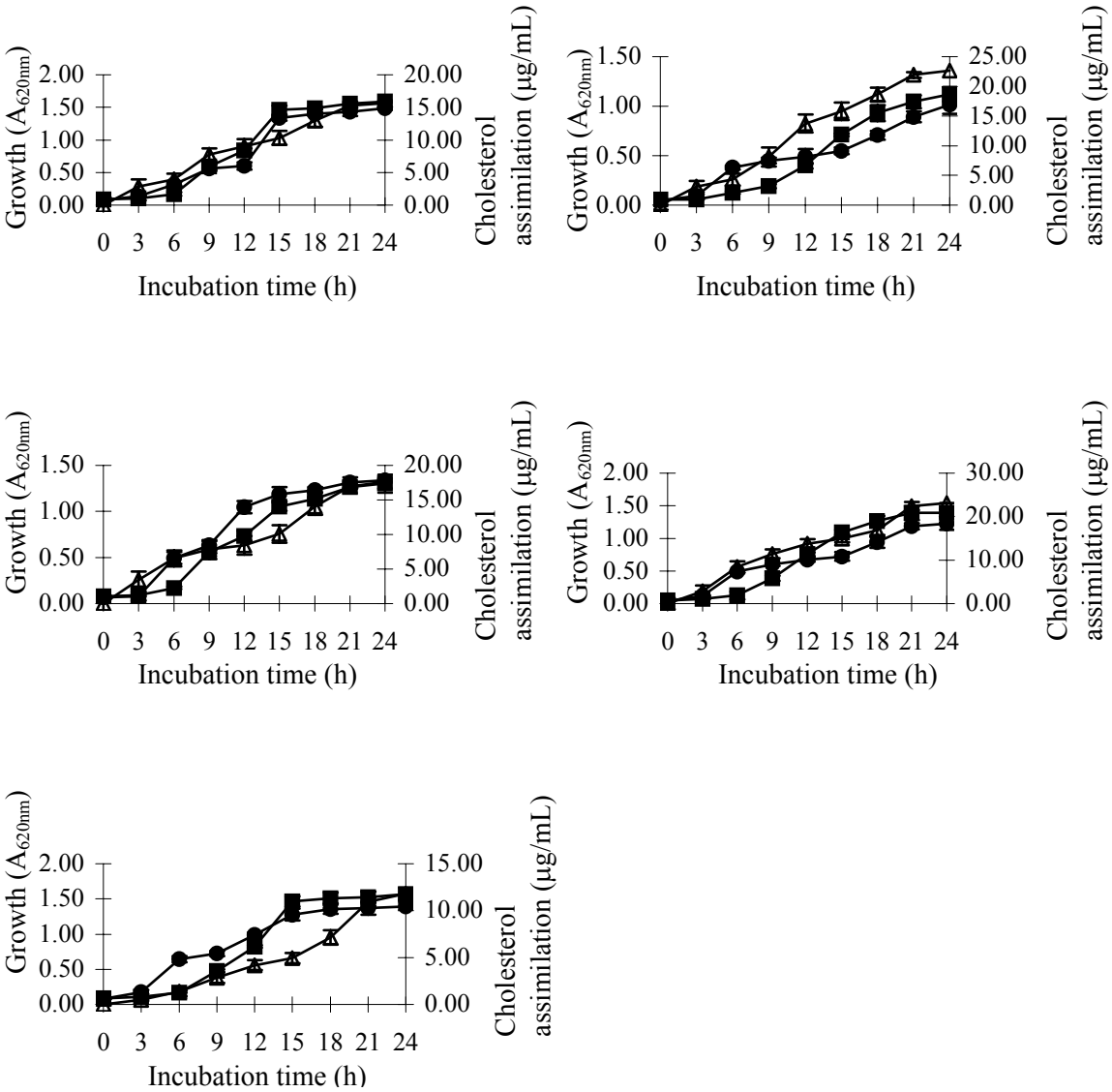


Fig. 3.5. Growth profiles in medium containing cholesterol (■), without cholesterol (●), and cholesterol assimilation patterns (△) for *B. longum* BB536 (a), *B. breve* ATCC 15698 (b), *B. infantis* 1912 (c), *B. infantis* ATCC 17930 (d) and *B. longum* 1941 (e). Results are expressed as mean ± standard error of means; n = 2.

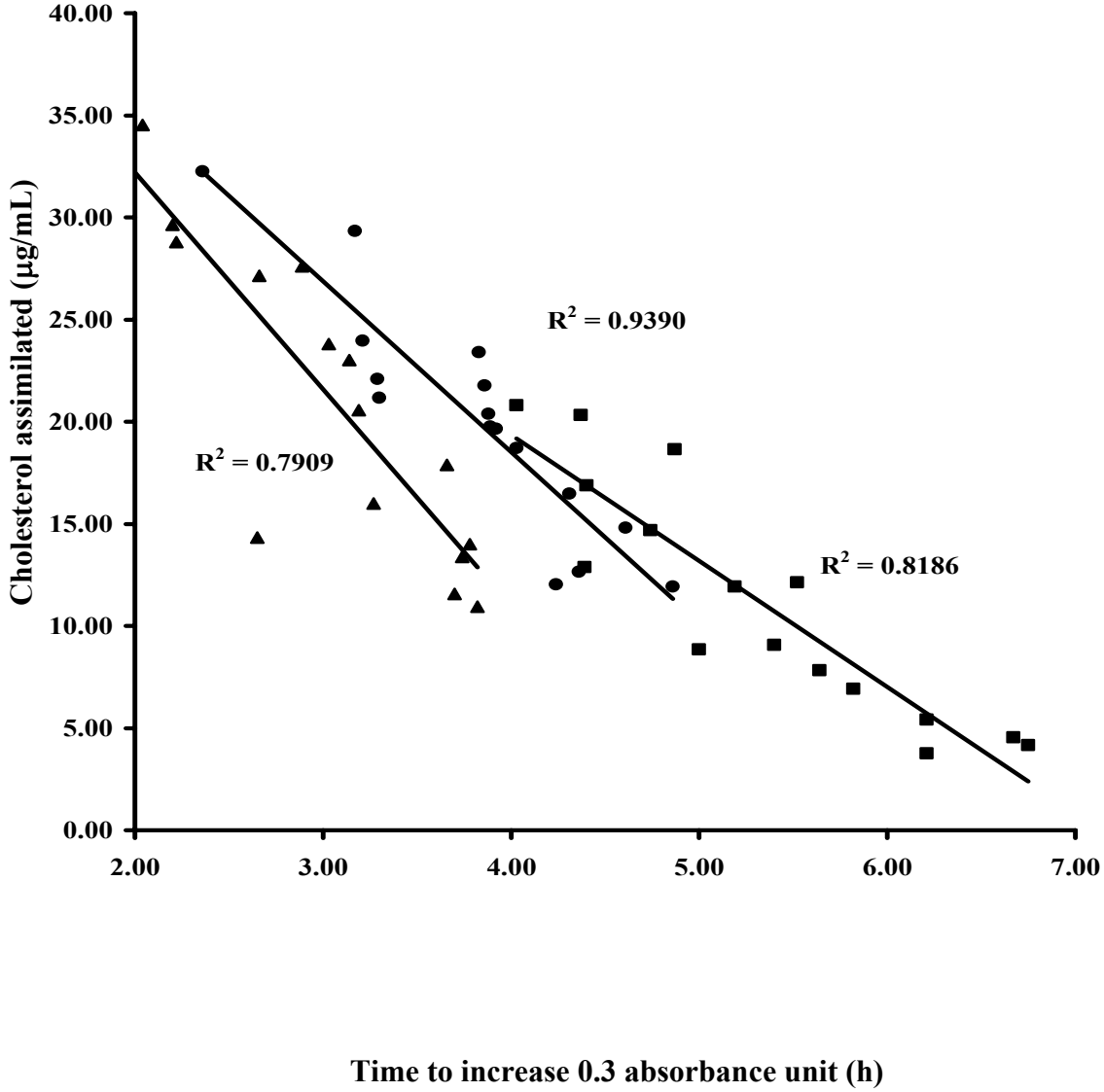


Fig. 3.6. Regression analyses of cholesterol assimilation and bile tolerance in MRS media containing 0.30 % oxgall (●), 0.3 % cholic acid (▲)and 0.3 % taurocholic acid (■).

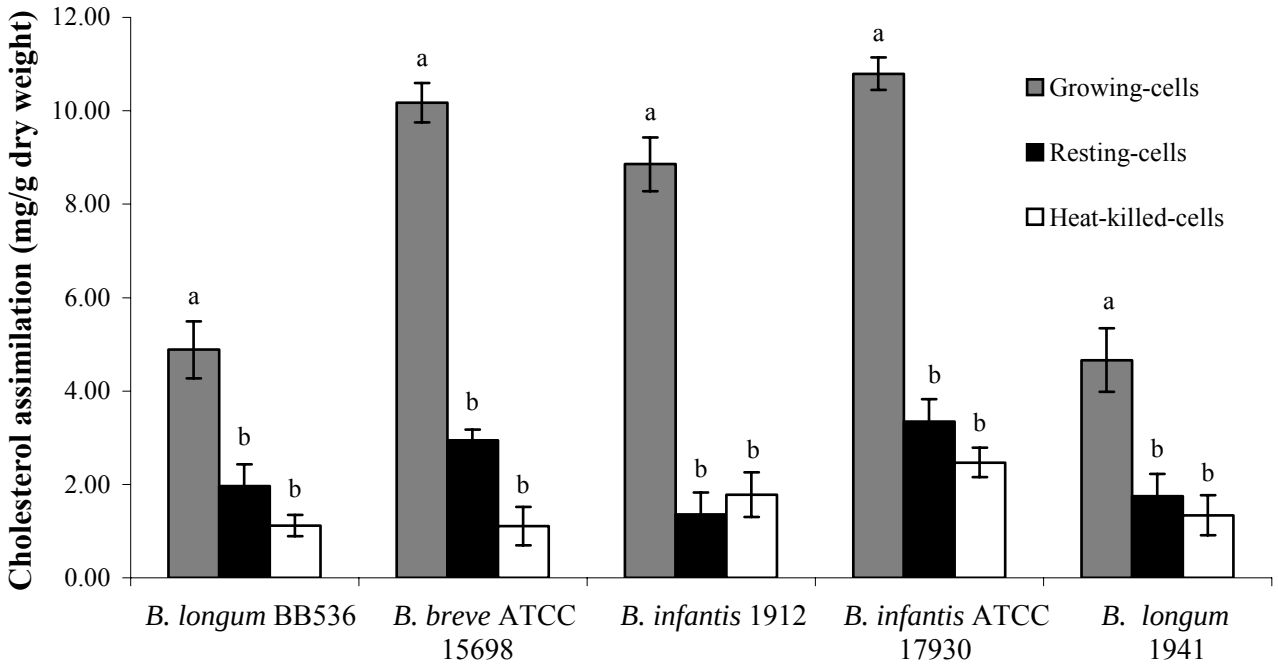


Fig. 3.7. Cholesterol removal by growing-, heat-killed- and resting-cells of *Bifidobacterium*. Cholesterol assimilation = $(C_1 – C_2)/(W_2-W_1)$, while for heat-killed- and resting-cells, cholesterol assimilation = $(C_1 – C_2)/(W_2)$ where C_1 and C_2 were the amount of cholesterol present in the fermentation broths at time = 0 and time = 20 h respectively, and W_1 and W_2 were the dry weight of the individual culture at time = 0 and time = 20 h respectively. Results are expressed as mean ± standard error of means; n = 2. [a,b]Significantly different ($P < 0.05$).

4.0 Bile salt deconjugation, bile salt hydrolase and cholesterol co-precipitation by *Lactobacillus* and *Bifidobacterium*

4.1 Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains

4.1.1 Introduction

Probiotics are defined as 'live microbial supplement that beneficially affects the host by improving its intestinal microbial balance' (Fuller, 1992), or in a more general content as 'living micro-organisms, which upon ingestion in certain numbers, exert health affects beyond inherent basic nutrition' (Guarner & Schaafsma, 1998). Milk fermented with lactobacilli was first demonstrated to exhibit hypocholesterolemic effects in humans as early as 1963 (Shaper, Jones & Kyobe, 1963; Mann, 1974). Various studies have shown that some lactobacilli could lower total plasma cholesterol and low-density-lipoprotein (LDL) cholesterol (Anderson & Gilliland, 1999; Sanders, 2000). In recent years, interest has risen in the possibility of using bile salt deconjugation by lactic acid bacteria to lower serum cholesterol level in hypercholesterolemic patients and prevent hypercholesterolemia in normal people (De Smet, Van Hoorde, Woestyne, Christiaens, & Verstraete, 1995). Probiotics such as *Lactobacillus acidophilus* were found to excrete bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), the enzyme that catalyses the hydrolysis of glycine- and taurine-conjugated bile salts into amino acid residues and free bile salts (bile acids). BSH was found to be present in several bacterial species of the gastrointestinal tract, such as *Lactobacillus* sp., *Bifidobacterium longum*, *Clostridium perfringens*, and *Bacteroides fragilis* ssp. *fragilis* (Corzo & Gilliland, 1999). Ahn, Kim, Lim, Baek, & Kim (2003) found precipitated halo and opaque granular white material on agar plugs around *L. acidophilus* colonies, which were confirmed to be cholate, chenodeoxycholate and deoxycholate, produced by the deconjugation of taurocholic acid, taurochenodeoxycholic acid and taurodeoxycholic acid.

The major route of cholesterol excretion from humans and other mammals is through faeces. Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo & Gilliland, 1999). Conjugated bile salts are secreted into the small intestine for absorption of dietary fat, hydrophobic vitamins and other fat soluble compounds. A small fraction of bile salts that are not absorbed is lost as free bile salts in faeces. Free bile salts were less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen (Center, 1993).

This section has been published: *Liong, MT and Shah, NP. 2005. Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains.* ***Int Dairy J*** *15: 391-398.*

At the physiological pH of the intestinal lumen, deconjugated bile salts can be transported through the epithelium (Wong, Oelkers, Craddock, & Dawson, 1994) and into the blood stream of the host, or precipitated. Thus, in a steady state situation, deconjugation of bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation (Reynier et al., 1981). Experiments with germ free rats have shown that bile salt deconjugation by *B. longum* increases bile salt excretion (Chikai, Nakao, & Uchida, 1987).

Up to now, the largest study conducted on the distribution and extent of BSH activity in lactic acid bacteria, involving more than 300 lactic acid bacteria strains from genera *Bifidobacterium* and *Lactobacillus*, and species *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus thermophilus* was reported by Tanaka, Doesburg, Iwasaki & Mierau (1999). *Lactobacillus* sp. from the stationary phase of static cultures reportedly had higher BSH activities occurring at low pH. It was hypothesized that high BSH deconjugation activity associated with the stationary phase of culture was a result of reduced pH levels in the medium (Corzo & Gilliland, 1999). Klaver & Van der Meer (1993) found that the removal of cholesterol from the medium was contributed from the precipitation of cholesterol with the free bile salts as pH decreased. Moreover, the disappearance of glycine-conjugated bile salt from the growth media was reportedly due to precipitation caused by acidic conditions (Zhu & Brown, 1990). However, a study by Noh, Kim, & Gilliland (1997) revealed that cholesterol was removed *in-vitro* by *L. acidophilus* ATCC 43121 and L1 when the pH was maintained at 6.0. With large amounts of cholesterol being removed by similar strains of *L. acidophilus* at pH 6.0, Brashears, Gilliland, & Buck (1998) hypothesized that cholesterol removal was not solely contributed to bile salt deconjugation and co-precipitation. The regulation of BSH activity by pH is still not clear although BSH activities were shown to be higher at lower pH values. Furthermore, different strains of the same bacterial species exhibited different BSH activity under similar pH levels (Lunden & Savage, 1990; Corzo & Gilliland, 1999).

The aims of this study were to examine bile salt deconjugation ability, BSH activity and cholesterol removal ability from co-precipitation with deconjugated bile by lactobacilli strains in order to select strains for cholesterol lowering properties.

4.1.2 Materials and Methods

4.1.2.1 Bacteria

Strains of *Lactobacillus acidophilus* and *L. casei* were in this study. Working cultures were prepared as described in Chapter 3.0, section 3.1.2.1.

4.1.2.2 Deconjugation of sodium glycocholate and sodium taurocholate

Ten-millilitre volumes of MRS broth were supplemented with 6 mM sodium glycocholate, 6 mM sodium taurocholate or a combination of sodium glycocholate and sodium taurocholate at 2.8 mM and 1.2 mM, respectively. Individual bile salts were added as 6 mM each, because it resembles the concentrations prevailing in the human small intestine (Brashears et al., 1998; De Boever and Verstraete, 1999), while bile mixtures contained 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate, because it resembles the molar ratio of the two salts in human bile (Sandine, 1979). Each strain was inoculated at 1% level and incubated anaerobically at 37 °C for 20 h. Bile salt deconjugation ability was based on release of deconjugated bile and the modified method of Irwin, Johnson & Kopalo (1944) was used to measure the amount of free cholic acid released by each organism. Briefly, 10 mL culture of each organism after the incubation period was adjusted to pH 7.0 with NaOH (1 N). Cells were centrifuged at 10000 x g (Microspin 24, Sorvall Instruments, Melbourne, Australia) at 4 °C for 10 min. Supernatant obtained was adjusted to pH 1.0 with HCl (10 N). One millilitre of the supernatant was added with 2 mL of ethyl acetate and the mixture was vortexed for 1 min. Two millilitre of the ethyl acetate layer was transferred into a glass tube and evaporated under nitrogen at 60 °C. The residue was immediately dissolved in 1 mL of NaOH (0.01 N). After complete mixing, 1 mL of furfuraldehyde (1%) and 1 mL of H_2SO_4 (16 N) were added, and the mixture was vortexed for 1 min before heating at 65 °C in a water bath for 10 min. After cooling, 2 mL of glacial acetic acid was added and the mixture was vortexed for 1 min. Absorbance was read at 660 nm (Pharmacia Novaspec II, Cambridge, England). The amount of cholic acid released was determined using cholic acid standard (Sigma Chemical Co., St. Louis, MO, USA). All experiments were replicated twice.

4.1.2.3 BSH assay and protein assay

BSH activity was measured by determining the amount of amino acids liberated from conjugated bile salts by lactobacilli strains as described by Tanaka, Hashiba, Kok & Mierau (2000), with several modifications. Briefly, cells grown in MRS broth for 20 h were centrifuged at 10000 x g at 4 °C for 10 min. The cell pellet was washed twice before

suspension into 10 mL of 0.1 M phosphate buffer (pH 7.0). The cell concentration was adjusted to OD value of 1 unit at 600 nm. Five millilitre of the cell suspension was sonicated for 3 min with constant cooling in ice, followed by centrifugation at 10000 x g at 4 °C for 10 min. To 0.1 mL of appropriately diluted supernatant obtained, 1.8 mL of 0.1 M sodium phosphate buffer (pH 6) and 0.1 mL of conjugated bile salt were added. Conjugated bile used were 6 mM sodium glycocholate, 6 mM sodium taurocholate or 6 mM conjugated bile salt mixture (glycocholic acid, glycochenodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid) (Sigma). The mixture was incubated at 37 °C for 30 min. Enzymatic reaction was terminated by adding 0.5 mL of trichloroacetic acid (15% wt vol^{-1}) to 0.5 mL of sample. The mixture was centrifuged and 0.2 mL of supernatant obtained was added to 1 mL of distilled water and 1 mL of ninhydrin reagent (0.5 mL of 1% ninhydrin in 0.5 M citrate buffer pH 5.5, 1.2 mL of 30% glycerol, 0.2 mL of 0.5 M citrate buffer pH 5.5). The preparation was vortexed and boiled for 14 min. After subsequent cooling, the absorbance at 570 nm was determined using glycine or taurine as standards. One unit of BSH activity was defined as the amount of enzyme that liberated 1 μmol of amino acid from substrate per min.

Protein concentrations were determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), with bovine serum albumin (Sigma) as the standard. All experiments were repeated twice.

4.1.2.4 Co-precipitation of cholesterol with deconjugated bile

Freshly prepared sterile MRS broth was supplemented with 6 mM sodium glycocholate, 6 mM sodium taurocholate, or 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate. Water-soluble cholesterol (polyoxyethanyl-cholesteryl sebacate) (Sigma) was used as previously described (Pereira & Gibson, 2002). It was filter sterilized and added to the broth at a final concentration of 70-100 μg mL^{-1}. The broth was inoculated at 1% level with each strain and incubated anaerobically at 37 °C for 20 h. The water soluble cholesterol had a solubility of 60 mg mL^{-1} and contained 30% cholesterol. Thus, initial cholesterol concentration varied between 70-100 μg mL^{-1} and was quantified separately for every batch. After the incubation period, cells were centrifuged (10000 x g, 4 °C, 10 min) and the remaining cholesterol concentration in the broth was determined using a modified colourimetric method as described by Rudel & Morris (1973). One millilitre of the aliquot was added with 1 mL of KOH (33% wt vol^{-1}) and 2 mL of absolute ethanol, vortexed for 1 min, followed by heating at 37 °C for 15 min. After cooling, 2 mL of distilled water and 3

mL of hexane were added and vortexed for 1 min. One millilitre of the hexane layer was transferred into a glass tube and evaporated under nitrogen. The residue was immediately dissolved in 2 mL of o-phthalaldehyde reagent. After complete mixing, 0.5 mL concentrated sulphuric acid was added and the mixture was vortexed for 1 min. Absorbance was read at 550 nm (Pharmacia Novaspec II, Cambridge, England) after 10 min. Co-precipitation of cholesterol with cholic acid formed was determined by the difference between cholesterol level in the control (inoculated MRS broth without bile) after the incubation period, and the final cholesterol level in the inoculated MRS broth with bile. All experiments were replicated twice.

4.1.2.5 Statistical analysis

Data analysis was carried out with SPSS Inc. software (version 10.0). One-way analysis of variance was used to study significant difference between means, with significance level at $\alpha = 0.05$. Tukey's-test was used to perform multiple comparisons between means. All data presented are mean values of two determinations and three replicates, unless stated otherwise.

4.1.3 Results

4.1.3.1 Bile salt deconjugation by lactobacilli

Bile salt deconjugation activity by strains of lactobacilli is shown in Table 4.1. Bile salt deconjugation was determined by the amount of cholic acid released, which ranged from 1.14 to 4.77 mM. All strains were able to deconjugate both sodium glycocholate and sodium taurocholate at varying degrees. In general, *L. acidophilus* showed better deconjugation ability as compared to *L. casei* in broth containing sodium glycocholate and sodium taurocholate. In broth containing sodium glycocholate, overall deconjugation was observed to be higher by strains *L. acidophilus* ATCC 33200, 4357, 4962 and *L. casei* ASCC 1521, which liberated more than 4.17 mM of cholic acid, while deconjugation was lowest by strains *L. acidophilus* ATCC 4356 and *L. casei* ASCC 1520 which released only 1.31 mM and 1.56 mM, respectively. All strains showed lower deconjugation of sodium taurocholate compared to sodium glycocholate. *L. acidophilus* also showed better deconjugation ability towards sodium taurocholate as compared to *L. casei*. Strains *L. acidophilus* ATCC 33200, 4357, 4962 and *L. casei* ASCC 1521 deconjugated highest level of sodium taurocholate with more than 3.27 mM cholic acid released. Strains *L. acidophilus* ATCC 4356, *L. casei* ASCC 1520 and 290 were found to be least capable of deconjugating sodium taurocholate. At concentrations that resemble the molar ratio of sodium glycocholate and sodium taurocholate

in human bile, *L. acidophilus* in general also showed higher cholic acid liberation, ranging from 1.88 to 2.96 mM as compared to that by *L. casei*, which ranged from 1.14 to 2.69 mM. *L. acidophilus* ATCC 33200, 4357, 4962 and *L. casei* ASCC 1521 showed highest level of deconjugation of both sodium glycocholate and sodium taurocholate at concentrations of 2.8 mM and 1.2 mM respectively, while *L. casei* ASCC 1520, 290 and CSCC 2607 showed lowest deconjugation ability towards both sodium glycocholate and sodium taurocholate at such concentrations.

4.1.3.2 BSH activity of lactobacilli

BSH activity obtained from cell extracts of lactobacilli strains is shown in Table 4.2. All strains showed varying degree of BSH activity towards both sodium glycocholate and sodium taurocholate. In general, *L. acidophilus* had higher total BSH activity compared to *L. casei* for both glycine- and taurine-conjugated bile. Most strains showed substrate preference towards sodium glycocholate compared to sodium taurocholate, with the exception of *L. acidophilus* ATCC 4356 that showed higher total BSH activity towards sodium taurocholate. Using bile salt mixture that contained glycocholic, glycochenodeoxycholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acid, all strains exhibited highest total BSH activity compared to individual conjugated bile, ranging from 1.60 to 1.99 U mL^{-1}. Similar to individual conjugated bile, *L. acidophilus* showed higher total BSH activity towards conjugated bile mixture compared to *L. casei*. *L. acidophilus* ATCC 33200, 4357 and *L. casei* 1521 showed highest total BSH activity towards individual glycine- and taurine-conjugated bile, and conjugated bile mixture. *L. acidophilus* ATCC 4356 exhibited lowest total BSH activity towards both sodium glycocholate and sodium taurocholate, while *L. casei* ASCC 1520 and 290 showed minimal total BSH activity towards taurine-conjugated bile and bile salt mixture. Specific activity of BSH did not correlate well with total BSH activity by most strains due to varying protein content in cell extracts. *L. acidophilus* ATCC 4962 and *L. casei* ATCC 15820 had high total BSH activity towards sodium glycocholate and exhibited high specific activity as well, while strain of *L. acidophilus* ATCC 4356 that had lowest total activity also showed lowest specific activity towards the glycine-conjugated substrate. However, *L. casei* ASCC 1520 showed low total BSH activity but high specific activity towards sodium taurocholate. It was found that most strains exhibited higher BSH specific activity towards mixture of conjugated bile as compared to individual conjugated bile.

4.1.3.3 Co-precipitation of cholesterol with deconjugated bile by lactobacilli

Co-precipitation of cholesterol with cholic acid as liberated from the deconjugation of sodium glycocholate and sodium taurocholate by lactobacilli is shown in Table 4.3. Cholesterol was co-precipitated with deconjugation of both sodium glycocholate and sodium taurocholate at varying levels. Precipitation of cholesterol upon the deconjugation of sodium glycocholate ranged from 0.60 to 4.70 μg mL^{-1} and was higher compared to sodium taurocholate, which ranged from 0.13 to 2.92 μg mL^{-1}. Deconjugation of sodium glycocholate by *L. acidophilus* showed higher amount of cholesterol co-precipitation compared to *L. casei.* Highest amount of cholesterol precipitated from the deconjugation of sodium glycocholate was by strains *L. acidophilus* ATCC 33200, 4357, 4962, *L. casei* ASCC 1521 and 15820, with more than 3.71 μg mL^{-1} of precipitation. Cholesterol was precipitated in lowest amount when sodium glycocholate was deconjugated by *L. casei* ASCC 1520 and 279, with only 0.60 μg mL^{-1} and 0.73 μg mL^{-1} of precipitation, respectively. Similar to sodium glycocholate, cholesterol precipitation with the deconjugated sodium taurocholate by *L. acidophilus* strains was higher compared to *L. casei* strains. Highest cholesterol precipitation was obtained upon deconjugation by strains *L. acidophilus* ATCC 33200, 4357 and *L. casei* ASCC 1521, while the lowest amount of cholesterol precipitation was obtained from deconjugation activity by strains *L. acidophilus* ATCC 4356, *L. casei* ASCC 1520, 279, 290 and CSCC 2607. At concentration that resembles the human bile, co-precipitation of cholesterol was lower compared to individual deconjugation of sodium glycocholate but higher than individual deconjugation of sodium taurocholate. At such concentration, more than 3.25 μg mL^{-1} of cholesterol was precipitated from deconjugation activity by strains *L. acidophilus* ATCC 33200, 4357, 4962 and *L. casei* ASCC 1521, while minimal amount of cholesterol was precipitated from the deconjugation activity with strains *L. casei* ASCC 1520 and 279.

4.1.4 Discussion

All strains of lactobacilli used were human derived and their capabilities to deconjugate bile and BSH activities were unknown, although they have been studied for other health beneficial effects. Our previous studies (Chapter 3.0, section 3.1.3.3) indicated that these strains were able to remove cholesterol *in-vitro* via several mechanisms, and we would like to examine the bile salt deconjugation properties and BSH activities of these strains, before further usage in *in-vivo* studies. Furthermore, very little attempt was made to investigate the specificity of deconjugation abilities of probiotic strains on taurine- and glycine-conjugated bile forms. Free bile acids formed by the deconjugation of conjugated

bile salts are less soluble and are less likely to be reabsorbed by the intestinal lumen compared to their conjugated counterpart, and are lost from the human body through faeces (Center, 1993). This could lead to higher metabolism of cholesterol and subsequently, the reduction of serum cholesterol (Reynier et al., 1981). Klaver & Van der Meer (1993) theorized that while bile salts were deconjugated and pH of the fermentation media dropped due to natural acid production by culture, cholesterol micelles destabilized and cholesterol co-precipitated with free bile acids. In this study, all lactobacilli strains studied could deconjugate both glycine- and taurine-conjugated bile salts into cholic acid. However, more glycine-conjugated bile salt was found to be efficiently deconjugated by both strains of *L. acidophilus* and *L. casei* than taurine-conjugated bile salt. There was a good correlation between bile salt deconjugation and BSH activity. Strains *L. acidophilus* ATCC 33200, 4357, 4962 and *L. casei* ASCC 1521 had highest BSH activity that led to highest deconjugation of sodium glycocholate and sodium taurocholate. Substrate preference towards sodium glycocholate by the enzyme resulted in higher liberation of cholic acid from sodium glycocholate than sodium taurocholate. This was supported by previous experiments resembling human intestinal pH of 6.5 and glycocholate to taurocholate ratio of 2:3, which found glycine conjugated bile salt to be more efficiently deconjugated by strains of *L. acidophilus* from both human and porcine origins than taurine conjugated bile salt (Corzo & Gilliland, 1999). Characterizing cholylglycine hydrolase from a bile-adapted strain of *Xanthomonas maltophilia*, Dean et al. (2002) found that the enzyme hydrolysed cholylglycine following the Michaelis-Menten kinetics and there was competitive inhibition by cholytaurine, as if both conjugated bile salts were hydrolysed at a single site. Using *Lactobacillus buchneri* JCM 1069 and *Lactobacillus kefir* BCCM 9480, it was found that BSH expressed substrate specificity based on the structure of the steroid moiety of the bile salt conjugate (De Smet et al., 1995; Moser & Savage, 2001). Since sodium glycocholate predominates the human intestine, Brashears et al. (1998) postulated that strains that prefer to deconjugate sodium glycocholate may have more potential to lower serum cholesterol concentrations if the deconjugation mechanism is important in decreasing serum cholesterol.

Molarities ratio of glycocholate per taurocholate in the gall bladder of the human adult was estimated to be 2.2: 3.0, and about 10 to 15 mmol of total conjugated bile salts were secreted into the gastrointestinal tract for each of six daily cycles (Hofmann, 1977). Since that the volume of the gall bladder is smaller than that of the gastrointestinal tract, the conjugated bile salts become diluted when entering into the upper part of the small intestine. It was estimated that the highest bile salt molarity throughout the small intestine is between 2.4 and 4.0 mM (Hofmann, 1977). A higher concentration was expected in the duodenum where the bile is secreted, and a lower concentration at the end of the ileum due to diffusion

mechanisms, microbial transformation and absorption throughout the intestinal wall (Corzo & Gilliland, 1999). Thus, with high deconjugation activity by *L. acidophilus* ATCC 33200, 4357, 4962 and *L. casei* ASCC 1521 toward sodium glycocholate and sodium taurocholate at concentrations that resemble the human bile, we postulate that these strains may exert good *in-vivo* deconjugation effects as observed from this *in-vitro* experiment.

Results on the BSH activity showed that all strains except *L. acidophilus* ATCC 4357 and *L. casei* ASCC 1520 exhibited higher BSH specific activity towards mixture of glycine- and taurine-conjugated bile, than individual conjugated bile. Despite that, the liberation of cholic acid from individual conjugated bile was higher than the mixture of conjugated bile. Although the reason may not be clear at the moment, De Boever, Wouters, Verschaeve, Berckmans, Schoeters & Verstraete (2000) previously reported that the supplementation of oxgall burdened the fermentation of gut microbiota in a concentration-dependent manner. A decrease in enzyme activity was noticed with increasing concentrations of oxgall. Thus, we postulate that the higher individual concentration of conjugated bile (6 mM) as compared to total concentration of bile mixture (4 mM), may have contributed to a lower BSH specific activity by most strains. Furthermore, De Boever & Verstaete (1999) found that 1 mM of cholic acid caused growth inhibition towards *L. plantarum* 80, while a higher concentration of 5 mM caused bacteriotoxicity. Thus, when more cholic acid was liberated from individual conjugated bile as compared to the liberation from the mixture of conjugated bile, higher burdening effects from the formation of end product via bile salt hydrolysis may have occurred. This may lead to a lower BSH specific activity from most strains studied to counteract the high concentrations of cholic acid. More studies are needed to investigate the correlation between BSH activity and end-product toxicity.

Our previous study (Chapter 3.0, section 3.1.3.3) indicated that the removal of cholesterol from fermentation media was contributed by cholesterol assimilation, binding to cell surface and incorporation into cellular membrane. Thus, co-precipitation of cholesterol with deconjugated bile was measured by the difference between the final cholesterol concentration in MRS broth supplemented with bile source and in MRS broth without bile source, as the comparison with difference of the final cholesterol concentration in uninoculated control would produce misleading interpretations. It was postulated that the co-precipitation of cholesterol with deconjugated bile was contributed to the pH of media. The optimum pH for BSH of *L. acidophilus* O16 was between 5.5 and 6.5 (Corzo & Gilliland, 1999) and 6.0 for *L. acidophilus* NCFM (Gilliland & Speck, 1977). At the normal pH of the upper intestinal tract (5.5-6.50), about 50 percent of free bile salts and a small amount of

glycine-conjugated bile salts were found to be protonated (non-ionized), while no protonation occurs in taurine-conjugated bile salt (Carey & Cahalane, 1988). Thus, at acidic pH, unconjugated bile salts are protonated and precipitated, while taurine-conjugated bile salts remain ionized in solution, and glycine-conjugated bile salts are partially precipitated without hydrolysis (Dashkevicz & Feighner, 1989). Our results showed that co-precipitation of cholesterol was less than 5% of total cholesterol used, and it occurred at pH ranging from 3.82 to 4.90. Our preliminary studies (data not shown) indicated that co-precipitation of cholesterol with deconjugated bile was minimal even at pH below 2.0, indicating that it would not be a major factor in controlling serum cholesterol because pH of intestine is unlikely to be lower than 6.0 (Brashears et al., 1998).

All lactobacilli strains studied were able to deconjugate both sodium glycocholate and sodium taurocholate. Substrate preference for BSH was more towards sodium glycocholate than sodium taurocholate, while *L. acidophilus* had better deconjugation ability and BSH activity than *L. casei*. *L. acidophilus* ATCC 33200, 4356, 4962 and *L. casei* ASCC 1521 showed highest bile salt deconjugation and BSH activity compared to other strains studied. These strains also showed highest deconjugation capability and BSH activity in experiments using concentrations of sodium glycocholate and sodium taurocholate that resemble the human bile, and pH levels that are similar to the pH of human intestine. More work is needed to evaluate their deconjugation activity in the *in-vivo* system.

Table 4.1

Deconjugation of sodium glycocholate and sodium taurocholate by *Lactobacillus*

Strains	Cholic acid released (mM)		
	Sodium glycocholate	Sodium taurocholate	Sodium glycocholate + sodium taurocholate
L. acidophilus ATCC 33200	4.40 ± 0.35[a, A]	3.41 ± 0.28[a, A]	2.96 ± 0.34[a, A]
L. acidophilus ATCC 4356	1.31 ± 0.19[c, A]	1.20 ± 0.27[b, A]	1.88 ± 0.25[bc, A]
L. acidophilus ATCC 4357	4.17 ± 0.11[a, A]	3.27 ± 0.38[a, AB]	2.56 ± 0.15 [ab, B]
L. acidophilus ATCC 4962	4.34 ± 0.24[a, A]	3.27 ± 0.30[a, A]	2.76 ± 0.26[ab, A]
L. casei ASCC 1520	1.56 ± 0.17[c, A]	1.24 ± 0.19[b, A]	1.14 ± 0.05 [c, A]
L. casei ASCC 1521	4.77 ± 0.51[a, A]	3.23 ± 0.55[a, AB]	2.69 ± 0.32[ab, A]
L. casei ASCC 279	1.78 ± 0.43[c, A]	1.43 ± 0.17[b, A]	1.90 ± 0.16[bc, A]
L. casei ASCC 290	1.90 ± 0.53[c, A]	1.20 ± 0.14 [b, A]	1.66 ± 0.15[bc, A]
L. casei ASCC 292	2.35 ± 0.45[bc, A]	1.36 ± 0.12[b, A]	2.05 ± 0.34[ab, A]
L. casei ATCC 15820	3.21 ± 0.22[a, A]	2.44 ± 0.15[ab, A]	2.07 ± 0.35[ab, A]
L. casei CSCC 2607	1.79 ± 0.12[c, A]	1.34 ± 0.22[b, A]	1.32 ± 0.41[bc, A]

Values are means of triplicates from two separate runs, n = 2. Deconjugation of glycine or taurine conjugated bile based on release of cholic acid. MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate; 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate.

[abc]Means within a column with different lowercase letter are significantly different ($P < 0.05$);

[AB]Means within a row with different uppercase letters are significantly different ($P < 0.05$).

Table 4.2

BSH activity of *Lactobacillus* on sodium glycocholate and sodium taurocholate

Strains	BSH activity								
	Sodium glycocholate			Sodium taurococholate			Conjugated bile mixture		
	Total protein (mg mL^{-1})	Total activity (U mL^{-1})	Specific activity (U mg^{-1})	Total protein (mg mL^{-1})	Total activity (U mL^{-1})	Specific activity (U mg^{-1})	Total protein (mg mL^{-1})	Total activity (U mL^{-1})	Specific activity (U mg^{-1})
L. acidophilus ATCC 33200	1.74 ± 0.27	1.81 ± 0.31	1.04 ± 0.42	1.74 ± 0.34	1.40 ± 0.21	0.81 ± 0.11	1.66 ± 0.76	1.98 ± 0.37	1.20 ± 0.67
L. acidophilus ATCC 4356	0.82 ± 0.22	0.45 ± 0.22	0.55 ± 0.13	1.29 ± 0.37	1.16 ± 0.28	0.90 ± 0.21	1.58 ± 0.25	1.84 ± 0.14	1.17 ± 0.19
L. acidophilus ATCC 4357	1.60 ± 0.46	1.73 ± 0.18	1.09 ± 0.44	2.29 ± 0.48	1.47 ± 0.23	0.64 ± 0.24	1.83 ± 0.52	1.91 ± 0.16	1.05 ± 0.40
L. acidophilus ATCC 4962	1.54 ± 0.28	1.74 ± 0.19	1.13 ± 0.16	1.32 ± 0.16	1.37 ± 0.34	1.04 ± 0.27	1.59 ± 0.27	1.99 ± 0.17	1.26 ± 0.21
L. casei ASCC 1520	1.66 ± 0.10	1.56 ± 0.14	0.94 ± 0.28	2.21 ± 0.43	1.17 ± 0.12	1.93 ± 0.23	1.48 ± 0.40	1.71 ± 0.16	1.15 ± 0.27
L. casei ASCC 1521	2.15 ± 0.13	1.71 ± 0.21	0.80 ± 0.14	1.38 ± 0.23	1.43 ± 0.14	1.03 ± 0.19	1.85 ± 0.28	1.96 ± 0.23	1.06 ± 0.18
L. casei ASCC 279	1.54 ± 0.14	1.66 ± 0.24	1.08 ± 0.17	1.26 ± 0.19	1.28 ± 0.34	1.02 ± 0.34	1.40 ± 0.25	1.84 ± 0.21	1.31 ± 0.28
L. casei ASCC 290	2.08 ± 0.56	1.60 ± 0.17	0.77 ± 0.27	1.42 ± 0.40	1.06 ± 0.23	0.75 ± 0.22	1.36 ± 0.34	1.60 ± 0.50	1.17 ± 0.40
L. casei ASCC 292	1.74 ± 0.14	1.69 ± 0.12	0.97 ± 0.26	1.38 ± 0.26	1.32 ± 0.13	0.96 ± 0.20	1.57 ± 0.15	1.91 ± 0.22	1.22 ± 0.23
L. casei ATCC 15820	1.53 ± 0.26	1.75 ± 0.32	1.15 ± 0.35	2.07 ± 0.29	1.36 ± 0.28	0.66 ± 0.13	1.29 ± 0.14	1.86 ± 0.31	1.45 ± 0.32
L. casei CSCC 2607	1.98 ± 0.66	1.60 ± 0.19	0.81 ± 0.39	1.28 ± 0.29	1.34 ± 0.31	1.04 ± 0.31	0.87 ± 0.22	1.68 ± 0.32	1.93 ± 0.32

Values are means of triplicates from two separate runs, n = 2. BSH activity from cell free extracts of lactobacilli strains grown on MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate; 6 mM conjugated bile mixture.

Table 4.3

Cholesterol precipitation with deconjugation of sodium glycocholate and sodium taurocholate by *Lactobacillus*

Strains	Sodium glycocholate		Sodium taurococholate		Sodium glycocholate + sodium taurocholate	
	Final pH	Cholesterol precipitated ($\mu g\ mL^{-1}$)	Final pH	Cholesterol precipitated ($\mu g\ mL^{-1}$)	Final pH	Cholesterol precipitated ($\mu g\ mL^{-1}$)
L. acidophilus ATCC 33200	4.53	$4.64 \pm 0.25^{a, A}$	4.48	$2.25 \pm 0.34^{ab, B}$	4.27	$3.25 \pm 0.41^{ab, AB}$
L. acidophilus ATCC 4356	4.75	$2.98 \pm 0.10^{ab, A}$	4.28	$0.67 \pm 0.14^{ab, B}$	4.52	$2.17 \pm 0.11^{bc, A}$
L. acidophilus ATCC 4357	3.93	$4.70 \pm 0.68^{a, A}$	4.23	$2.81 \pm 0.22^{a, C}$	4.88	$3.54 \pm 0.14^{ab, B}$
L. acidophilus ATCC 4962	4.02	$4.05 \pm 0.76^{a, A}$	4.49	$1.63 \pm 0.42^{ab, A}$	4.79	$3.81 \pm 0.39^{a, A}$
L. casei ASCC 1520	4.27	$0.60 \pm 0.15^{b, A}$	4.21	$0.74 \pm 0.15^{ab, A}$	4.14	$0.71 \pm 0.60^{c, A}$
L. casei ASCC 1521	4.20	$3.71 \pm 0.80^{a, A}$	3.82	$2.92 \pm 0.94^{a, A}$	4.06	$3.31 \pm 0.17^{a, A}$
L. casei ASCC 279	4.48	$0.73 \pm 0.10^{b, A}$	4.65	$0.41 \pm 0.12^{b, A}$	4.40	$0.79 \pm 0.13^{c, A}$
L. casei ASCC 290	4.89	$1.25 \pm 0.15^{b, A}$	4.19	$0.13 \pm 0.09^{b, B}$	4.57	$1.33 \pm 0.19^{c, A}$
L. casei ASCC 292	4.90	$2.86 \pm 0.21^{ab, A}$	4.17	$1.22 \pm 0.19^{ab, B}$	4.58	$2.88 \pm 0.10^{abc, A}$
L. casei ATCC 15820	4.24	$3.76 \pm 0.20^{a, A}$	3.86	$1.13 \pm 0.78^{ab, A}$	4.01	$2.96 \pm 0.24^{abc, A}$
L. casei CSCC 2607	4.35	$1.13 \pm 0.38^{b, A}$	3.91	$0.63 \pm 0.22^{ab, A}$	4.05	$1.50 \pm 0.13^{c, A}$

Values are means of triplicates from two separate runs, n = 2. MRS broth supplemented with cholesterol and 6 mM sodium glycocholate; 6 mM sodium taurocholate; 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate.

^{abc}Means within a column with different lowercase letter are significantly different ($P < 0.05$).

^{AB}Means within a row with different uppercase letters are significantly different ($P < 0.05$).

4.2 Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties

4.2.1 Introduction

The gastrointestinal tract consists of over 400 different strains of bacteria in the mammalian host. Among those, lactobacilli and bifidobacteria have attracted much attention with regard to their potential probiotic effects (Collins & Gibson, 1999). Probiotics are defined as 'live microbial supplement that beneficially affects the host by improving its intestinal microbial balance' (Fuller, 1992). Bifidobacteria are characterized as Gram-positive, non-spore forming, obligate anaerobes and non-motile bacilli, which sometimes could be observed in the form of club-shaped or spatulate. Bifidobacteria have been associated with several health benefits including synthesis of vitamin-B, lowering of blood ammonia levels, decreased duration of diarrhoea, growth inhibition of exogenous organisms and stimulation of the immune system (Mitsuoka, 1992). The intestinal tract of a breast-fed infant contains approximately 90% of the microbial population as bifidobacteria, which produce immune orientated by-products that protect the infant from pathogenic bacteria. Analysis of human faecal samples showed that the population of bifidobacteria in the human colon reaches 10^9 to 10^{10} cfu/g (Benno et al., 1989).

Elevated levels of blood and dietary cholesterol are considered as a major risk factor for coronary heart diseases and colon cancer. For each 1 mmol higher than normal cholesterol level, the risk of coronary heart disease was approximately 35 percent greater, while coronary death was 45 percent higher. Small reduction in serum cholesterol of 1 per cent was also found to reduce risk of coronary heart disease by 2 to 3 percent (Manson et al., 1992). There appears to be a relationship between consumption of dairy products containing bifidobacteria and the lowering of serum cholesterol levels in humans. Different hypotheses have been advanced to explain the possibility of hypocholesterolemic effects by probiotics, including assimilation (Gilliland, Nelson & Maxwell, 1985), co-precipitation (Klaver & van der Meer, 1993) and enzymatic hydrolysis of conjugated bile (Corzo & Gilliland, 1999).

This section has been published: *Liong, MT and Shah, NP. 2005. Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties.* ***Food Res Int*** *38: 135-142.*

Bile, composed mainly of bile salts, is produced by liver cells, and is secreted into the duodenum via the bile duct. Bile salts are glycine and taurine conjugates of bile acids, and act as natural ionic detergents. In the intestine, bile salts play an important role in emulsifying lipids, which enable intra-luminal lipolysis and absorption of lipolytic products by enterocytes. Cholic acid, one of the most common free bile acids in the intestine, is produced mostly by the deconjugation of free bile salts, such as taurocholic acid and glycocholic acid (Kurdi et al., 2003). The free bile acids are further modified by various intestinal microorganisms to produce secondary bile acids, such as deoxycholic acid and lithocholic acid (Baron & Hylemon, 1997). The deconjugation of bile salts in the small intestine and the excretion of the resulting free bile acids can result in a reduction of serum cholesterol because the replacement of bile salts would require the utilization of some cholesterol in the body. Free bile salts are less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen (Center, 1993).

Gram positive bacteria have been reported to be capable of hydrolysing the amide bond of conjugated bile salts, liberating free bile salts with lower detergent properties (Knarreborg et al., 2002). Bifidobacteria were found to excrete bile salt hydrolase (BSH; cholylglycine hydrolase; EC 3.5.1.24), the enzyme that catalyses the hydrolysis of glycine and/or taurine conjugated bile salts into amino acid residues and free bile salts (Tahri et al., 1995). BSH was found to be present in several bacterial strains of the gastrointestinal tract, such as *Lactobacillus* sp., *Bifidobacterium longum*, *Clostridium perfringens*, and *Bacteroides fragilis* ssp. *fragilis* (Corzo & Gilliland, 1999). Tanaka et al. (1999) conducted the largest study on the distribution and extent of BSH activity in lactic acid bacteria, involving more than 300 lactic acid bacteria strains from the genera of *Bifidobacterium* and *Lactobacillus*, and the strains *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Streptococcus thermophillus*. The function of this enzyme in the producing bacterium is still unclear although various hypotheses have been proposed, including utilization of the liberated amino acid by the producing bacterium and increase in resistance to the toxic levels of bile salts in the gastrointestinal environment (De Smet et al., 1995). When fed to germ-free rats, *Bifidobacterium longum* deconjugated bile salts and caused an increase in the excretion of free bile salts in the faeces (Chikai, Nakao & Uchida, 1987).

Gram-positive bacteria of static cultures reportedly had higher BSH activities occurring at low pH. It was hypothesized that high BSH deconjugation activity associated with the stationary phase of culture was a result of reduced pH levels in the medium (Corzo & Gilliland, 1999). Removal of cholesterol and the disappearance of glycine-conjugated bile salt from the growth media were contributed by precipitation that was caused by decreasing

pH levels (Klaver & Van der Meer, 1993). Contrarily, a study by Noh, Kim & Gilliland (1997) revealed that cholesterol was removed *in-vitro* by lactic acid bacteria when the pH was maintained at 6.0. With large amounts of cholesterol being removed by those similar strains at pH 6.0, Brashears, Gilliland & Buck (1998) hypothesized that cholesterol removal was not solely related to bile salt deconjugation and co-precipitation. The regulation of BSH activity by pH is still equivocal, although BSH activities were shown to be higher in lower pH values. Furthermore, several strains of the same bacterial species reportedly exhibited different BSH activity under similar pH levels (Lunden & Savage, 1990; Corzo & Gilliland, 1999).

In this study, we examined the bile salt deconjugation ability, BSH activity and cholesterol removal from co-precipitation with deconjugated bile by five bifidobacteria strains in order to select strains with cholesterol lowering properties.

4.2.2 Materials and Methods

4.2.2.1 Bacteria

Strains of *Bifidobacterium* were used in this study. Working cultures were prepared as described in section 3.2.2.1

4.2.2.2 Deconjugation of sodium glycocholate and sodium taurocholate

The ability of *Bifidobacterium* to deconjugate bile was evaluated using sodium glycocholate and sodium taurocholate as described in section 4.1.2.2.

4.2.2.3 BSH assay and protein assay

The BSH activity of *Bifidobacterium* was quantified based on the liberation of amino acid as described in section 4.1.2.3.

4.2.2.4 Co-precipitation of cholesterol with deconjugated bile

The co-precipitation of cholesterol from deconjugation of bile by *Bifidobacterium* was determined as described in section 4.1.2.4.

4.2.2.5 Co-precipitation of cholesterol with bile at different pH levels

Co-precipitation of cholesterol with bile was determined over a range of pH 1.0 to 7.0 in 0.1 M sodium acetate buffer (pH 1.0 to 5.0) or 0.1 M sodium phosphate buffer (pH 6.0 to 7.0). Cholic acid (10 mM) was used as the deconjugated bile, while 5 mM sodium glycocholate and 5 mM sodium taurocholate were used as the conjugated bile. Filter-sterilized cholesterol (70-100 μg/ml) was added to 10 mL of bile solution in buffer at various pH levels and incubated at 37 °C for 20 h. After the incubation period, the mixture was centrifuged and the remaining cholesterol concentration in the supernatant was determined using the method as described in section 3.1.2.4.

4.2.2.6 Statistical analysis

Data were statistically analysed as described in section 4.1.2.5

4.2.3 Results

4.2.3.1 Bile salt deconjugation by bifidobacteria

Bile salt deconjugation by bifidobacteria strains, as determined by the amount of cholic acid released, is shown in Table 4.4. All strains were able to deconjugate sodium glycocholate and sodium taurocholate, liberating cholic acid ranging from 0.92 to 3.22 mM. Although deconjugation activities among strains were insignificant statistically, however, it appeared that bifidobacterial strains showed preference towards deconjugation of sodium glycocholate compared to sodium taurocholate. *B. infantis* ATCC 17930 and 1912 deconjugated highest amount of individual glycine- and taurine-conjugated bile compared to other strains studied, while *B. longum* 536 and *B. longum* 1941 showed lowest deconjugation of glycine- and taurine-conjugated bile, respectively. All strains showed varying degree of deconjugating activity at concentrations that resemble the molar ratio of sodium glycocholate and sodium taurocholate in the human bile. Under such a molar ratio, *B. infantis* 1912 released highest amount of cholic acid, while *B. longum* 1941 liberated lowest amount of deconjugated bile.

4.2.3.2 BSH activity of bifidobacteria

BSH activity obtained from cell extracts of bifidobacteria strains is shown in Table 4.5. All strains showed varying degree of BSH activity towards both sodium glycocholate and sodium taurocholate, ranging from 0.83 to 1.37 U/ml. Although BSH activities were not significant statistically, similar trend with the deconjugation activities were found. All strains

appeared to show higher substrate specificity toward glycine-conjugated bile than taurine-conjugated bile. However, *B. longum* 536 showed highest BSH activity towards sodium glycocholate despite having lower deconjugation ability compared to the other strains studied, while *B. longum* 1941 similarly exhibited lowest BSH activity. *B. infantis* ATCC 17930 and 1912 had highest BSH activity with respect to sodium taurocholate, compared to *B. longum* 1941 which had the lowest. Using bile salt mixture that contained glycocholic, glycochenodeoxycholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acid, all strains exhibited higher total BSH activity compared to sodium taurocholate (except for *B. longum* 536), but lower BSH activity compared to sodium glycocholate (except for *B. infantis* 17930). Specific activity of BSH did not correlate well with total BSH activity by most strains due to varying protein content in cell extracts. *B. longum* 536 had highest total BSH activity for sodium glycocholate but exhibited lowest specific activity. Similarly, *B. infantis* 17930 showed highest total BSH activity with sodium taurocholate and conjugated bile mixture but exhibited low specific activity.

4.2.3.3 Co-precipitation of cholesterol with deconjugated bile by bifidobacteria

Co-precipitation of cholesterol with cholic acid produced from the deconjugation of sodium glycocholate and sodium taurocholate by bifidobacteria is shown in Table 4.6. All strains showed varying degree of cholesterol co-precipitation after deconjugation of both glycine- and taurine-conjugated bile, ranging from 0.46 to 4.38 μg/ml. More cholesterol was precipitated from the deconjugation of sodium glycocholate compared to sodium taurocholate, by all strains of bifidobacteria studied. Highest amount of cholesterol (> 3.02 μg/ml) precipitated from the deconjugation of sodium glycocholate and sodium taurocholate was by *B. infantis* 17930 and 1912. Lowest amount of cholesterol was precipitated by *B. longum* 536 when conjugated bile was used in the concentrations that resemble the human bile. Under such bile concentrations, more cholesterol was co-precipitated by *B. breve* 15698 and *B. infantis* 17930 compared to individual taurine-conjugated bile, but less co-precipitation was observed when compared to glycine-conjugated bile from deconjugation activity by all bifidobacteria strains.

4.2.3.4 Co-precipitation of cholesterol with conjugated and deconjugated bile at various pH

We further determined the effects of pH on cholesterol co-precipitation with cholic acid, and investigated the possibility of cholesterol co-precipitation with conjugated bile at various pH levels. Co-precipitation of cholesterol with cholic acid, sodium glycocholate and

sodium taurocholate at various pH levels is shown in Figure 4.1. Highest co-precipitation was observed with cholic acid, the deconjugated bile, followed by sodium glycocholate and lowest with sodium taurocholate. Co-precipitation of cholesterol with cholic acid increased rapidly with decreasing pH levels below 5.0, with maximum co-precipitation of 3.71 μg/ml was obtained at pH 1.0. Small amount of cholesterol co-precipitated with sodium glycocholate, which showed higher co-precipitation (0.59 to 0.83 μg/ml) below pH 4.0. Co-precipitation of cholesterol with sodium taurocholate was minimal (0.02 to 0.14 μg/ml) despite higher co-precipitation at pH 2.0 to 1.0.

4.2.4 Discussion

The major route of cholesterol excretion from humans and other mammals is through faeces. Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo & Gilliland, 1999). Deconjugation of bile salts in a mammalian host takes place in the small and large intestines. In a steady state situation, deconjugation of bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation (Reynier et al., 1981). In recent years, the possibility of using bile salt deconjugation by bifidobacteria to lower serum cholesterol levels in hypercholesterolemic patients or to prevent hypercholesterolemia in normal individuals has received increased attention. In this study, all bifidobacteria strains could deconjugate both sodium glycocholate and sodium taurocholate. However, more sodium glycocholate was deconjugated compared to sodium taurocholate. This was consistent with higher substrate specificity by BSH for glycine-conjugated bile compared with taurine-conjugated one. BSH reportedly had a broad substrate range and can hydrolyse not only human bile, but also tauroursodeoxycholic acid and taurohyodeoxycholic acid. However, the highest level of activity was observed with glycochenodeoxycholic acid, and had higher affinities for glycine-conjugated bile acids than for taurine-conjugated bile acids (Tanaka et al., 2000). Previous studies have also reported that glycine-conjugated bile salt was more efficiently deconjugated by *L. acidophilus* strains than taurine-conjugated bile salt (Corzo & Gilliland, 1999). BSH from *Clostridium perfringens* had higher substrate specificity for glycine-conjugates than for taurine-conjugates (Gopal & Hylemon, 1988), and BSH from *L. buchneri* JCM 1069 and *L. kefir* BCCM 9480 expressed substrate specificity for glycine-conjugates, based on the structure of the steroid moiety of the bile salt conjugate (De Smet et al., 1995). BSH from *B. longum* was found to exhibit high levels of homology with BSH of various lactobacilli and *C. perfringens*, and with a hypothetical protein of *Bacillus subtilis* (Tanaka et al., 2000). Another hypothesis was stated to explain such

preference towards sodium glycocholate. It was reported that glycine-conjugated bile salts were more toxic as compared to taurine-conjugated counterparts, and bifidobacteria expressed BSH as a protective mechanism against the toxicity of bile acids (De Smet et al., 1995; Grill, Perrin & Schneider, 2000). Thus, higher BSH was expressed in the presence of glycine-conjugated bile than that of taurine-conjugates, contributing to higher deconjugation of sodium glycocholate than sodium taurocholate.

Based on genetic data of *B. longum*, Tanaka et al. (2000) found that BSH is an intracellular enzyme, and no enzyme activity was found to be present in the supernatants of overnight cultures. Similarly, previous reports found that the BSH enzyme of lactic acid bacteria and bifidobacteria was located intracellularly (Lunden & Savege, 1990; Grill et al., 1995). The enzyme was released either by sonication or by other cell disruption methods, or by lysis in assays performed with whole cells. Sonication of cells under acidic conditions has been found to result in loss of enzyme activity (Shah & Jelen, 1990), while mechanical rupturing minimized loss of activity (Shah & Lankaputhra, 1997). Thus in this study, BSH activity was determined from cell extracts that were obtained from mechanical cell disruption using a homogenizer. Total BSH activity did not correlate well with protein concentration in the cell extracts, and thus did not produce linear correlation with specific activity. This may be due to several reasons such as the sensitivity of BSH to oxygen and different homogenization efficiency (Tanaka et al., 1999).

Benno et al. (1989) reported that bifidobacteria are one of the most predominate culture existing in the human colon, and possessed higher BSH activity than other probiotics. Screening experiments using 30 strains of bifidobacteria revealed that only 2 strains did not show BSH activity, and all positive strains contained constitutive intracellular BSH enzymes (Kim et al., 2004). Brashears et al. (1998) reported that the human intestine is predominated by sodium glycocholate. Ratio of glycocholate per taurocholate in the gall bladder of the human adult was estimated to be 2.2 to 3.0 M, and the highest bile salt molarity throughout the small intestine is between 2.4 and 4.0 mM (Hofmann, 1977). *B. infantis* 17930 and 1912 were of human origin and showed highest deconjugation ability for both sodium glycocholate and sodium taurocholate. These strains also deconjugated highest amount of sodium glycocholate and sodium taurocholate at concentrations of 2.8 mM and 1.2 mM, respectively, which resemble the concentrations of the human bile. Thus, we postulate that these strains may survive better in the human gastrointestinal environment to predominate the colon and subsequently enhance bile salt deconjugation *in-vivo*.

Previous studies showed that the co-precipitation of cholesterol with deconjugated bile was correlated with the pH of media (Tanaka et al., 2000). The pH optimum for BSH activity at 37 °C was between 5.0 and 7.0, and maximum activity occurred at pH 6.0, at a temperature between 40 and 45 °C. The enzyme was stable at pH values from 4.0 to 8.0, but inactivated at pH values above 8.0 and below 4.0 (Tanaka et al., 2000). Our result showed that co-precipitation of cholesterol occurred at pH ranging from 3.78 to 4.96. The solubility of cholic acid was found to decrease with decreasing pH values, because cholic acid is insoluble in pH less than 5.0 due to its pK of 5.0 to 6.0 (Brashears et al., 1998). Theoretically, cholesterol co-precipitation would increase with decreasing pH values. However, our study showed that co-precipitation of cholesterol was not entirely pH dependent. Final pH of medium with deconjugated sodium taurocholate and inoculated with *B. infantis* 17930 and *B. longum* 536 was 4.32 and 3.81, respectively, while amount of cholesterol precipitated was 3.08 μg/ml and 0.33 μg/ml, respectively. Similarly, the deconjugation of sodium glycocholate and sodium taurocholate at concentrations that resemble the human bile by *B. infantis* 17930 and *B. infantis* 1912 had final pH of 4.29 and 4.05, respectively. However, levels of cholesterol precipitated were 4.15 μg/ml and 2.58 μg/ml, respectively.

We further determined as to whether the pH was the sole factor contributing to cholesterol co-precipitating with deconjugated bile. The pK_a of taurine and glycine conjugated bile salts, and of unconjugated bile salts, are pH 1.9, 3.9 and 5.0, respectively. At decreasing pH levels, taurine-conjugated bile salts will remain ionized in solution, glycine-conjugated bile salts will be partially precipitated without hydrolysis and conjugated bile salts will be protonated and precipitated (Dashkevicz & Feighner, 1989). Our results found that cholesterol precipitation was highest with cholic acid at pH below 5.0, minimal co-precipitation occurred with sodium glycocholate at pH below 4.0 and co-precipitation with sodium taurocholate was negligible, even at pH 1.0. Since BSH has an isoelectric point between 4.4 and 4.6 (Kim et al., 2004), and pH of intestine is unlikely to be lower than 6.0 (Brashears et al., 1998), we postulate that co-precipitation of cholesterol with sodium taurocholate would not be a major factor in controlling serum cholesterol. Based on these findings, we hypothesize that the *in-vitro* removal of cholesterol upon deconjugation activity on sodium glycocholate and sodium taurocholate was contributed by several factors: a) co-precipitation with deconjugated bile (cholic acid) at pH levels below 5.0; b) higher substrate preference of BSH for glycine-conjugates, which led to higher cholesterol co-precipitation with the deconjugated bile upon deconjugation of sodium glycocholate; and c) co-precipitation with sodium glycocholate at pH levels below 4.0.

All bifidobacteria strains studied were able to deconjugate both glycine- and taurine-conjugated bile, although more cholic acid was released from the deconjugation of sodium glycocholate compared to sodium taurocholate. This was consistent with higher substrate preference of BSH for glycine-conjugated bile than taurine-conjugates. Experiments were carried out using concentrations of sodium glycocholate and sodium taurocholate that resemble the human bile, and pH levels that were similar to the pH of the human intestine. Results indicated that the human origin strain of *B. infantis* 17930 had highest bile salt deconjugation ability and BSH activity, and therefore may exert effective deconjugation activity *in-vivo*. However, more work are needed to elucidate this.

Table 4.4

Deconjugation of sodium glycocholate and sodium taurocholate by bifidobacteria strains[a,b]

Strains	Cholic acid released (mM)[c,d]		
	Sodium glycocholate	Sodium taurocholate	Sodium glycocholate + sodium taurocholate
B. breve ATCC 15698	1.71 ± 0.16 a, A	1.26 ± 0.23 a, A	1.50 ± 0.32 a, A
B. infantis 1912	3.02 ± 0.34 a, A	2.46 ± 0.11 a, A	2.89 ± 0.16 a, A
B. infantis ATCC 17930	3.22 ± 0.51 a, A	2.15 ± 0.43 a, A	2.52 ± 0.23 a, A
B. longum 1941	2.01 ± 0.55 a, A	0.92 ± 0.51 a, A	0.96 ± 0.22 a, A
B. longum 536	1.68 ± 0.23 a, A	1.24 ± 0.15 a, A	1.15 ± 0.13 a, A

[a]Results are expressed as mean ± standard error of means; values are means of triplicates from two separate runs; n = 2.

[b]Deconjugation of glycine or taurine conjugated bile based on released of cholic acid.

[c]MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate; 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate.

[d]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [A]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 4.5

BSH activity of bifidobacteria strains on sodium glycocholate and sodium taurocholate[a]

Strains	BSH activity[b,c]								
	Sodium glycocholate			Sodium taurococholate			Conjugated bile mixture		
	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)
B. breve ATCC 15698	1.36 ± 0.22 a, A	1.12 ± 0.34 a, A	0.83 ± 0.21 a, A	1.65 ± 0.16 a, A	0.91 ± 0.13 a, A	0.55 ± 0.17 a, A	1.12 ± 0.20 a, A	1.02 ± 0.51 a, A	0.91 ± 0.27 a, A
B. infantis 1912	1.20 ± 0.28 a, A	1.13 ± 0.20 a, A	0.94 ± 0.20 a, A	1.48 ± 0.24 a, A	1.00 ± 0.23 a, A	0.67 ± 0.14 a, A	1.80 ± 0.49 a, A	1.01 ± 0.15 a, A	0.56 ± 0.19 a, A
B. infantis ATCC 17930	1.06 ± 0.18 a, A	1.20 ± 0.14 a, A	1.14 ± 0.13 a, A	1.82 ± 0.34 a, A	1.05 ± 0.12 a, A	0.58 ± 0.18 a, A	2.08 ± 0.22 a, A	1.39 ± 0.22 a, A	0.67 ± 0.18 a, A
B. longum 1941	1.22 ± 0.19 a, A	1.10 ± 0.24 a, A	0.91 ± 0.31 a, A	1.13 ± 0.15 a, A	0.83 ± 0.15 a, A	0.74 ± 0.36 a, A	1.75 ± 0.58 a, A	1.01 ± 0.26 a, A	0.58 ± 0.24 a, A
B. longum 536	2.20 ± 0.73 a, A	1.37 ± 0.29 a, A	0.63 ± 0.18 a, A	1.65 ± 0.41 a, A	0.96 ± 0.18 a, A	0.58 ± 0.21 a, A	2.15 ± 0.22 a, A	0.87 ± 0.29 a, A	0.41 ± 0.16 a, A

[a]Results are expressed as mean ± standard error of means; values are means of triplicates from two separate runs; n = 2.

[b]BSH activity from cell free extracts of *Bifidobacterium* grown on MRS-LC broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate; 6 mM conjugate bile mixture.

[c]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [A]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 4.6

Cholesterol precipitation with deconjugation of sodium glycocholate and sodium taurocholate by bifidobacteria strains[a,b,c]

Strains	Sodium glycocholate		Sodium taurococholate		Sodium glycocholate + sodium taurocholate	
	Final pH	Cholesterol precipitated (μg/ml)	Final pH	Cholesterol precipitated (μg/ml)	Final pH	Cholesterol precipitated (μg/ml)
B. breve ATCC 15698	4.31	2.33 ± 0.44 ab, A	4.32	1.08 ± 0.10 ac, A	4.08	1.38 ± 0.56 bc, A
B. infantis 1912	4.17	3.44 ± 0.26 ab, A	3.78	3.02 ± 0.61 ab, A	4.05	2.58 ± 0.15 ab, A
B. infantis ATCC 17930	4.48	4.38 ± 0.79 a, A	4.32	3.08 ± 0.32 a, A	4.29	4.15 ± 0.28 a, A
B. longum 1941	4.96	2.42 ± 0.25 ab, A	4.24	1.01 ± 0.35 bc, A	4.57	2.17 ± 0.10 bc, A
B. longum 536	4.59	1.29 ± 0.16 b, A	3.81	0.33 ± 0.15 c, A	4.49	0.46 ± 0.29 c, A

[a]Results are expressed as mean ± standard error of means; values are means of triplicates from two separate runs; n = 2.

[b]MRS broth supplemented with cholesterol and 6 mM sodium glycocholate; 6 mM sodium taurocholate; 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate.

[c]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

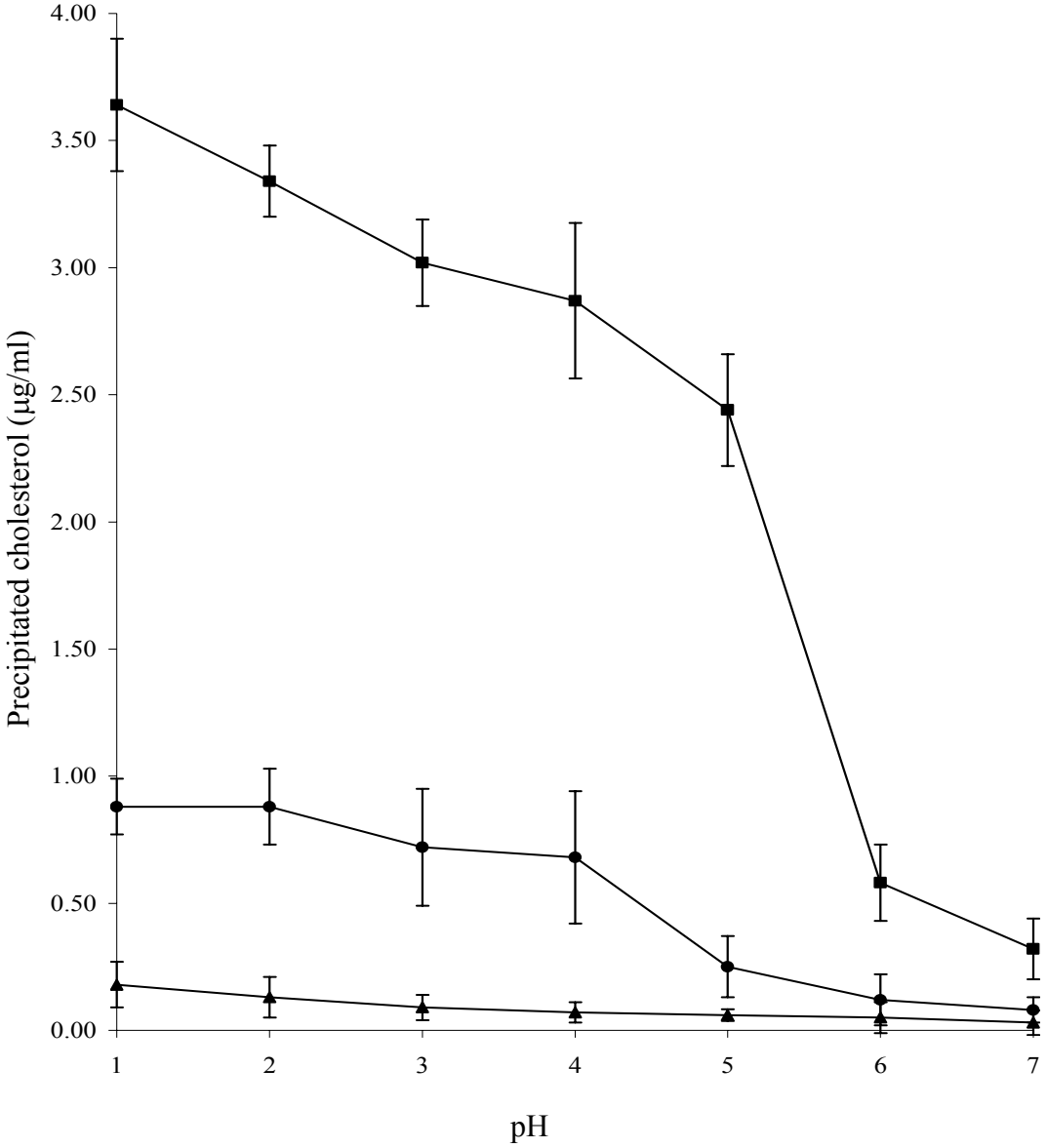


Fig. 4.1. The effect of different pH on the co-precipitation of cholesterol with 10 mM cholic acid (■), 5 mM sodium glycocholate (●) or 5 mM sodium taurocholate (▲). Error bars represent standard error of means. Each value is the mean of two independent trials.

5.0 Optimization of cholesterol removal, evaluation of growth properties and production of organic acids by *Lactobacillus* and *Bifidobacterium* in the presence of prebiotics

5.1 Optimization of Cholesterol Removal by *Lactobacillus casei* ASCC 292 in the Presence of Prebiotics by Response Surface Methodology

5.1.1 INTRODUCTION

Probiotics are defined as 'live microbial supplement that beneficially affects the host by improving its intestinal microbial balance' (Fuller, 1992). Over the years, lactobacilli have been associated with the improvement of lactose intolerance, increase in natural resistance to infectious disease in gastrointestinal tract, suppression of cancer, improved digestion and reduction in serum cholesterol level (Gibson and Roberfroid, 1995). Studies have shown that a small reduction in serum cholesterol of 1 per cent could reduce risk of coronary heart disease by 2 to 3 percent (Manson et al., 1992). We have previously shown that cholesterol was removed by strains of lactobacilli in laboratory media (Chapter 3.0, section 3.1.3.3). Various *in-vivo* studies have reported that some lactobacilli could lower total cholesterol and low-density-lipoprotein (LDL) cholesterol (Anderson and Gilliland, 1999; Sanders, 2000). A prebiotic is a food ingredient that are neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract, and selectively used as a substrate for beneficial bacteria in the colon (Collins and Gibson, 1999). Most widely researched prebiotics fall in the group of oligosaccharides, especially oligofructose (Niness, 1999). Feeding rats with a diet supplemented with oligofructose lowered plasma concentrations of VLDL, triacylglycerol and phospholipids (Roberfroid and Delzenne, 1998), and reduced postprandial triglyceridemia (Kok and Delzenne, 1998).

Another approach to gut microflora management is the use of synbiotics, where probiotics and prebiotics are used in combination. The concept of synbiotic has been widely studied, mostly to improve survivability of probiotics in both *in-vitro* and *in-vivo* experiments (Collins and Gibson, 1999; Cummings et al., 2001), and to modulate colonic microbial populations using animal models (Flickinger et al., 2000; Gibson and Roberfroid, 1995). However, to our knowledge, there is no information on suitable combinations of probiotics and prebiotics specifically targeting removal of cholesterol using *in-vitro* models, although a limited number of studies addressed the use of prebiotics or synbiotics to reduce serum cholesterol, regulate hepatic lipogenesis and lipid metabolisms (Kok and Delzenne, 1998; Suskovic et al., 2001). Yet, all these studies involved *in-vivo* experiments, where the true interaction patterns of synbiotics to reduce cholesterol are poorly understood.

This section has been published: *Liong, MT and Shah, NP. 2005. Optimization of cholesterol removal by probiotics in presence of prebiotics using response surface methodology.* ***Appl Environ Microbiol*** *71: 1745-1753.*

Response surface methodology (RSM) is a statistical and mathematical method that involves main and interactions effects to account for curvature, to improve optimal process settings and to troubleshoot process problems and weak points (Montgomery, 1996). It has been successfully utilized to optimize compositions of microbiological media, conditions of enzyme hydrolysis, parameters for food preservation and fermentation processes (Lee and Chen, 1997). Previous studies have used conventional methods (such as one factor at one time) to evaluate the *in-vitro* performance of probiotics and/or prebiotics to remove cholesterol. These methods, however, require a large number of experiments to describe the effect of individual factors and were time consuming. Besides, no established statistical method was introduced to distinguish the interaction effects from main effects. Furthermore, up to now, there has been no reported study on the use of RSM to remove or reduce cholesterol using neither *in-vitro* experiments nor animal models. Thus, the aims of this study were to optimize cholesterol removal by using *L. casei* ASCC 292 in the presence of FOS and maltodextrin, through the response surface approach. This information will provide better understanding of the interactions involved in cholesterol reduction for *in-vivo* experiments.

5.1.2 MATERIALS AND METHODS

5.1.2.1 Bacteria and media preparation

L. casei ASCC 292 is a human derived strain obtained from the Australia Starter Culture Research Centre (ASRC) (Werribee, Australia). The organism was grown in sterile de Mann, Rogosa, Sharpe (MRS) broth using 1% inoculum and 20 h incubation at 37 °C, and was transferred successively three times in MRS broth prior to use. At the end of the fermentation period, the culture was centrifuged, and the cell pellet washed twice with distilled water. The supernatant was discarded and 0.1 M phosphate buffer (pH 6.8) containing 2.0 % (w/v) of food grade cryoprotectant Unipectin™ RS 150 (Savannah Bio Systems, Balwyn East, Australia) was added. The mixture was vortexed, and freeze-dried at -18 °C for 48 h.

Six types of commercially available prebiotics were used including sorbitol (Sigma Chemical Co., St. Louis, MO, U.S.A.), mannitol (Sigma), maltodextrin (Grain Processing Corp., Muscatine, IA, U.S.A.), hi-amylose maize (Starch Australasia Ltd., Lane Cove, NSW, Australia), inulin (Orafti Pty. Ltd., Tienen, Belgium) and FOS (Orafti). Hi-amylose maize contained > 70% amylose, and 32.5% total dietary fibre. Inulin used was Raftiline ST with a purity of 92% and an average degree of polymerization (DP) of 10. FOS used was Raftilose P95 that had a purity of 95% with an average DP of 4.

All prebiotics and freeze-dried cells of *L. casei* ASCC 292 were used at concentrations as per the experimental design explained later (Table 5.1). Prebiotics media were inoculated with freeze-dried cells of *L. casei* ASCC 292, at appropriate levels as described in the experimental design.

5.1.2.2 Cholesterol removal

The removal of cholesterol by *L. casei* ASCC 292 was determined as described in Chapter 3.0, section 3.1.2.4.

5.1.2.3 Growth of *L. casei* ASCC 292 in presence of prebiotics

The growth was determined using the plate count method. Bacilli generally divide in one plane, and can produce chains of cells due to the failure to separate completely. Thus, at the end of the fermentation time, fermentation broth containing probiotic cultures sonicated for 5 s to disrupt clumps of lactobacilli (Bermudez et al., 2001) before serial dilutions were performed. MRS agar was used for plating and the plates were incubated anaerobically at 37 °C for 24 h. Growth was calculated as $\log_{10}$ colony forming units (CFU/ml) and expressed as percentage difference between initial growth values obtained at time = 0 and at the end of the incubation period.

5.1.2.4 Mean doubling time

The mean doubling time was calculated as described previously (Shin et al., 2000). The specific growth rate (μ) of the cultures was obtained using the following equation:

$$\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$$

where X_2 and X_1 are the cell density at time t_2 and t_1, respectively. The mean doubling time (T_d) was calculated as:

$$T_d = \ln 2/\mu$$, and expressed in min.

5.1.2.5 Utilization of substrate and growth yield

The utilization of substrate was determined as the difference between initial concentration of prebiotics and final concentration after the incubation period. Broths containing *L. casei* ASCC 292 were centrifuged at 2714 x g at 4 °C for 15 min, and the supernatant was used to determine the concentration of residual prebiotics. As both

substrates are oligosaccharides, their residual concentration was determined by colourimetry after hexose hydrolysis with phenol-sulphuric acid (Dubois et al., 1956). Utilization of the substrate was expressed in percentage of the initial concentration over final concentration. Growth yield was expressed as growth per gram of substrate utilized. The growth was obtained using the pour plate method as described earlier, and expressed as the difference between initial $\log_{10}$ CFU/ml values at time = 0 and at the end of the incubation period.

5.1.2.6 Short chain fatty acids determination

The fermentation of prebiotics was determined by measuring short chain fatty acids as the end products of fermentation using high performance liquid chromatography (HPLC, Varian Australia Pty. Ltd., Mulgrave, Australia). At the end of the incubation period, fermentation broths containing *L. casei* ASCC 292 and the prebiotics used were centrifuged at 2714 x g at 4 °C for 15 min, and the supernatant was prepared for HPLC analysis using a method as described previously (Dubey and Mistry, 1996). SCFA were expressed as the total acetic, butyric and propionic acids.

5.1.2.7 Experimental design and statistical analyses

Screening experiments to select prebiotics were performed with seven independent factors namely, *L. casei* ASCC 292 (X_1), sorbitol (X_2), mannitol (X_3), maltodextrin (X_4), hi-amylose maize (X_5), inulin (X_6) and FOS (X_7), using a two level partial factorial design 2^{7-2} resulting in 64 experimental runs (including duplicates) and 5 middle point runs (Table 5.1). First order empirical equation was used to exclude insignificant factors and to generate steepest ascent, which led to optimization, by a rotatable central composite design (CCD). The treatment combinations were allocated in 2 blocks and all experiments were carried out in 2 days. The first block, representing the first day of the experiment, contained the factorial runs accompanied by 4 center runs. The second block, representing the second day of the experiment, contained the axial runs accompanied by 2 center runs. These modeling and statistical analyses were performed using the Design Expert version 5.07 software (Stat-Ease Corp., Minneapolis, MN, U.S.A.).

5.1.3 RESULTS AND DISCUSSION

5.1.3.1 Screening of prebiotics and steepest ascent

Response surfaces are often influenced by various factors. The primary purpose of screening experiments is to select important main effects from less important ones. In this

study, screening was used to generate first degree order equation and to test significance of factors. Complete replication of a 2^7 factorial design would involve 128 experimental runs. However, only seven degree of freedoms would be needed to estimate main effects, and 21 degree of freedoms would estimate two-factor interaction effects, while the remaining 99 degree of freedoms would estimate error or/and three or higher-factor interaction effects (Cox and Reid, 2000). Thus, a partial two-level factorial design (2^{7-2}) was applied in this study. Partial factorial designs are capable of identifying important factors and to determine interaction effects between factors, using less number of experimental runs as compared to full factorial design without loss of information on main factor effects and their interactions (Montgomery, 1996). Results from the two level partial factorial design are shown in Table 5.1, while analysis of variance (ANOVA) for the evaluation of the first-order model is shown in Table 5.2. ANOVA showed that the model used was suitable, with only 4.63% total variation that was not explained by the model, and the lack-of-fit test was insignificant. The first-order model generated for screening was linear, with the presence of curvature being insignificant. The removal of cholesterol was significantly influenced by concentrations of probiotic (X_1), maltodextrin (X_4) and FOS (X_7), while the other prebiotics were found to have insignificant influence. Thus, only these three factors were used for further optimization experiments. From this first degree order model, a first-order equation (coded term) was generated for response of cholesterol removal (Y), with factors probiotic (X_1), FOS (X_2), and maltodextrin (X_3):

$$Y = 24.58 + 7.13\ X_1 + 3.58\ X_2 + 5.49\ X_3.$$

From the equation and coefficient estimate, probiotic level (X_1) produced greatest effect and was used as the fundamental scale in the next step, steepest ascent. This determined the path of steepest ascent and a movement was generated along that path until no improvement occurred. The steepest ascent design was based on the increase of 0.50 (% wt/vol) concentrations for X_1. This produced 5 design units (0.50/0.10 = 5). Thus, the movement for X_2 was 2.51 design units [(3.58/7.13)(5) = 2.51], and that for X_3 was 3.85 design units [(5.49/7.13)(5) = 3.85]. The following steepest ascent coordinates were generated as shown in Table 5.3. Steepest ascent coordinates showed that the removal of cholesterol decreased after the fourth step, with the highest value of 55.16 μg/ml, from the combination of probiotic (1.70% wt/vol), FOS (4.78% wt/vol) and maltodextrin (6.79% wt/vol). This combination was used as middle points for optimization experiments.

5.1.3.2 Optimization of cholesterol removal

The optimization of cholesterol removal was performed using CCD with fixed middle point of probiotic (1.70% wt/vol), FOS (4.80% wt/vol) and maltodextrin (6.80% wt/vol), and alpha of ±1.682 to produce design rotatability (Araujo and Brereton, 1996). The design matrix for CCD and responses are shown in Table 5.4, while the adequacy and fitness were evaluated by ANOVA and regression coefficients (Table 5.5). The ANOVA results indicated that the quadratic regression to produce the second-order model was significant. The lack-of-fit test was insignificant and only 4.60% of the total variation was not explained by the model. This suggested that the model accurately represented data in the experimental region. This also indicated that second-order terms were sufficient and higher-order terms were not necessary. Probiotic, maltodextrin and FOS levels were significant for the removal of cholesterol. It must be noted that the *t* value of the quadratic term of FOS (X_2^2) was higher than others (Table 5.5), indicating that the second-order regression of FOS was the strongest effect. The intercept c is the estimated response at the center point, with the coded values of X_1, X_2 and X_3 at 0, 0 and 0, respectively.

The effect of each factors was further assessed using perturbation plots to show how the response changes as each factor moves from the chosen reference point, with all other factors held constant at reference values (Oh et al., 1995). In this study, as one particular chosen factor was assessed, the other factors were held constant at the optimum point. We defined the response surface model as obtained from Table 5.5 as $\hat{Y} = f(X_1, X_2, X_3)$, and (X^*_1, X^*_2, X^*_3) to be the optimum points of the factors, which in our experiments would be 0.018, 0.062, -0.081. Thus, the perturbation effect of X_1 was defined as:

$$\hat{Y}(X_1) = f(X_1, X^*_2, X^*_3).$$

Similarly, the perturbation effects of X_1 and X_2 would be:

$$\hat{Y}(X_2) = f(X^*_1, X_2, X^*_3)$$

$$\hat{Y}(X_3) = f(X^*_1, X^*_2, X_3)$$

The perturbation effect curves produced with the vertical axis representing $\hat{Y}(X_j)$ and the horizontal axis representing X_j. In this study, all X_j values have common coded levels, and thus the horizontal axis would represent the common coded levels. By overlying all perturbation curves, we get the perturbation plot (Oh et al., 1995). Figure 5.1 shows the perturbation plot of the factors used in this study. Although all factor showed a significant quadratic effect, the curve with the most prominent change was the perturbation curve of FOS, compared to the other factors that were fixed at their maximum levels. Thus, we believe that FOS was the most significant factor that contributed to the removal of cholesterol and had the most pronounced quadratic effect. The probiotic showed least

prominent change as compared to the other two factors, however, it still showed a significant quadratic effect.

The best explanatory equation to fit the second-order model and subsequently produce the response surface was expressed as:

$$Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3$$

where $c \ldots c_{23}$ are regression coefficients and X_1, X_2, X_3 are the coded independent factors. Here, the second-order regression model involved three factors, thus producing 3 linear, 3 quadratic and 3 interaction terms. The response surface was generated (Figure 5.2) based on the second-order equation:

$$Y_0 = 50.61 + 0.0081X_1 + 0.75X_2 - 0.61X_3 - 4.65X_1^2 - 8.10X_2^2 - 5.55X_3^2 - 3.01X_1X_2 - 4.22X_1X_3 - 3.69X_2X_3$$

An optimum point was produced with optimum cholesterol removal obtained at 50.66 μg/ml. The combination that produced the optimum point was (X_1, X_2, X_3) = (0.018, 0.062, -0.081). The original levels that correlated with those coded values were found to be probiotic at 1.71% wt/vol, FOS at 4.95% wt/vol and maltodextrin at 6.64% wt/vol. From the coefficient estimates (Table 5.5), all interaction terms were found to be significant. It must be noted that the coefficient estimate of the interaction terms of (X_1, X_3) and (X_2, X_3) had negative signs (X_{13} = -4.22, X_{23} = -3.69). These negative signs may imply that for an increase of the response, the coded levels of (X_1, X_3) and (X_2, X_3) must have different signs, either one must be higher than zero and the other lower than zero (Oh et al., 1995). From the three dimensional plot of probiotic and maltodextrin (Figure 5.2B), it was found that when optimum point was achieved, the coded levels of X_1 and X_3 were 1.71 and -0.081, respectively. The same applied for maltodextrin and FOS interactions (Figure 5.2C), with optimum being achieved at 0.062 and -0.081 for X_2 and X_3, respectively. However, it must be noted that the interactions of X_1 and X_2 also showed a negative sign, but the response surface showed that the optimum was achieved at X_1 = 0.018 and X_2 = 0.062, which would produce a positive sign instead. This may be due to other terms that may dominate this particular interaction term (Oh et al., 1995). Considering that the lack-of-fit test was insignificant, other higher terms would not have contributed to this, thus, we postulate that the linear term might have played a role.

All these predictions by the regression model were further ascertained by a validation experiment. We compared the cholesterol removal patterns over a 24 h period using four different media: the optimum medium (probiotic 1.71% wt/vol, FOS 4.95%

wt/vol and maltodextrin 6.64% wt/vol), the center-point medium (probiotic 1.70% wt/vol, FOS 4.80% wt/vol and maltodextrin 6.80% wt/vol), the high-point medium (probiotic 2.40% wt/vol, FOS 7.20% wt/vol and maltodextrin 8.80% wt/vol) and the low-point medium (probiotic 1.00% wt/vol, FOS 2.40% wt/vol and maltodextrin 4.80% wt/vol). The cholesterol removal curves are shown in Figure 5.3. Although the exact cholesterol removal quantities were different from the predictions, the patterns were in tandem with predictions by the model. The highest level of cholesterol was removed from the optimum medium, and that from the center-point medium. The least amount of cholesterol was removed from both high-point and low-point media, as supported by the response surface of cholesterol removal (Figure 5.2). All these indicated that the model produced was reliable to optimize *in-vitro* cholesterol removal that may be used to benefit human physiological health.

5.1.3.3 Growth, substrate utilization and end-product of fermentation of prebiotics

We further studied the growth, substrate utilization, growth yield, mean doubling time and the end-product of fermentation of prebiotics, at the experimental regions used to obtain optimum removal of cholesterol. The statistical analyses with coefficient estimates and the significance of each response model are presented in Table 5.6.

The response surface of growth (Y_1) was generated based on the coded factor equation using the coefficients in Table 5.6. The growth increased with increasing probiotic level from 1.00% wt/vol to 1.69% wt/vol. A further increase in concentrations of probiotic beyond 1.69% wt/vol generated a decrease in the growth. Similarly, an increment in FOS and maltodextrin concentrations from 2.40% wt/vol to 4.86% wt/vol and 4.80% wt/vol to 6.82% wt/vol, respectively, increased the growth, but further increase in the prebiotics concentration generated a decrease in the growth. All factors studied showed significant quadratic effects, as shown by the P-values of the coefficient estimates. Other than main quadratic effects, interaction between probiotic and maltodextrin produced strongest influence on the growth, while the interaction between probiotic and FOS was insignificant. The response surface of growth showed similar patterns with the response surface of removal of cholesterol, indicating a strong correlation between removal of cholesterol and growth. We have previously shown that *in-vitro* cholesterol assimilation was growth associated (Chapter 3.0, section 3.1.3.4). Knowing this, it would be beneficial to maintain or improve viability of *L. acidophilus* ASCC 292 in *in-vivo* models in order to favour cholesterol removal as well.

The response surfaces of substrate utilization (Y_2) and growth yield (Y_3) are shown in Figures 5.4 and 5.5 and were generated based on the second-order coefficients (Table 5.6). Only maltodextrin produced significant quadratic effect; the substrate utilization increased with increasing maltodextrin concentration from 4.80 to 6.56% wt/vol, but a further increase produced a decrease in the substrate utilization. A maltodextrin-like oligosaccharide was reported to have a slower rate of fermentation compared to FOS, and was more fermentable to the distal part of the large intestine (Flickinger et al., 2000). Probiotic and FOS did not produce significant quadratic effect, but showed significant linear correlations (Figure 5.4). The interaction effects showed that only the interaction between probiotic and FOS was significant. At higher FOS concentration (7.20% wt/vol), substrate utilization increased with increasing concentration of probiotic (1.00 to 2.40% wt/vol). Contrarily, at lower FOS concentration (2.40% wt/vol), substrate utilization decreased with increasing concentration of the probiotic. This may be due to competition for substrate with increasing cell numbers at low substrate levels. It must be noted that substrate utilization increased with increasing probiotic and FOS concentrations, despite a decrease in growth and cholesterol removal at these experimental regions. Thus, cholesterol removal may be growth associated, but both cholesterol removal and growth were not influenced solely by utilization of FOS. This is supported by the lower growth yield at these regions (Figure 5.5). Although maltodextrin did not show an overall significant interaction effects with probiotic or FOS, it generated a significant main quadratic effect. Our results indicated that interactions between probiotic and maltodextrin might have stronger influence on growth and cholesterol removal at regions that were not contributed by interaction of probiotic and FOS. It appears that maltodextrin served as an alternative substrate when FOS was insufficient to increase the growth and subsequently removal of cholesterol. This is important in synbiotic preparation as an *in-vivo* adjunct; FOS may be used solely, or used in lower concentrations but coupled with maltodextrin.

The mean doubling time was used as a measure of the effectiveness of specific carbon source in modulating growth rate (Bruno et al., 2002). In this study, the quadratic model for mean doubling time (Y_4) was generated using the coefficients as stated in Table 5.6. The mean doubling time decreased minimally at low FOS level (2.40% wt/vol) compared to the prominent changes at higher FOS level (7.20% wt/vol). It must be noted that the substrate utilization increased with increasing probiotic concentrations at higher FOS level (Figure 5.4), thus resulting in a decrease in the mean doubling time. This indicated that fermentation of FOS was more effective at higher FOS concentration. However, the mean doubling time was found to be lowest at the combination of 2.40% wt/vol probiotic and 2.40% wt/vol FOS, and that of 2.40% wt/vol probiotic and 4.80% wt/vol maltodextrin,

despite the lowest substrate utilization, growth and removal of cholesterol at those regions. Considering that the concentration of probiotic was at its highest but FOS and maltodextrin concentrations were at their lowest, it appears logical that the growth would be faster (lower mean doubling time) but overall the percentage of growth would be small, as compared to lower probiotic concentrations or higher prebiotic concentrations. Thus, although cholesterol removal was found to be lower, there are possibilities that cholesterol removal may be faster in these regions before cholesterol-removing activities reached a plateau. Further studies would be needed to confirm these phenomena.

The major products of metabolism of prebiotics are short chain fatty acids (SCFA), carbon dioxide and hydrogen, and bacterial cell mass (Cummings et al., 2001). Although much work has been done on SCFA production and the significance of the individual acids, no particular pattern of SCFA production from prebiotic fermentation has emerged as yet. The SCFA (Y_5) was obtained as a total of individual fatty acids, namely acetic, butyric and propionic acids. Only the probiotic produced significant quadratic effects on SCFA production, and so did the interactions between (X_1, X_2) and (X_1, X_3). The response surface (Figure 5.6) generated from the second-order coefficients (Table 5.6) showed a close correlation with substrate utilization (Figure 5.4). This indicated that the production of SCFA from the fermentation of FOS was closely associated with the uptake of the substrate. However, it must be noted that increasing the concentration of probiotic at higher level of FOS (7.20% wt/vol) generated a decrease in SCFA production, but increased the substrate utilization. The hydrolysis of FOS was repressed by products of their hydrolysis (Kaplan and Hutkins, 2003). Thus, we postulate that an increase in substrate utilization in those experimental regions would generate higher concentration of hydrolysis products, and subsequently repress further SCFA production.

5.1.3 Conclusions

Optimal cholesterol removal from media was 50.66 μg/ml in presence of 1.71% wt/vol probiotic, 4.95% wt/vol FOS and 6.64% wt/vol maltodextrin. The validation experiment showed that RSM was reliable in developing a model, optimizing factors, and in analysing interaction effects. Analyses of growth, substrate utilization, yield, mean doubling time and production of SCFA showed that cholesterol removal was growth associated. FOS was the preferred substrate for growth, cholesterol removal, and the production of SCFA, while maltodextrin was alternatively used for these purposes when FOS was insufficient. The information

gathered in this study will benefit the development of a synbiotic product that will particularly target cholesterol removal. *In-vivo* experiments are required to ascertain the cholesterol lowering capacity of *L. casei* ASCC 292 in presence of prebiotics.

TABLE 5.1. Treatment combinations and response for screening experiments.

Standard order	Coded factor level[a]							Response[b]
	Probiotic	Sorbitol	Mannitol	Maltodextrin	Hi-amylose maize	Inulin	FOS	Y Cholesterol removed
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	(μg/ml)
1	-1	-1	-1	-1	-1	1	1	28.44
2	1	-1	-1	-1	-1	-1	-1	36.10
3	-1	1	-1	-1	-1	-1	-1	22.11
4	1	1	-1	-1	-1	1	1	39.32
5	-1	-1	1	-1	-1	-1	1	26.62
6	1	-1	1	-1	-1	1	-1	39.17
7	-1	1	1	-1	-1	1	-1	20.75
8	1	1	1	-1	-1	-1	1	39.74
9	-1	-1	-1	1	-1	-1	-1	18.76
10	1	-1	-1	1	-1	1	1	39.79
11	-1	1	-1	1	-1	1	1	27.82
12	1	1	-1	1	-1	-1	-1	36.46
13	-1	-1	1	1	-1	1	-1	17.96
14	1	-1	1	1	-1	-1	1	39.64
15	-1	1	1	1	-1	-1	1	23.91
16	1	1	1	1	-1	1	-1	31.62
17	-1	-1	-1	-1	1	1	-1	19.28
18	1	-1	-1	-1	1	-1	1	38.47
19	-1	1	-1	-1	1	-1	1	25.89
20	1	1	-1	-1	1	1	-1	34.53
21	-1	-1	1	-1	1	-1	-1	18.80
22	1	-1	1	-1	1	1	1	40.06
23	-1	1	1	-1	1	1	1	28.91
24	1	1	1	-1	1	-1	-1	36.72
25	-1	-1	-1	1	1	-1	1	24.62
26	1	-1	-1	1	1	1	-1	33.65
27	-1	1	-1	1	1	1	-1	19.58
28	1	1	-1	1	1	-1	1	39.16
29	-1	-1	1	1	1	1	1	24.33
30	1	-1	1	1	1	-1	-1	31.15
31	-1	1	1	1	1	-1	-1	18.23
32	1	1	1	1	1	1	1	37.29
33	0	0	0	0	0	0	0	31.67
34	0	0	0	0	0	0	0	27.92
35	0	0	0	0	0	0	0	27.40
36	0	0	0	0	0	0	0	30.94
37	0	0	0	0	0	0	0	32.08

[a]Probiotic: 0.10-0.30% wt/vol; Sorbitol: 0.50-1.50% wt/vol; Mannitol: 0.50-1.50% wt/vol; Maltodextrin: 0.50-1.50% wt/vol; Hi-amylose maize: 0.50-1.50% wt/vol; Inulin: 0.50-1.50% wt/vol; FOS: 0.50-1.50% wt/vol.

[b]All factorial points are means of duplicate values.

TABLE 5.2. Analysis of variance and coefficient estimates for the evaluation of the first-order model.

Source of variation	Sum of squares	DF[a]	Mean square	F-value	P-value
Model[b]	6354.22	9	706.02	132.88	0.0001
Curvature	0.31	1	0.31	0.058	0.8099
Residual	308.17	58	5.31		
Lack-of-fit	153.00	22	6.95	1.61	0.0988
Pure error	155.17	36	4.31		
Correlation total	6662.70	68			

Factor:	Coefficient estimate	DF	Standard error	t-value	P-value
Probiotic (X_1)	7.13	1	0.29	24.73	0.0001[c]
Maltodextrin (X_4)	5.49	1	0.29	19.05	0.0001[c]
FOS (X_7)	3.58	1	0.29	12.41	0.0001[c]

[a]DF: degree of freedom.

[b]$R^2 = 0.9537$.

[c]Significant at alpha 0.05.

TABLE 5.3. Steepest ascent coordination path for all chosen factors in coded and natural levels.

Step		Coded factors			Natural factors[a]			Cholesterol removed (μg/ml)
		ξ_1	ξ_7	ξ_4	X_1	X_2	X_3	
1)	Base	0	0	0	0.20	1.00	1.00	25.61
	Δ	5	2.51	3.85	(5)(0.1) = 0.5	(2.51)(0.50) = 1.26	(3.85)(0.50) = 1.93	
2)	Base + Δ	5	2.51	3.85	0.70	2.26	2.93	37.66
3)	Base + 2Δ	10	5.02	7.70	1.20	3.52	4.86	50.31
4)	Base + 3Δ	15	7.53	11.55	1.70	4.78	6.79	55.16
5)	Base + 4Δ	20	10.04	15.40	2.20	6.04	8.72	51.88
6)	Base + 5Δ	25	12.55	19.25	2.70	7.30	10.65	47.24

[a]X_1: probiotic (% wt/vol), X_2: FOS (% wt/vol), X_3: maltodextrin (% wt/vol).

TABLE 5.4. Central composite design (CCD) combination matrix using coded levels and responses.

Standard run	Block[a]	Probiotic (X_1)	FOS (X_2)	Maltodextrin (X_3)	Responses[b]					
					Cholesterol removed (μg/ml)	Growth (%)	Substrate utilization (%)	Yield (growth/g substrate utilized)	Mean doubling time (min)	SCFA (mM)
1	1	-1	-1	-1	14.30	7.38	40.32	47.98	244.86	86.93
2	1	1	-1	-1	29.77	19.61	28.67	19.88	218.99	29.72
3	1	-1	1	-1	29.84	16.25	53.24	36.37	265.36	103.15
4	1	1	1	-1	32.42	27.79	50.56	34.80	188.54	50.77
5	1	-1	-1	1	29.84	27.65	49.39	41.30	247.70	74.91
6	1	1	-1	1	27.58	15.22	30.74	23.78	214.76	45.67
7	1	-1	1	1	29.77	21.62	51.37	35.75	265.37	71.93
8	1	1	1	1	16.33	9.94	52.63	36.56	215.11	81.23
9	1	0	0	0	48.98	43.46	50.38	60.93	242.68	60.10
10	1	0	0	0	45.39	43.03	59.20	58.02	246.22	66.44
11	1	0	0	0	49.53	47.53	53.22	63.69	246.13	67.80
12	1	0	0	0	45.78	40.42	53.43	60.27	242.06	66.36
13	2	-1.682	0	0	44.61	30.96	51.85	44.04	265.78	98.70
14	2	1.682	0	0	43.28	29.52	50.39	43.21	196.65	39.42
15	2	0	-1.682	0	33.20	27.80	33.36	38.44	242.94	33.27
16	2	0	1.682	0	35.17	28.29	64.63	23.95	243.23	60.58
17	2	0	0	-1.682	43.05	29.06	22.37	62.66	243.34	41.73
18	2	0	0	1.682	39.77	28.42	34.66	85.15	243.12	50.66
19	2	0	0	0	50.78	48.11	50.57	64.53	242.41	59.61
20	2	0	0	0	52.50	56.06	51.12	65.65	244.81	63.79

[a]1, first day of experiment; 2, second day of experiment.

[b]All factorial and axial points are means of duplicates.

TABLE 5.5. Analysis of variance of the second-order model[a] and coefficient estimates for the response Y_0 and factors X_1, X_2 and X_3.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model[b]	1778.16	9	197.57	20.76	0.0001
Residual	82.18	5	16.44		
Lack-of-Fit	70.47	5	14.09	3.71	0.1140
Pure error	15.19	4	3.80		
Total	1860.34	14	214.01		
Factor[c]	**Coefficient estimate**	**DF**	**Standard error**	**t-value**	**P-value**
Intercept	c = 50.61	1	1.27		
X_1	c_1 = 0.0081	1	0.83	0.0096	0.0003[d]
X_2	c_2 = 0.75	1	0.83	0.89	0.0001[d]
X_3	c_3 = -0.61	1	0.83	-0.73	0.0001[d]
X_1^2	c_{11} = -4.65	1	0.81	-5.72	0.0003[d]
X_2^2	c_{22} = -8.10	1	0.81	-9.96	0.0001[d]
X_3^2	c_{33} = -5.55	1	0.81	-6.82	0.0001[d]
X_1X_2	c_{12} = -3.01	1	1.09	-2.76	0.0222[d]
X_1X_3	c_{13} = -4.22	1	1.09	-3.87	0.0038[d]
X_2X_3	c_{23} = -3.69	1	1.09	-3.38	0.0081[d]

[a] $Y_0 = 50.61 + 0.0081X_1 + 0.75X_2 - 0.61X_3 - 4.65X_1^2 - 8.10X_2^2 - 5.55X_3^2 - 3.01X_1X_2 - 4.22X_1X_3 - 3.69X_2X_3$.
[b] $R^2 = 0.9540$.
[c] X_1: probiotic (% wt/vol), X_2: FOS (% wt/vol), X_3: maltodextrin (% wt/vol).
[d] Significant at alpha 0.05.

TABLE 5.6. Regression coefficients of the second-order equation[a] for the five responses[b].

Coefficient	Y_1	Y_2	Y_3	Y_4	Y_5
c	47.96	52.58	64.12	244.81	62.07
c_1	-0.20	-2.50[c]	-3.50	-22.12[c]	-16.78[c]
c_2	0.48	8.15[c]	-1.01	0.63	8.48
c_3	0.17	2.34[c]	2.65	1.82	1.33
c_{11}	-7.93[c]	-0.12	-9.75[c]	-5.93[c]	5.04[c]
c_{22}	-8.71[c]	-0.88	-14.14[c]	-1.74	-2.79
c_{33}	-8.46[c]	-8.12[c]	0.96	-1.69	-3.04
c_{12}	0.0079	3.61[c]	5.61	-8.53[c]	5.42[c]
c_{13}	-5.99[c]	-0.38	1.62	2.44	11.21[c]
c_{23}	-3.54[c]	-1.37	0.49	3.50	-0.59
R^2	0.9794	0.9437	0.9064	0.9682	0.9551
P-value	0.0001	0.0001	0.0012	0.0001	0.0001

[a] $Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3$.
[b] Y_1 = growth (%), Y_2 = susbtrate utilization (%), Y_3 = Yield (growth per g of substrate utilized), Y_4 = mean doubling time (min), Y_5 = SCFA (mM).
[c] Significant at alpha = 0.05.

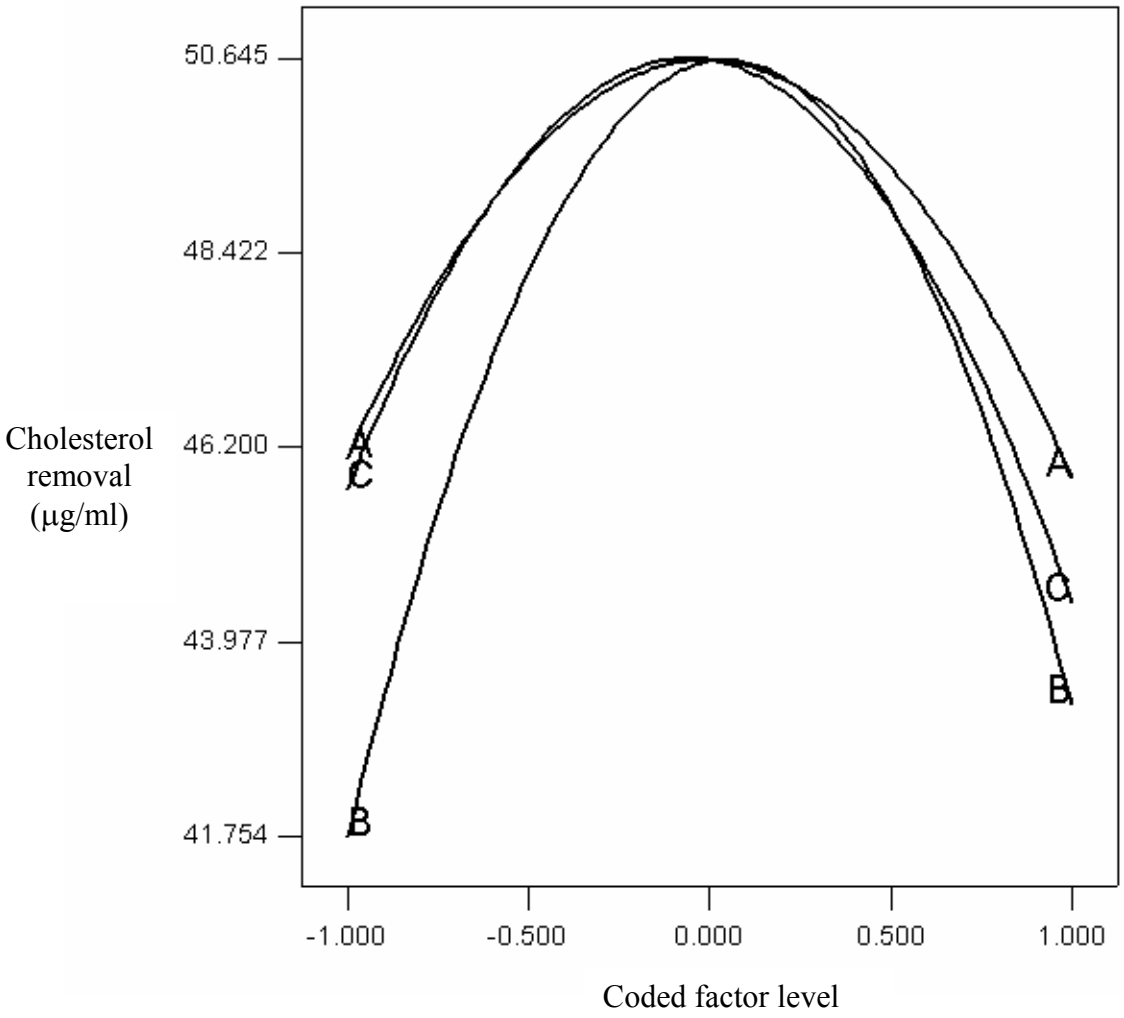
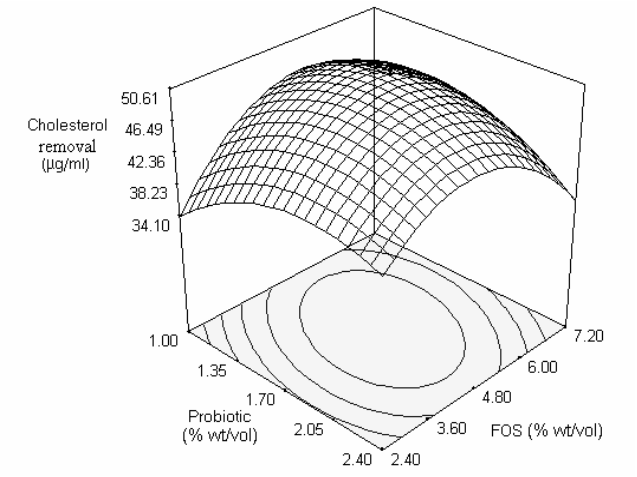
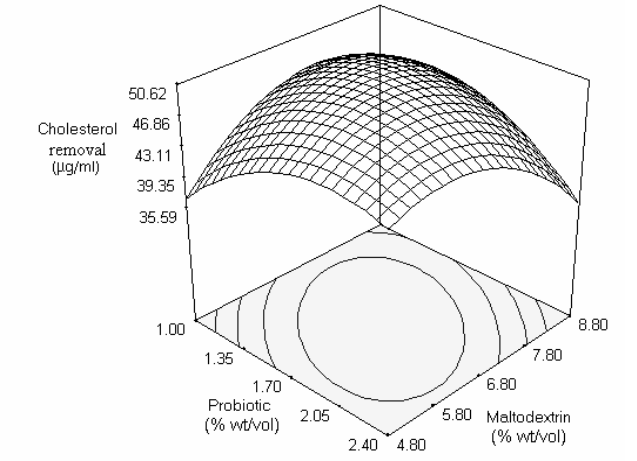


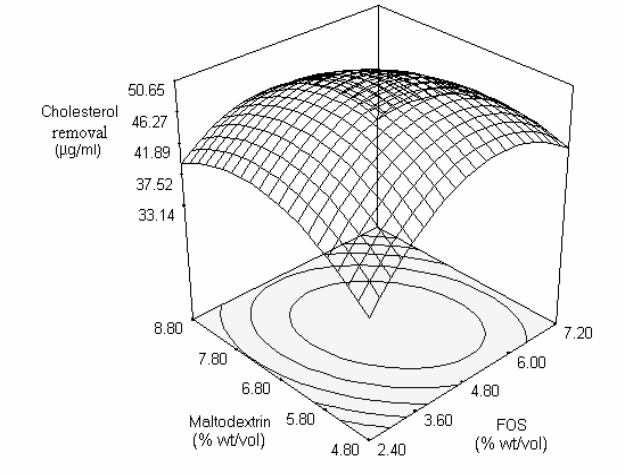
FIG. 5.1. Perturbation plot of probiotic (A), FOS (B) and maltodextrin (C).



(A)



(B)



(C)

FIG. 5.2. Response surface for cholesterol removal (µg/ml) from the effects of probiotic and FOS at 6.64% wt/vol maltodextrin (A), probiotic and maltodextrin at 4.95% wt/vol FOS (B), and FOS and maltodextrin at 1.71% wt/vol probiotic (C).

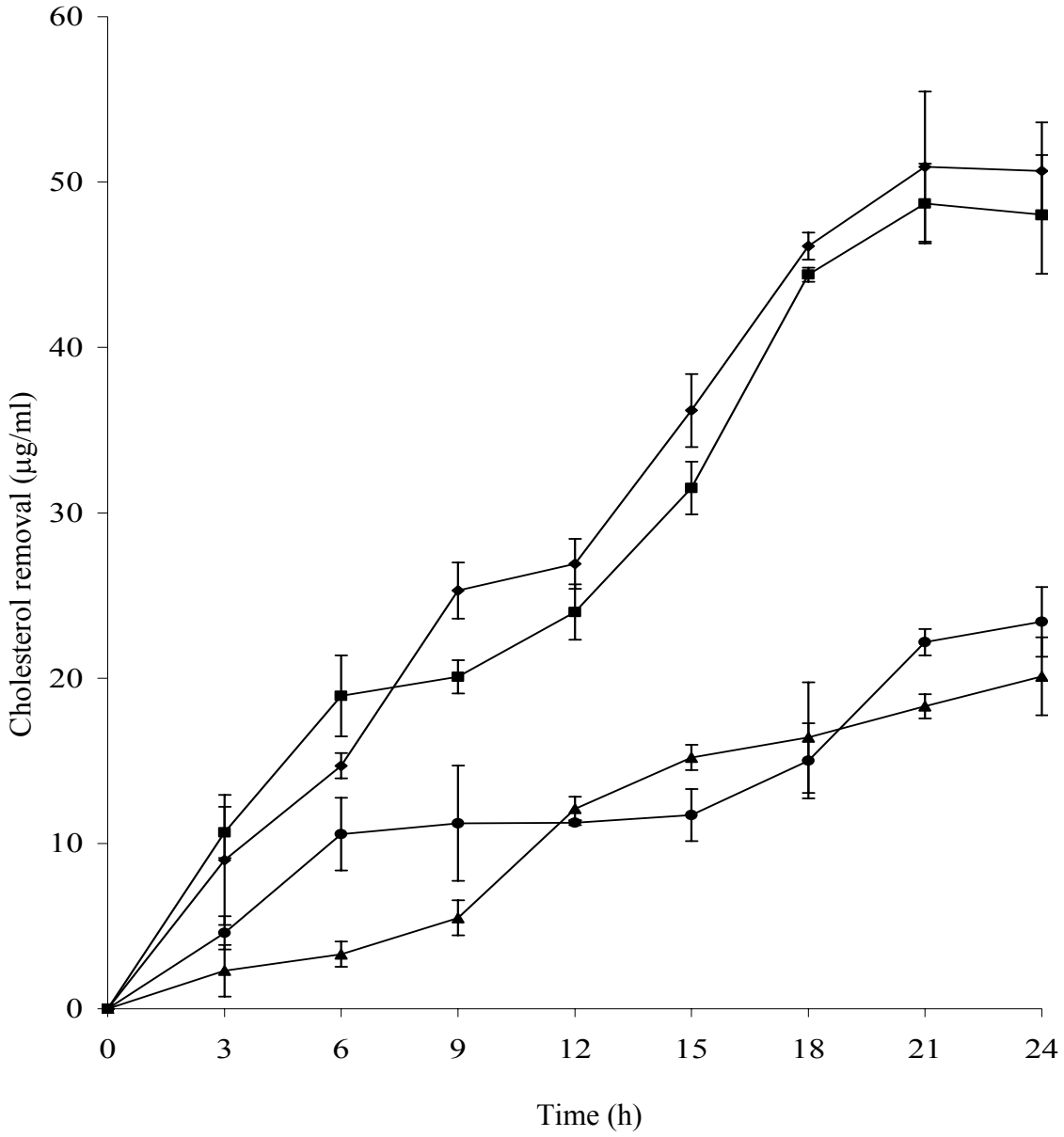


FIG. 5.3. Cholesterol removal by *L. casei* ASCC 292 in the optimum (♦), center-point (■), high-point (●) and low-point (▲) media, for the validation experiments. Factors combination for optimum medium were: probiotic 1.71% wt/vol, FOS 4.95% wt/vol and maltodextrin 6.64% wt/vol. Center-point medium were: probiotic 1.70% wt/vol, FOS 4.80% wt/vol and maltodextrin 6.80% wt/vol. High-point medium were: probiotic 2.40% wt/vol, FOS 7.20% wt/vol and maltodextrin 8.80% wt/vol, and low-point medium were probiotic 1.00% wt/vol, FOS 2.40% wt/vol and maltodextrin 4.80% wt/vol. Error bars represent standard error of means; n = 2.

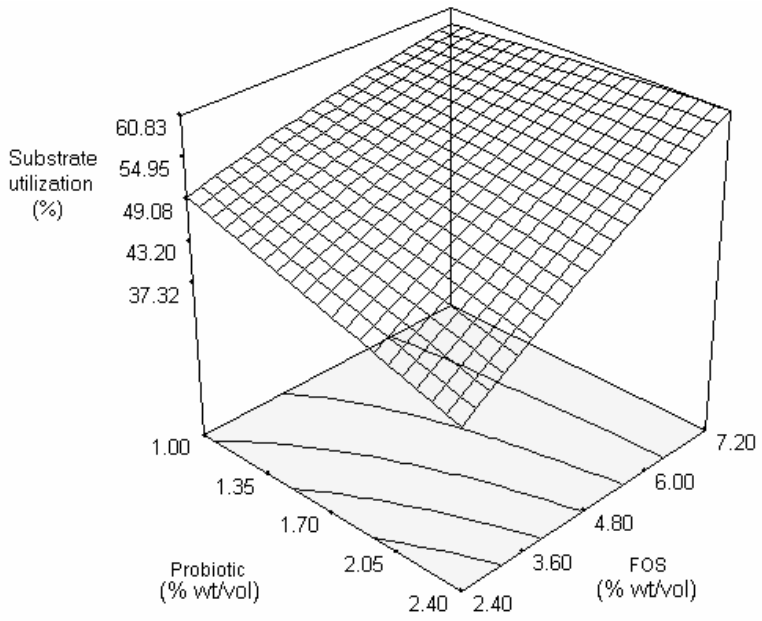


FIG. 5.4. Response surface for substrate utilization (%) from the effects of probiotic and FOS at 6.64% wt/vol maltodextrin.

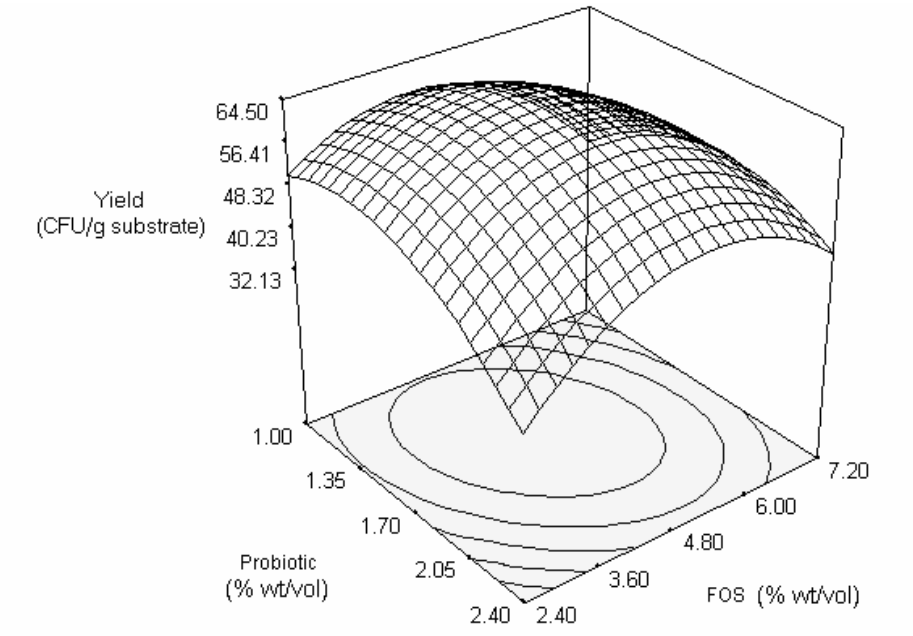


FIG. 5.5. Response surface for growth yield (CFU/g substrate) from the effects of probiotic and FOS at 6.64% wt/vol maltodextrin.

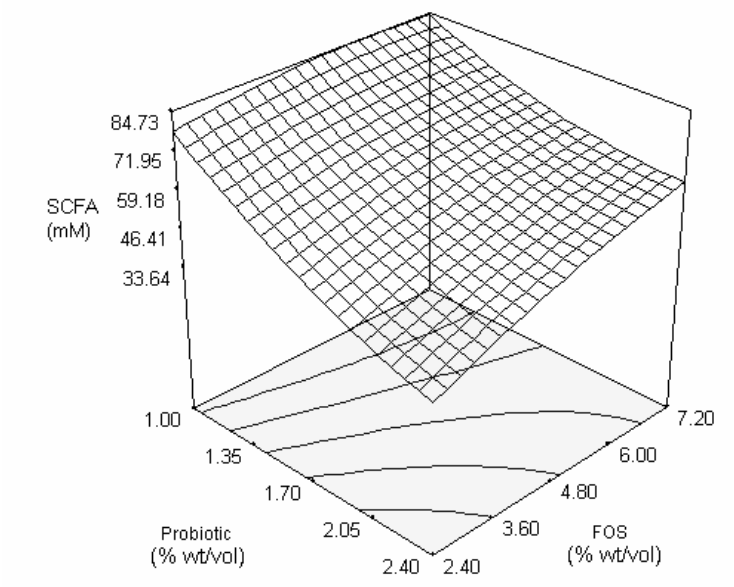


FIG. 5.6. Response surface for SCFA production (mM) from the effects of probiotic and FOS at 6.64% wt/vol maltodextrin.

5.2 Optimization of Growth of *Lactobacillus casei* ASCC 292 and Production of Organic Acids in the Presence of Fructooligosaccharide and Maltodextrin

5.2.1 Introduction

Probiotics are defined as 'viable microorganisms that have beneficial effects when digested to prevent and treat specific pathologic conditions' (Havenaar and Huis in't Veld 1992). The principle of using beneficial bacteria for conquering pathogens and balancing the gut environment has been recognized for many years. In fact, probiotics have been used for as long as people have eaten fermented foods (Rolfe 2000). However, Metchnikoff (1907) first time suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract. Probiotic microorganisms mainly consist of lactobacilli, streptococci, enterococci, lactococci and bifidobacteria. Over the years, *Lactobacillus casei* has been associated with improvement of intestinal disturbance, balancing of intestinal bacteria, lowering of faecal enzymes and inhibition of superficial bladder cancer (Spanhaak and others 1998).

The term prebiotic was introduced by Gibson and Roberfroid (1995) as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon." For a prebiotic to be classified as a food ingredient, it must neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract, and be a selective substrate for beneficial bacteria in the colon and alter the colonic microflora towards a healthier composition (Collins and Gibson 1999).

Most widely researched prebiotics fall in the group of oligosaccharides, especially oligofructose. The IUB-IUPAC joint commission on biochemical nomenclature and the AOAC defined oligofructose as fructose oligosaccharide or fructooligosaccharide (FOS) containing 2 to 10 monosaccharide residues linked by glycosidic bonds (Niness 1999). Maltodextrins are maltooligosaccharides with the degree of polymerization (DP) ranging from 3 to 9 (Flickinger 2000). Colonic bacteria generally ferment the undigested carbohydrate into hydrogen, carbon dioxide, and organic acids such as acetate, propionate and butyrate. Strains of *L. casei* reportedly utilized xylitol to produce organic acids such as lactic and acetic acids (Badet and others 2004).

This section has been published: *Liong, MT and Shah, NP. 2005. Optimization of growth of Lactobacillus casei ASCC 292 and production of short-chain fatty acid in the presence of fructooligosaccharide and maltodextrin.* ***J Food Sci*** *70: M113-M120.*

The organic acids may have beneficial effects on human glucose and lipid metabolism. Acetate can reduce the concentration of serum fatty acids, which are an important factor in lowering glucose utilization by tissues and inducing insulin resistance. Long-term dietary supplementation with propionate was shown to decrease blood glucose in rats and in humans, while butyrate is mostly used by colonocytes as an energy source (Rizkalla and others 2000).

The term synbiotic is used when a product contains both probiotics and prebiotics. A combination of prebiotic and probiotic has synergistic effects promoting growth of existing strains of beneficial bacteria in the colon, as well as improving the survival, implantation and growth of newly added probiotic strains. Considering that the word alludes to synergism, this term was debated to be reserved for products in which prebiotic compounds selectively favour probiotic organisms (Schrezenmeir and De Vrese 2001), such as the combination of FOS with a bifidobacterial strain or lactitol in conjunction with lactobacilli (Gibson and Roberfroid 1995).

Response surface methodology (RSM) is a statistical method that uses quantitative data from appropriate experiments to determine and simultaneously solve multivariate equations. Response surface models may involve just main effects and interactions or they may also have quadratic and possibly cubic terms to account for curvature. RSM designs are used to find improved or optimal process settings, troubleshoot process problems and weak points, and make a product or process more robust against external and non-controllable influences (Montgomery 1996). RSM has been successfully utilized to optimize the composition of microbiological media (Lee and Chen 1997) and study the alteration patterns of bacterial cellular fatty acid composition (Guerzoni and others 2001). Conventional methods (such as one factor at one time) require a large number of experiments to describe the effect of individual factors, are time consuming and a proper statistical method was not established to distinguish the interaction effects from main effects (Logothetis and Wynn 1989).

Although the beneficial effects of both synbiotics and organic acids have been widely documented (Swanson and others 2002b; Pereira and others 2003; Hsu and others 2004), the interaction patterns of such combinations are not well understood. Thus, the aims of this study were to optimize the growth of *L. casei* ASCC 292 using various inoculum sizes in the presence of FOS and maltodextrin, and to analyse the combined effect of inoculum size and prebiotics on organic acids production patterns.

5.2.2 Materials and Methods

5.2.2.1 Bacteria and media preparation

The bacteria and prebiotics used were prepared as described in section 5.1.2.1.

5.2.2.2 Growth of *L. casei* ASCC 292 in presence of prebiotics

The growth was determined using the pour plate method as described in section 5.1.2.3. The growth was expressed as the difference in $\log_{10}$ CFU/mL between the initial count obtained at time = 0 and that at the end of the incubation period.

5.2.2.3 Determination of organic acids

Organic acids were determined using HPLC as described in section 5.1.2.6. The HPLC system was equipped with a UV/Vis detector (Varian, Walnut Creek, CA, USA) set at 220 nm. An Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) was maintained at 65°C, while the degassed mobile phase (0.009 M H_2SO_4) was used at a flow rate of 0.6 mL/min.

5.2.2.4 Experimental design and statistical analyses

The response surface methodology was applied with three independent factors namely, *L. casei* ASCC 292 (X_1), FOS (X_2) and maltodextrin (X_3) to generate a central composite design (CCD) matrix. The treatment combinations were designed in 2 blocks and all the experiments were carried out in 2 days. The first block, representing the first day of experiment, contained the factorial runs accompanied by 4 center runs. The second block, representing the second day of experiment, contained the axial runs accompanied by 2 center runs. This matrix generated 20 experimental runs including 5 middle point runs. All experimental points are presented as the mean values of a triplicate determination.

Preliminary data using the conventional screening (data not shown) indicated that the best concentrations for growth were: 1.70% w/v of inoculum size, 4.80% w/v of FOS and 6.80% w/v of maltodextrin. Thus, these values were set to zero as a coded level. Factors were coded according to the following equation:

$$X_i = (x_i - X_0)/\Delta X_i$$

where X_i is the coded value of an independent factor; x_i is the real value of the factor; X_0 is the real value of the factor at center point; and ΔX_i is the step change value (Kong and others 2004). The actual factor levels and their corresponding coded levels are shown in Table 5.7.

To generate a CCD matrix with three factors, an alpha value of ±1.682 was used, and the complete design matrix is presented in Table 5.7. All modeling and statistical analyses were performed using the Design Expert version 5.07 software (Stat-Ease Corp., Minneapolis, MN, U.S.A.).

A validation experiment was conducted to confirm the validity and reproducibility of the model. The growth was assessed using the optimum-point, center-point, high-point and low-point media that were produced from the prediction. These actual results were compared with the predicted values by the model.

5.2.3 Results

5.2.3.1 Optimization of growth

We previously conducted a screening experiment using the conventional method. Eleven strains of human-derived lactobacilli were screened for their acid and bile tolerance capabilities. Out of these, one strain of *L. acidophilus* and one strain of *L. casei* was selected for further optimization to develop a synbiotic. Our preliminary data also showed that inoculum size, maltodextrin and FOS were significant factors that influenced growth (section 5.1.3.1). Thus, *L. casei* ASCC 292 and the prebiotics maltodextrin and FOS were selected for optimization. The optimization was performed using CCD with fixed middle point of inoculum size (1.70% w/v), FOS (4.80% w/v) and maltodextrin (6.80% w/v), and alpha value of ±1.682. The design matrix and the response of growth (Y_0) are presented in Table 5.7. The adequacy and fitness of the model were evaluated by ANOVA, and is presented in Table 5.8. The regression analyses revealed that both linear and cubic terms were insignificant, while the quadratic model was most suitable. This suggested that the model represented the data accurately in the experimental region ($P < 0.0001$). This was supported by insignificant lack-of-fit. The coefficient of regression showed that only 2.88% of total variation was not explained by the model. All these indicated that second-order terms were sufficient and higher-order terms were not necessary.

The influence of the factors studied on the growth of *L. casei* ASCC 292 is presented in Table 5.9. The linear effects of all factors were insignificant, while the quadratic effects of all factors were found to be significant. Considering that the quadratic effects had similar probability values, it must be noted that the individual *t* value of maltodextrin was highest, indicating strongest quadratic effect. Interaction term of inoculum size and FOS was not significant at alpha 0.05, while the interactions between inoculum size and maltodextrin, and FOS and maltodextrin were significant on the growth of *L. casei* ASCC 292. It must be also

noted that the *t* value of inoculum size-maltodextrin was the highest among all interaction terms, indicating the strongest interaction effect.

Factor effects were further studied using perturbation plots, as described previously (Oh and others 1995). These plots illustrate the changes of each factor as it moves from the chosen reference point, with all other factors held constant at the reference value. In this study, as one particular chosen factor was assessed, the other factors were held constant at the optimum point. We defined the response surface model as obtained from Table 5.9 as $Y = f(X_1, X_2, X_3)$. The optimum points of the factors are defined as (X^*_1, X^*_2, X^*_3), which in our experiments would be (-0.047, 0.007, 0.065). Thus, the perturbation effect of X_1 is defined as:

$$Y(X_1) = f(X_1, X^*_2, X^*_3).$$

Similarly, the perturbation effects of X_2 and X_3 would be:

$$Y(X_2) = f(X^*_1, X_2, X^*_3)$$

$$Y(X_3) = f(X^*_1, X^*_2, X_3)$$

The perturbation effect curves were produced with the vertical axis representing $Y(X_j)$ and the horizontal axis representing X_j. In this study, all X_j have common coded levels, and thus the horizontal axis would represent the common coded levels. By overlying all perturbation curves, we obtain the perturbation plot. Figure 5.7 shows the perturbation plot of all the factors studied. All factors showed a significant quadratic effect, with all curves showing very prominent changes.

The best explanatory equation to fit the second-order model and subsequently produce the response surface was expressed as:

$$Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3$$

where $c \ldots c_{23}$ are regression coefficients and X_1, X_2, X_3 are the coded independent factors. The response surface was produced by plotting two factors at a time, with the vertical axis representing growth ($\log_{10}$ CFU/mL) and the other two horizontal axes representing the actual levels of two explanatory factors. Thus, in each plot, the factor that was not represented by the two horizontal axes was fixed at its actual optimum level. In this study, the second-order regression model involved three factors, generating 3 linear, 3 quadratic and 3 interaction terms. Response surface was produced (Figures 5.8A and 5.8B) based on the second-order equation:

$$Y_0 = 1.85 - 0.010X_1 + 0.0094X_2 + 0.026X_3 - 0.27X_1^2 - 0.30X_2^2 - 0.29X_3^2 - 0.036X_1X_2 - 0.24X_1X_3 - 0.10X_2X_3.$$

An optimum point was produced with optimum growth obtained at 1.855 $\log_{10}$CFU/mL. The combination that produced the optimum point was (X_1, X_2, X_3) = (-0.047, 0.007, 0.065). The original levels that correlated with those coded values were found to be 67% w/v of inoculum size, 4.82% w/v of FOS and 6.93% w/v of maltodextrin. From the coefficient estimates (Table 5.9), the interaction terms of X_1-X_3 and X_2-X_3 were found to be significant. It must be noted that the coefficient estimate of these interaction terms had negative signs (X_{13} = -4.08, X_{23} = -3.46). These negative signs may suggest that for an increase in the response, the coded levels of X_1, X_3 and X_2, X_3 must have different signs, either one must be higher than zero and the other lower than zero. From the three dimensional plot of inoculum size and maltodextrin (Figure 5.8A), it was found that when optimum point was achieved, the coded levels of X_1 was –0.047 and X_3 was 0.065. However, it must also be noted that the interactions of X_2 and X_3 also showed a negative sign, but response surface showed that optimum was achieved at X_2 = 0.007 and X_3 = 0.065, which would produce a positive sign instead. This may be due to other terms that may dominate this particular interaction term (Oh and others 1995). Considering that the lack-of-fit test was insignificant, other higher terms would not have contributed to this, thus, we postulate that the linear term might have played a role.

All these predictions by the regression model were further ascertained by a validation experiment. We compared the growth of *L. casei* ASCC 292 over a 24 h period using four different media namely, the optimum-point medium (inoculum size: 1.67% w/v; FOS: 4.82% w/v; maltodextrin: 6.93% w/v), the center-point medium (inoculum size: 1.70% w/v; FOS: 4.80% w/v; maltodextrin: 6.80% w/v), the high-point medium (inoculum size: 2.40% w/v; FOS: 7.20% w/v; maltodextrin: 8.80% w/v) and the low-point medium (inoculum size: 1.00% w/v; FOS: 2.40% w/v; maltodextrin: 4.80% w/v). Growths as predicted from the model using optimum-point, center-point, high-point and the low-point media were 1.86, 1.85, 0.64 and 0.59 $\log_{10}$ CFU/mL, respectively. Our validation results (Figure 5.9) showed that the growths obtained from the optimum-point, center-point, high-point and the low-point media were 1.85, 1.76, 0.66 and 0.62 $\log_{10}$ CFU/mL, respectively. This produced an error of 0.54%, 4.86%, 3.13% and 5.08%, respectively for the four media as compared to the prediction. The small error proved the validity of the model and the reproducibility of the prediction.

5.2.3.2 Production of organic acids

The response obtained for lactic, acetic, propionic, butyric and formic acid is presented in Table 5.7, while the statistical analyses with coefficient estimates and the significance of each response model are presented in Table 5.10.

The response surface of lactic acid (Y_1) is shown in Figure 5.10, and was generated based on the following coded factor equation:

$$Y_1 = 74.71 - 16.57X_1 + 1.11X_2 + 0.51X_3 - 1.66X_1^2 + 7.20X_2^2 - 2.06X_3^2 + 4.92X_1X_2 + 10.45X_1X_3 - 1.97X_2X_3.$$

The response surface indicated that lactic acid was highly influenced by the changes in inoculum size. Increasing inoculum size caused a reduction in lactic acid production, and this was more prominent at lower concentrations of the prebiotics. In contrary, increasing concentration of maltodextrin at higher inoculum size contributed to an increase in lactic acid production (Figure 5.10). This may be contributed by the fact that higher population of cells requires larger amount of substrate and subsequently produce higher amount of lactic acid as the end-product. It is interesting to note that at low levels of inoculum size, lactic acid production was highest (Figure 5.10), while the growth at these regions was lowest (Figure 5.8).

The response surface of acetic acid (Y_2) is shown in Figure 5.11, and was generated based on the following coded factor equation:

$$Y_2 = 26.72 - 3.81X_1 + 3.50X_2 + 1.48X_3 - 2.23X_1^2 - 1.42X_2^2 + 0.47X_3^2 - 1.46X_1X_2 + 3.54X_1X_3 - 3.04X_2X_3.$$

The highest amount of acetic acid was obtained from the predicted inoculum size, concentration of FOS and maltodextrin at 1.00% w/v, 2.40% w/v and 4.80% w/v, respectively. At the optimum point of growth, 27.00 m*M* of acetic acid was produced.

Similar to lactic acid, production of acetic acid reduced with increasing inoculum size (Figure 5.11). Increasing concentration of FOS contributed to the increased acetic acid production, as can be seen from the ascending response surface and the significant linear effect of FOS. At lower concentration of maltodextrin (Figure 5.11A), acetic acid production reduced with increasing inoculum size, while at higher concentration of maltodextrin (8.80% w/v), the production of acetic acid appeared to increase with increasing inoculum size from 1.00% w/v to 1.71% w/v and reached the highest amount (28.60 m*M*). Further increase in inoculum size beyond that caused a decrease in production of acetic acid. This indicated that the production of acetic acid was strongly influenced by inoculum size, which was supported

by the significant individual linear and quadratic effect. Lower concentration of substrate (maltodextrin) may not be sufficient for an increase in bacterial population, and subsequently contributed to a decrease in acetic acid production. However, higher concentration of maltodextrin appeared only suitable for bacterial population with inoculum size up to 1.71% w/v, and not beyond that level. Production of acetic acid was lowest when both the prebiotics were at their lowest concentration, and increased with increasing concentrations of both substrates (Figure 5.11B).

The response surface of propionic acid (Y_3) is shown in Figure 5.12, and was generated based on the following coded factor equation:

$$Y_3 = 28.89 - 11.76X_1 + 4.62X_2 - 0.40X_3 + 7.54X_1^2 - 1.29X_2^2 - 3.37X_3^2 + 6.43X_1X_2 + 6.93X_1X_3 + 2.55X_2X_3.$$

The production of propionic acid decreased with increasing inoculum size (Figure 5.12). FOS exhibited more linear effect, which was supported by the insignificant quadratic effect (Table 5.10). Although all interaction effects were significant, only the interaction of inoculum size-FOS (Figure 5.12A) and inoculum size-maltodextrin (Figure 5.12B) showed prominent response surface changes, with inoculum size being the dominant factor.

The response surface of butyric acid (Y_4) and formic acid (Y_5) are shown in Figures 5.13 and 5.14, and were generated based on the following coded factor equations:

$$Y_4 = 8.02 - 1.22X_1 + 0.36X_2 + 0.25X_3 - 0.83X_1^2 - 0.63X_2^2 - 0.71X_3^2 + 0.45X_1X_2 + 0.74X_1X_3 - 0.10X_2X_3$$

$$Y_5 = 16.66 - 1.61X_1 + 0.31X_2 - 2.20X_3 - 3.16X_1^2 - 2.11X_2^2 - 2.60X_3^2 - 0.004X_1X_2 + 1.47X_1X_3 - 0.20X_2X_3.$$

The response surfaces illustrated significant quadratic effect for all interaction terms. Optimal or near optimal regions were found. Maximum production of butyric acid (Figure 5.13) was predicted to be at 8.51 m*M*, obtained from the combination of 1.10% w/v of the inoculum size, 4.80% w/v of FOS and 6.25% w/v of maltodextrin, while maximum production of formic acid (Figure 5.14) was predicted to be at 16.60 m*M*, obtained from the combination of 2.11% w/v of the inoculum size, 5.03% w/v of FOS and 5.86% w/v of maltodextrin. At the optimum point for growth, the production of butyric and formic acid was predicted to be at 8.08 m*M* and 16.43 m*M*, respectively. Response surfaces produced indicated that the region for optimum growth (Figure 5.8) was similar to that of the maximum or near-maximum for both butyric acid and formic acid (Figures 5.13 and 5.14). It appears that the production of these acids, under the influence of FOS and maltodextrin was growth associated.

5.2.4 Discussion

Our results showed that the increased of inoculum size beyond 1.67% w/v decreased the growth of *L. casei* ASCC 292. This could be explained by substrate limitation with increasing cell numbers. However, increasing the concentration of FOS and maltodextrin beyond 4.82% w/v and 6.93% w/v, respectively, also caused a reduction in the growth. It was previously reported that the uptake of FOS and its hydrolysis by *L. paracasei* 1195 were induced by higher oligosaccharides and repressed by the products of their hydrolysis (Kaplan and Hutkins 2003). We postulate that an increase in hydrolysis of FOS and maltodextrin beyond 4.82% and 6.93% w/v, respectively, would have generated significant amount of end-products that repressed the growth of *L. casei* ASCC 292. Kaplan and Hutkins (2003) also reported inhibited uptake of FOS, as a result of excess glucose, fructose, or sucrose in the media, indicating that these sugars may be transported by the FOS transport system.

Hydrolysis of prebiotic in the human intestine is affected by a number of bacterial cell-associated hydrolases that can hydrolyse a range of carbohydrates. Fermentation yields metabolizable energy for microbial growth and maintenance and also metabolic end-products. In adult humans, the principal end-products are organic acids together with gasses including CO_2, CH_4 and H_2 (Topping and Clifton 2001). Thus, we further analysed the production patterns of organic acids as influenced by different inoculum sizes and various concentrations of FOS and maltodextrin.

Our results showed that at lowest inoculum size, production of lactic acid was highest. Amrane and Prigent (1998) observed an inhibition effect due to lactic acid addition at different levels in the range of 0–50 g/l on the growth rate of *Lb. helveticus*, and total growth inhibition at 5 m*M* of undissociated lactic acid. The inhibition effect of lactic acid was also suggested to be associated with total lactic acid, lactates, or undissociated lactic acid (Adolf and others 2002). As high concentration of lactic acid was produced in our experimental regions, we anticipate that inhibition of growth occurred in those regions.

Majority of lactic acid bacteria utilizes sugar via the Embden-Meyerhof-Parnas pathway and yield more than 85% lactic acid, or the hexose monophosphate shunt and yield 50% lactic acid, as well as ethanol, acetic acid and carbon dioxide (Schwan 1998). Lactic acid bacteria have the genes that mediate the conversion of maltose and maltodextrins to glucose and glucose-1-phosphate, with maltose phosphorylase and maltodextrin phosphorylase, respectively. When glucose was added to maltose-grown lactococci, the amount of enzyme in cell extract decreased rapidly (Nilsson and Radstrom 2001). Thus, it appears that at high concentrations of maltodextrin, inoculum size beyond 1.71% w/v may

have generated inhibitory amount of glucose, which was illustrated with a decrease in acetic acid production. This was also supported by the decrease in growth at high concentration of maltodextrin, and at an inoculum size larger than 1.67% w/v.

The general reaction of organic acids production and overall stoichiometry for a hexose has been summarized as: $59C_6H_{12}O_6 + 38H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3\ CH_2COOH + 18\ CH_3\ CH_2\ CH_2COOH + 96\ CO_2 + 268\ H^+$ + heat + additional bacteria. Normal faecal organic acids are found to be in order as predicted from the equation, with acetate > propionate ≥ butyrate (Topping and Clifton 2001). However, our study showed that lower amount of acetic acid was produced than propionic acid. It appears that the prebiotics used either enhanced the production of propionic acid or reduced the production of acetic acid. Our results here showed that the production of propionic acid was strongly improved by maltodextrin but was least influenced by FOS.

Response surfaces of butyric acid and acetic acid showed similarity, indicating a good correlation between the productions of both acids. Miller and Wolin (1996) found that free radioactive acetate was easily incorporated into acetyl-S coenzyme A and then into butyrate. Acetate formed from $^{14}CO_2$ or CO_2 produced from the 3 and 4 carbons of glucose was also incorporated into butyrate. Asanuma and others (2003) found that the rate of butyrate production increased with an increase in acetate concentration in the medium, and the authors confirmed that acetate stimulates butyrate production by acting as a CoA acceptor in the butyryl-CoA or acetate CoA transferase reaction.

5.2.5 Conclusions

The growth of *L. casei* ASCC 292 was optimized at the inoculum size of 1.67% w/v, concentration of FOS at 4.82% w/v and that of maltodextrin at 6.93% w/v. RSM was reliable in developing a model, optimization of factors, and analysis of interaction effects. Analyses of response surface proposed that: a) growth was repressed at high concentrations of FOS and maltodextrin, possibly by end-product inhibition; b) lactic and acetic acids were enhanced by increasing inoculum size, but growth was inhibited at high levels of lactic acid; c) the production of acetic acid was possibly inhibited by end-product from hydrolysis of maltodextrin; d) fermentation of maltodextrin enhanced the production of propionic acid; e) the production of butyric acid was related to that of acetic acid.

Table 5.7 Combination matrix of the central composite design using coded levels and responses

Standard run	Block[a]	Factors[b]			Responses[c]					
		Inoculum size (X_1)	FOS (X_2)	Maltodextrin (X_3)	Growth (log_{10}CFU/mL)[d]	Acetic acid (m*M*)	Butyric acid (m*M*)	Propionic acid (m*M*)	Formic acid (m*M*)	Lactic acid (mM)
1	1	-1	-1	-1	0.351	19.65	7.64	59.64	12.59	107.05
2	1	1	-1	-1	0.933	15.41	4.01	10.30	11.19	56.69
3	1	-1	1	-1	0.695	42.47	8.52	52.16	13.21	107.60
4	1	1	1	-1	1.056	19.84	4.76	26.17	12.59	59.52
5	1	-1	-1	1	1.183	27.66	7.56	39.69	4.06	94.03
6	1	1	-1	1	0.724	25.02	4.93	15.72	9.34	68.06
7	1	-1	1	1	1.029	25.78	6.08	40.07	4.67	69.31
8	1	1	1	1	0.506	29.85	7.21	44.17	9.13	80.44
9	1	0	0	0	1.650	28.92	8.52	32.66	16.78	71.06
10	1	0	0	0	1.841	28.01	8.17	30.26	17.10	78.56
11	1	0	0	0	1.805	27.69	8.37	31.74	16.77	77.40
12	1	0	0	0	1.729	27.79	8.07	30.50	16.13	76.26
13	2	-1.682	0	0	1.324	26.03	7.47	65.20	2.56	101.34
14	2	1.682	0	0	1.263	10.23	2.86	26.33	11.05	34.15
15	2	0	-1.682	0	1.189	15.20	4.96	13.11	9.22	85.60
16	2	0	1.682	0	1.210	25.64	6.46	28.48	10.30	99.96
17	2	0	0	-1.682	1.243	23.01	4.75	13.97	10.67	58.86
18	2	0	0	1.682	1.216	28.54	6.26	15.85	6.09	74.34
19	2	0	0	0	1.827	23.28	7.31	29.02	17.66	78.61
20	2	0	0	0	1.947	28.57	8.53	26.69	16.74	70.15

[a]1, first day of experiment; 2, second day of experiment.

[b]X_1 = (inoculum size – 1.70)/0.70; X_2 = (FOS – 4.80)/1.50; X_3 = (maltodextrin – 6.80)/2.00.

[c]All experimental points are means of triplicates; n = 3.

[d]Growth was expressed as the difference in log_{10} CFU/mL between the initial count obtained at time = 0 and that at the end of the incubation period.

Table 5.8 Analysis of variance of the second-order model[a] for the response Y_0 and factors X_1, X_2 and X_3[b].

Source	Sum of squares	DF	Mean square	F-value	P-value
Regression:					
Linear	0.012	3	0.0041	0.017	0.9969
Quadratic	3.53	6	0.59	50.48	< 0.0001[d]
Cubic	0.015	4	0.0038	0.21	0.9205
Model[c]	3.54	9	0.39	33.77	< 0.0001[d]
Residual	0.10	9	0.012		
Lack-of-Fit	0.076	5	0.015	2.13	0.2417
Pure error	0.029	4	0.0071		
Correlation total	4.01	19			

[a]$Y_0 = 1.85 - 0.010X_1 + 0.0094X_2 + 0.026X_3 - 0.27X_1^2 - 0.30X_2^2 - 0.29X_3^2 - 0.036X_1X_2 - 0.24X_1X_3 - 0.10X_2X_3$.

[b]X_1: inoculum size (% w/v), X_2: FOS (% w/v), X_3: maltodextrin (% w/v).

[c]$R^2 = 0.9712$.

[d]Significant at alpha 0.05.

Table 5.9 Coefficient estimates for the response Y_0[a] and factors X_1, X_2 and X_3[b].

Factor[c]	Coefficient estimate	DF	Standard error	t-value	P-value
Intercept	c = 1.85	1	0.044		
X_1	$c_1 = -0.010$	1	0.029	-0.36	0.7293
X_2	$c_2 = 0.0095$	1	0.029	0.32	0.7534
X_3	$c_3 = 0.026$	1	0.029	0.90	0.3900
X_1^2	$c_{11} = -0.27$	1	0.028	-9.42	< 0.0001[c]
X_2^2	$c_{22} = -0.30$	1	0.028	-10.59	< 0.0001[c]
X_3^2	$c_{33} = -0.29$	1	0.028	-10.22	< 0.0001[c]
X_1X_2	$c_{12} = -0.036$	1	0.038	-0.94	0.3736
X_1X_3	$c_{13} = -0.24$	1	0.038	-6.31	0.0001[c]
X_2X_3	$c_{23} = -0.10$	1	0.038	-2.75	0.0225[c]

[a]$Y_0 = 1.85 - 0.010X_1 + 0.0094X_2 + 0.026X_3 - 0.27X_1^2 - 0.30X_2^2 - 0.29X_3^2 - 0.036X_1X_2 - 0.24X_1X_3 - 0.10X_2X_3$.

[b]X_1: inoculum size (% w/v), X_2: FOS (% w/v), X_3: maltodextrin (% w/v).

[c]Significant at alpha 0.05.

Table 5.10 Regression coefficients of the second-order equation[a] for the five responses[b].

Coefficient	Y_1	Y_2	Y_3	Y_4	Y_5
c	74.74	26.72	28.89	8.02	16.66
c_1	-16.57[c]	-3.81[c]	-11.76[c]	-1.22[c]	1.61[c]
c_2	1.11	3.50[c]	4.62[c]	0.36	0.31
c_3	0.51	1.48	-0.40	0.25	-2.20[c]
c_{11}	-1.66	-2.23[c]	7.54[c]	-0.83[c]	-3.16[c]
c_{22}	7.20[c]	-1.42	-1.29	-0.63[c]	-2.11[c]
c_{33}	-2.06	0.47	-3.37[c]	-0.71[c]	-2.60[c]
c_{12}	4.92	-1.46	6.43[c]	0.45	-0.004
c_{13}	10.45[c]	3.45[c]	6.93[c]	0.74[c]	1.47[c]
c_{23}	-1.97	-3.04[c]	2.55[c]	-0.10	-0.20
R^2	0.9063	0.8642	0.9891	0.9260	0.9474
P-value	0.0012	0.0055	0.0001	0.0004	0.0001

[a]$Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3$.

[b]Y_1 = lactic acid (m*M*), Y_2 = acetic acid (m*M*), Y_3 = propionic acid (m*M*), Y_4 = butyric acid (m*M*), Y_5 = formic acid (m*M*).

[c]Significant at alpha = 0.05.

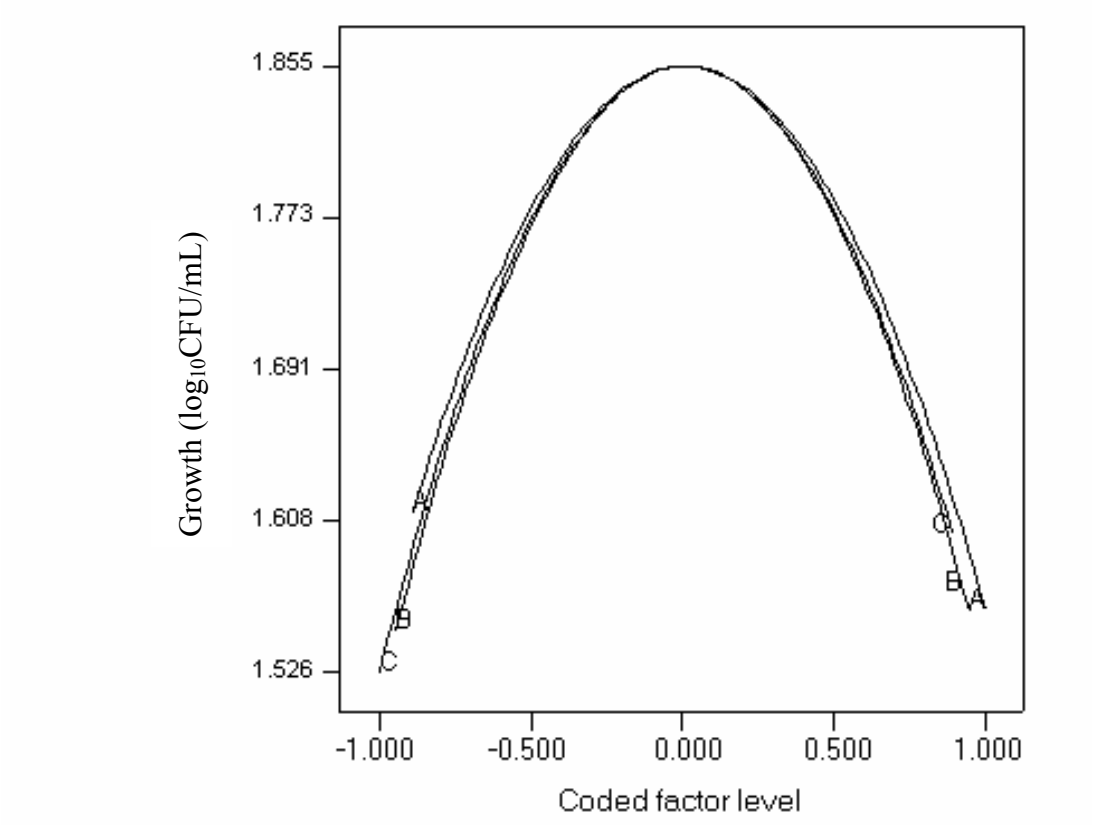
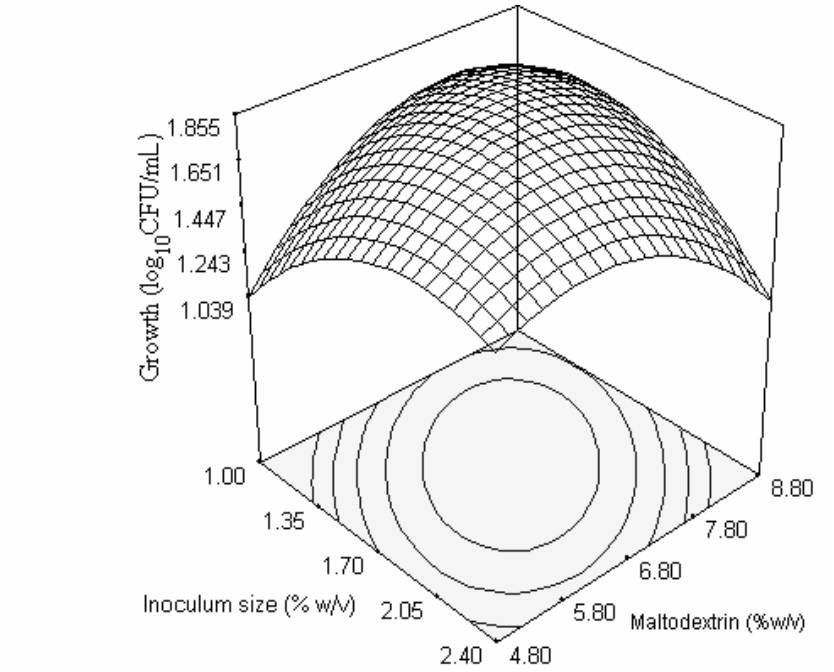
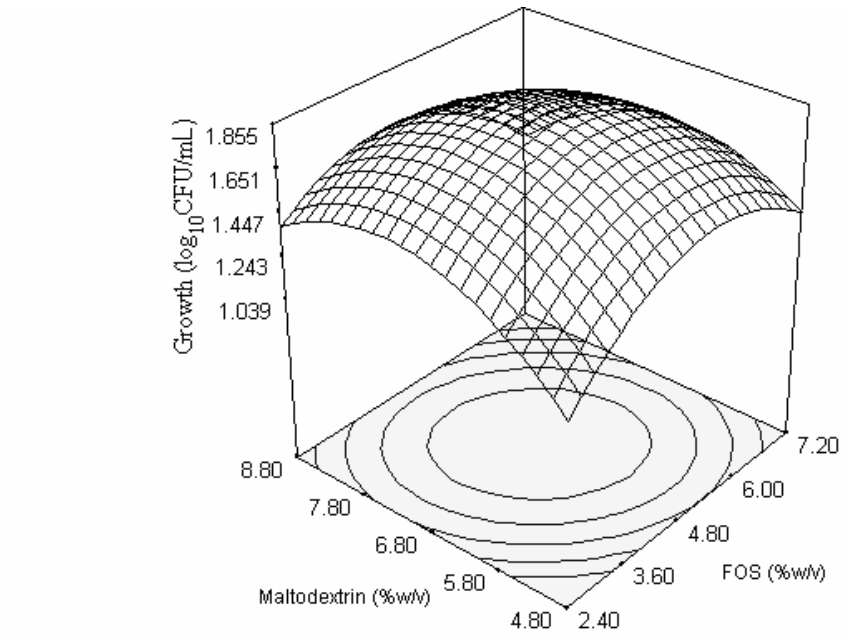


Figure 5.7 Perturbation plot of inoculum size (A), FOS (B) and maltodextrin (C). Growth was expressed as the difference in $\log_{10}$ CFU/mL between the initial count obtained at time = 0 and that at the end of the incubation period.



(A)



(B)

Figure 5.8 Response surface for growth (log_{10}CFU/mL) from the effects of (A) inoculum size and maltodextrin at 4.82% w/v of FOS, and (B) FOS and maltodextrin at 1.67% w/v of inoculum size. Growth was expressed as the difference in log_{10} CFU/mL between the initial count obtained at time = 0 and that at the end of the incubation period.

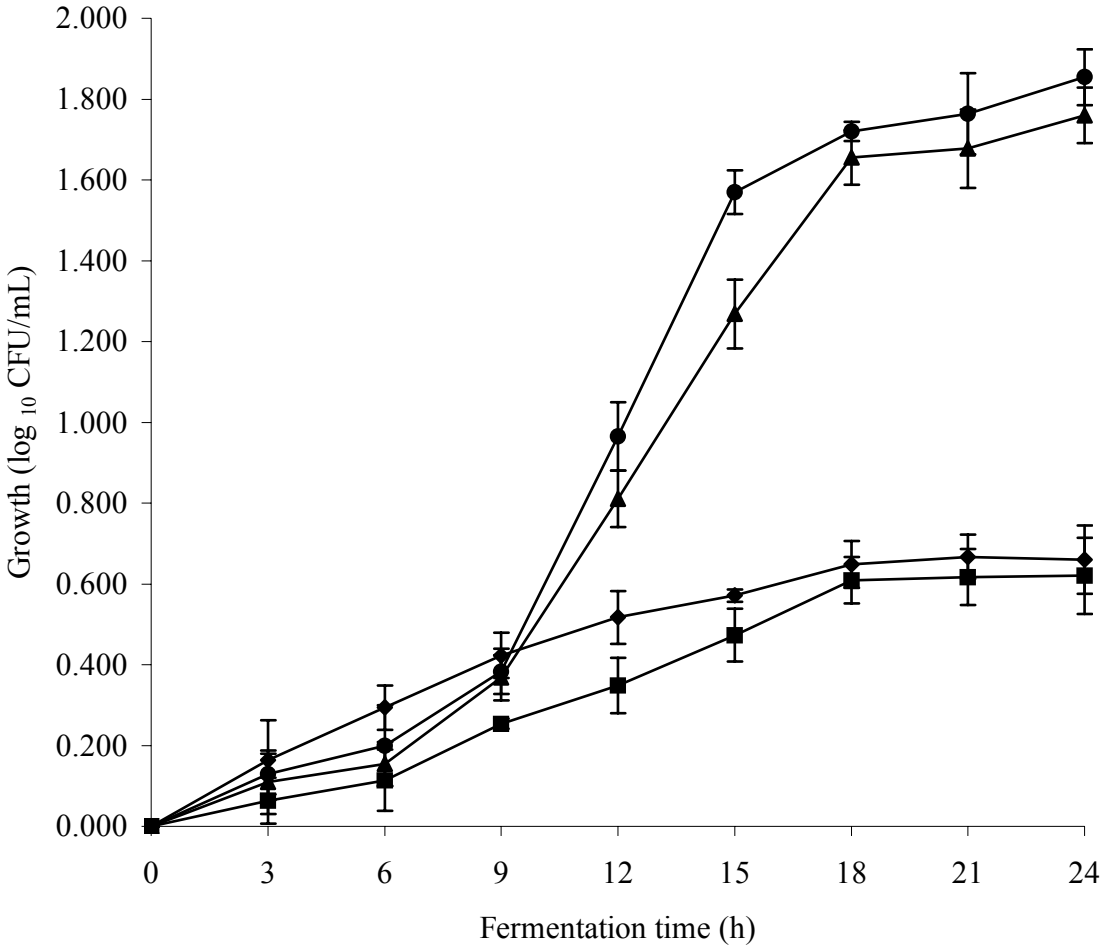


Figure 5.9 Validation experiment for growth of *L. casei* ASCC 292 in the optimum-point (●), center-point (▲), high-point (♦) and low-point (■) media. Factors combination for optimum medium were: 1.67% w/v inoculum size, 4.82% w/v FOS and 6.93% w/v maltodextrin. Center-point medium were: 1.70% w/v inoculum size, 4.80% w/v FOS and 6.80% w/v maltodextrin. High-point medium were: 2.40% w/v inoculum size, 7.20% w/v FOS and 8.80% w/v maltodextrin, and low-point medium were: 1.00% w/v inoculum size, 2.40% w/v FOS and 4.80% w/v maltodextrin. Error bars represent standard error of means; n = 3. Growth was expressed as the difference in $\log_{10}$ CFU/mL between the initial count obtained at time = 0 and that at the end of the incubation period.

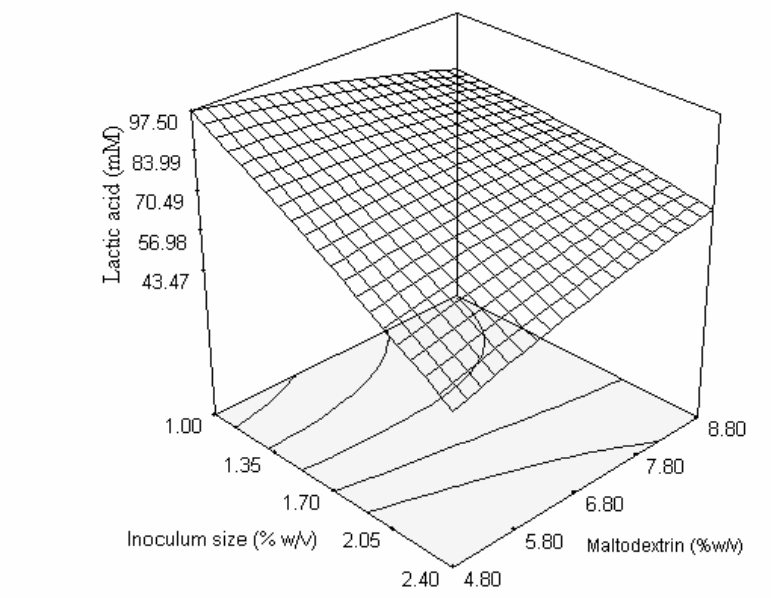
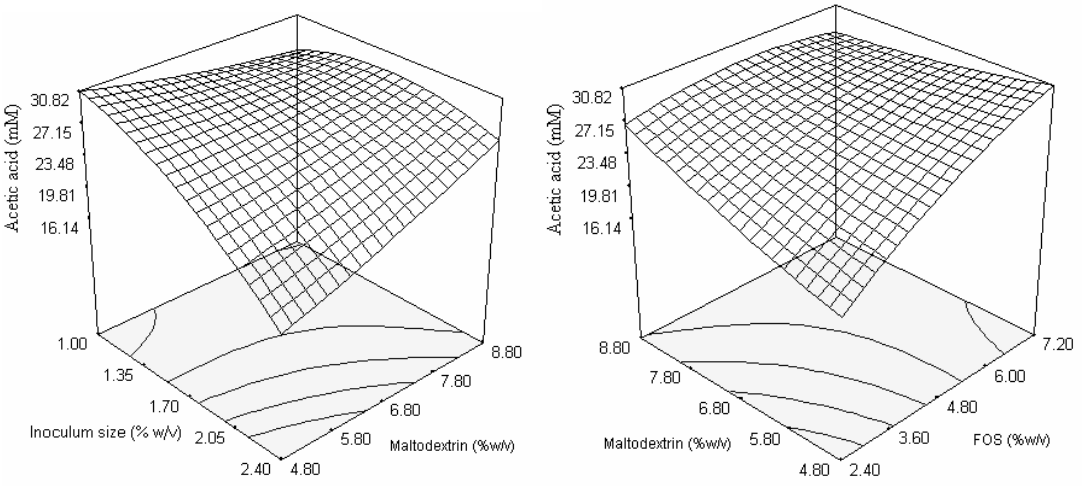
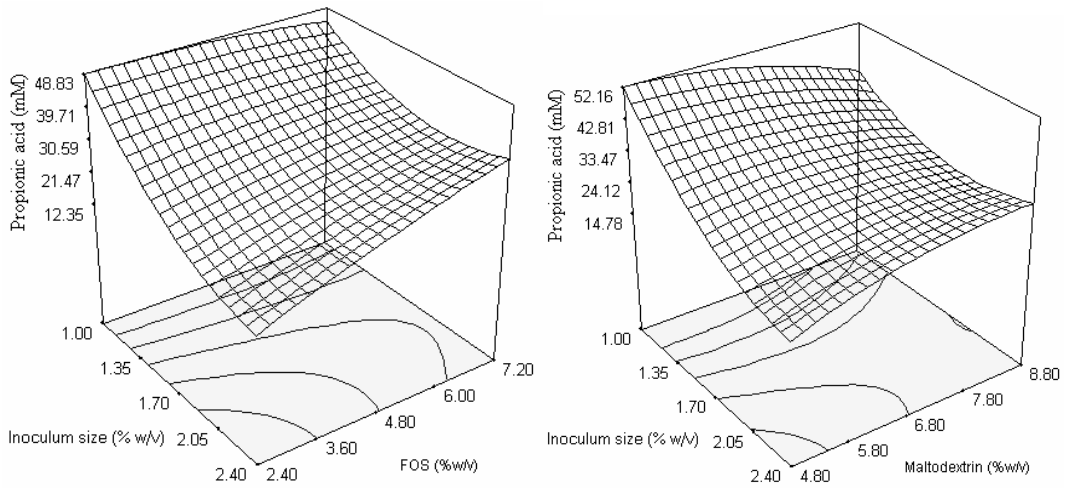


Figure 5.10 Response surface for the production of lactic acid (m*M*) from the effects of inoculum size and maltodextrin at 4.82% w/v of FOS.



(A) (B)

Figure 5.11 Response surface for the production of acetic acid (m*M*) from the effects of (A) inoculum size and maltodextrin at 4.82% w/v of FOS, and (B) FOS and maltodextrin at 1.67% w/v of inoculum size.



(A) (B)

Figure 5.12 Response surface for the production of propionic acid (m*M*) from the effects of (A) inoculum size and FOS at 6.93% w/v of maltodextrin, and (B) inoculum size and maltodextrin at 4.82% w/v of FOS.

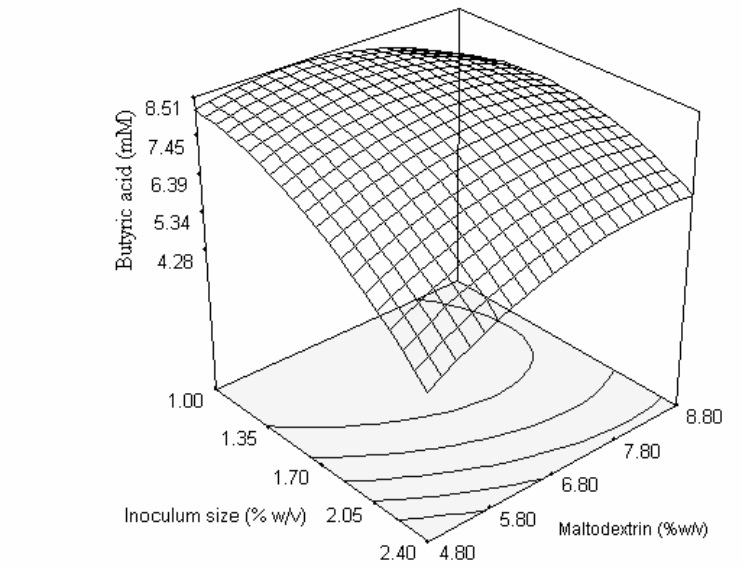


Figure 5.13 Response surface for the production of butyric acid (m*M*) from the effects of inoculum size and maltodextrin at 4.82% w/v of FOS.

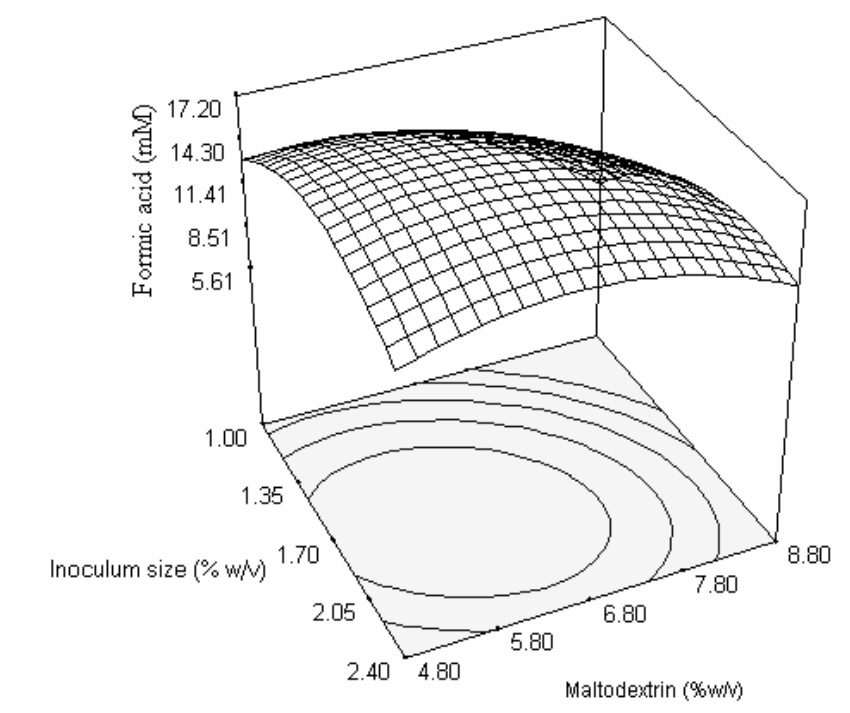


Figure 5.14 Response surface for the production of formic acid (m*M*) from the effects of inoculum size and maltodextrin at 4.82% w/v of FOS.

5.3 Optimization of cholesterol removal, growth and fermentation patterns of *Lactobacillus acidophilus* ATCC 4962 in presence of mannitol, FOS and inulin: a response surface methodology approach

5.3.1 INTRODUCTION

Interest in the usage of probiotics for human health dated back to 1908 when Metcnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (O'Sullivan *et al.* 1992). More recently, probiotics have been defined as 'cultures of live microorganisms that, applied in animals or humans, benefit the host by improving properties of indigenous microflora' (Arihara and Itoh, 2000). They mainly consist of lactobacilli, streptococci, enterococci, lactococci and bifidobacteria. Over the years, lactobacilli have been associated with the improvement of lactose intolerance, increase in natural resistance to infectious disease in gastrointestinal tract, suppression of cancer, improved digestion and reduction in serum cholesterol level (Gibson and Roberfroid, 1995). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks (Lourens-Hattingh and Viljoen, 2001). Various studies reported that lactobacilli could lower total cholesterol and low-density-lipoprotein (LDL) cholesterol (Anderson and Gilliland, 1999; Sanders, 2000).

Prebiotics are defined as nondigestable substances that exert biological effect on humans by selectively stimulating the growth or bioactivity of beneficial microorganisms either present, or therapeutically introduced to the intestine (Tomasik and Tomasik, 2003). Several non-starchy polysaccharides such as fructooligosaccharides, lactulose and β-cyclodextrin have been considered as prebiotics.

Recently, polyols such as mannitol, sorbitol and xylitol have been included to the prebiotics group (Klahorst, 2000). Prebiotics have been linked with cholesterol reducing effects. It was previously found that hepatocytes isolated from oligofructose-fed rats had a slightly lower capacity to synthesize triacylglycerol from radiolabeled acetate. This led to the hypothesis that decreased de novo lipogenesis in the liver, through lipogenic enzymes, is the key to reduction of VLDL-triglyceride secretion in rats fed with oligosaccharides (Robertfroid and Delzenne, 1998). Administration of oligofructose was postulated to modify lipogenic enzyme gene expression, observed by a 50% reduction of activity of acetyl-CoA carboxylase, malic enzyme and ATP citrate lyase (Delzenne and Kok, 2001).

This section has been published: *Liong, MT and Shah, NP. 2005. Optimization of cholesterol removal, growth and fermentation patterns of Lactobacillus acidophilus ATCC 4962 in presence of mannitol, FOS and inulin: a response surface methodology approach.* ***J Appl Microbiol*** *98: 1115-1126.*

Probiotics and prebiotics simultaneously present in a product are called either synbiotics or eubiotics. Such a combination aids survival of the administered probiotic and facilitates its inoculation into the colon. Additionally, the prebiotic induces growth and increases activity of positive endogenic intestinal flora (Tomasik and Tomasik, 2003). Experiments with rats showed that synbiotics protect the organism from carcinogens significantly better than either prebiotics or probiotics individually (Gallaher and Khil, 1999). However, there is little information on suitable combinations of probiotics and prebiotics specifically targeting removal or lowering of cholesterol.

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. It also has important applications in design, development and formulation of new products, as well as improvement of existing product designs (Myers and Montgomery, 1995). Response surface models may involve main effects and interactions or have quadratic and possibly cubic terms to account for curvature. It has been successfully utilized to optimize compositions of microbiological media (Oh *et al*. 1995), improving fermentation processes (Lee and Chen, 1997) and product development (Gomes and Malcata, 1998). Conventional methods (such as one factor at one time) have been applied previously to evaluate the *in-vitro* performance of probiotics and/or prebiotics to remove cholesterol. However, these methods require a large number of experiments to describe the effect of individual factors, were time consuming, and no statistical method was established to distinguish the interaction effects from main effects. Thus, the aim of this study was to optimize cholesterol removal by using *L. acidophilus* ATCC 4962 in the presence of mannitol, FOS and inulin, through the approach of response surface.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Bacteria and media preparation

L. acidophilus ATCC 4962 was used in this study. Working cultures and types of prebiotics used were described in section 5.1.2.1. All prebiotics were used at concentrations as per the experimental design and were prepared in phosphate buffer (0.1 M, pH 6.0) containing ammonium citrate (2.0 g L^{-1}), sodium acetate (5.0 g L^{-1}), magnesium sulphate (0.1 g L^{-1}), manganese sulphate (0.05 g L^{-1}), dipotassium phosphate (2.0 g L^{-1}) and Tween 80 (1.0 mL L^{-1}). Freeze-dried cells of *L. acidophilus* ATCC 4962 were inoculated at appropriate levels as described in the experimental design.

5.3.2.2 Cholesterol removal

Removal of cholesterol was determined as described in section 5.1.2.2.

5.3.2.3 Growth of *L. acidophilus* ATCC 4962 in the presence of prebiotics

Growth was determined using the pour plate method as described in section 5.1.2.3. Growth was calculated as log10 colony forming units (CFU mL^{-1}) and expressed as percentage difference between initial growth values obtained at time = 0 and at the end of the incubation period.

5.3.2.4 Mean doubling time

Mean doubling time was determined as described in section 5.1.2.4.

5.3.2.5 Short chain fatty acids (SCFA) determination

SCFA was determined using HPLC as described in section 5.1.2.6.

5.3.2.6 Experimental design and statistical analyses

The design of experiment and analyses of data were performed using a response surface methodology approach as described in section 5.1.2.7.

5.3.3 RESULTS

5.3.3.1 Screening of factors and steepest ascent

Results from the two-level partial factorial design are shown in Table 5.11, while analysis of variance (ANOVA) for the evaluation of the first-order model is shown in Table 5.12. ANOVA showed that the model used was suitable, lack-of-fit test was insignificant with only 9.58% total variation that was not explained by the model. Removal of cholesterol was significantly influenced by inoculum size of *L. acidophilus* ATCC 4962 (X_1), mannitol (X_3), FOS (X_6) and inulin (X_7), while the other prebiotics were found to have insignificant influence and were not included in the ANOVA. Thus, further optimization processes will only involve these four factors. A first-order equation (coded term) was generated from this first-degree order model, for response of cholesterol removal (Y), with the significant factors now redefined as inoculum size (X_1), mannitol (X_2), FOS (X_3) and inulin (X_4):

$$Y = 33.28 + 3.50X_1 + 1.17X_2 + 0.83X_3 + 1.17X_4$$

From the equation and coefficient estimate, inoculum size (X_1) produced greatest effect and was used as the fundamental scale in the next step, steepest ascent. In this study, the steepest ascent design was based on the increase of 0.50 (% w/v) concentrations for X_1. This produced 5 design units (0.50/0.10 = 5). Thus, movement for X_2 was 1.67 design units [(1.17/3.50)(5) = 1.67], for X_3 was 1.19 design units [(0.83/3.50)(5) = 1.19] and for X_4 was 2.53 design units [(1.17/3.50)(5) = 2.53]. The following steepest ascent coordinates were generated as shown in Table 5.13. Steepest ascent coordinates showed that removal of cholesterol decreased after the fifth step, with highest value of 50.938 μg mL^{-1}, from the combination of inoculum size (2.20% w/v), mannitol (4.36% w/v), FOS (3.40% w/v) and inulin (6.08% w/v). This combination was used as the middle point for optimization experiments.

5.3.3.2 Optimization of cholesterol removal

Optimization was performed using CCD with fixed middle point of inoculum size (2.20% w/v), mannitol (4.30% w/v), FOS (3.40% w/v) and inulin (6.00% w/v). Design matrix for CCD and responses are shown in Table 5.14, while the adequacy and fitness were evaluated by ANOVA and regression coefficients (Table 5.15). ANOVA results indicated that the quadratic regression to produce the second-order model was significant. Lack-of-fit test was insignificant and a good coefficient regression was obtained. Inoculum size, mannitol, FOS and inulin significantly influenced cholesterol removal.

The effect of each factors were further assessed using perturbation plots, to show how the response changes as each factor moves from the chosen reference point, with all other factors held constant at reference values (Oh *et al*. 1995). In this study, as one particular chosen factor was assessed, the other factors were held constant at the optimum point. Figure 5.15 shows the perturbation plot of the factors used in this study. Although all factor showed significant quadratic effect, the curve with the most prominent change was the perturbation curve of inoculum size, compared to the other factors that were fixed at their maximum levels. Thus, we believe that inoculum size was the most significant factor that contributed to the removal of cholesterol with the most obvious quadratic effect. Although the P-values of both FOS and inulin showed similar levels of significance, it could be clearly seen from the perturbation plot that the response curve of inulin was less prominent than that of FOS.

The best explanatory equation to fit the second-order model and subsequently produce the response surface was expressed as:

$$Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_4X_4 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{44}X_4^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{14}X_1X_4 + c_{23}X_2X_3 + c_{24}X_2X_4 + c_{34}X_3X_4$$

where $c \ldots c_{23}$ are regression coefficients and X_1, X_2, X_3, X_4 are the coded independent factors. Here, the second-order regression model involved four factors, thus producing four linear, four quadratic and six interaction terms. Response surface was generated (Figure 5.16) based on the second-order equation:

$$Y_0 = 56.58 + 6.38X_1 - 0.63X_2 - 1.49X_3 - 1.19X_4 - 7.34X_1^2 - 6.42X_2^2 - 5.97X_3^2 - 5.75X_4^2 - 0.72X_1X_2 + 0.34X_1X_3 - 0.034X_1X_4 + 1.51X_2X_3 - 0.50X_2X_4 - 1.01X_3X_4$$

An optimum point was produced with optimum cholesterol removal obtained at 58.142 μg mL^{-1}. The combination that produced the optimum point was (X_1, X_2, X_3, X_4) = (0.437, -0.082, -0.115, -0.092). The original levels that correlated with those coded values were found to be inoculum size at 2.64% w/v, mannitol at 4.14% w/v, FOS at 3.28% w/v and inulin at 5.82% w/v.

All these predictions by the regression model were further ascertained by a validation experiment. We compared the cholesterol removal patterns over a 24 h period using four different media: the optimum medium (inoculum size: 2.60% w/v; mannitol: 4.10% w/v; FOS: 3.30% w/v; inulin: 5.80% w/v), the center-point medium (inoculum size: 2.20% w/v; mannitol: 4.30% w/v; FOS: 3.40% w/v; inulin: 6.00% w/v), the high-point medium (inoculum size: 3.20% w/v; mannitol: 6.30% w/v; FOS: 4.40% w/v; inulin: 8.00% w/v) and the low-point medium (inoculum size: 1.20% w/v; mannitol: 2.30% w/v; FOS: 2.40% w/v; inulin: 4.00% w/v). The cholesterol removal curves are shown in Figure 5.17. Although the exact cholesterol removal quantities were different from the predictions, the patterns were in tandem with predictions by the model. Highest cholesterol was removed from the optimum medium, and lower from the center-point medium. Least cholesterol was removed from both high-point and low-point media, as supported by the response surface of cholesterol removal (Figure 5.16).

5.3.3.3 Growth, mean doubling time and production of SCFA

We further studied patterns of growth, mean doubling time and production of SCFA from the fermentation of prebiotics, at the experimental regions used to obtain optimum removal of cholesterol. The response obtained using the CCD is shown in Table 5.16. The statistical analyses with coefficient estimates and the significance of each response model are presented in Table 5.17.

The response surface of growth (Y_1) is shown in Figure 5.18, and was generated based on the following coded factor equation:

$$Y_1 = 41.97 + 2.49X_1 - 0.12X_2 - 1.49X_3 - 3.35X_4 - 3.90X_1^2 - 4.05X_2^2 - 2.77X_3^2 - 0.50X_4^2 - 0.22X_1X_2 + 1.66X_1X_3 + 1.63X_1X_4 + 0.89X_2X_3 - 0.08X_2X_4 + 0.53X_3X_4$$

The response surface clearly indicated that an optimum point (45.21%) was produced with X_1, X_2, X_3 and X_4 at 2.23% w/v, 4.21% w/v, 3.04% w/v and 4.00% w/v, respectively. Growth increased with increasing inoculum size level from 1.20% w/v to 2.23% w/v. Further increase in concentrations of inoculum size beyond 1.69% w/v generated a decrease in growth. Similarly, increasing concentrations of mannitol and FOS from 2.30% w/v to 4.21% w/v and 2.40% w/v to 3.04% w/v, respectively, increased growth, but further increase in the prebiotics concentration generated a decrease in growth. Inulin produced highest growth at its lowest concentration of 4.00% w/v, and produced lowest growth at its highest concentration of 8.00% w/v. It appeared that growth of *L. acidophilus* ATCC 4962 was influenced by inulin in a linear manner, while inoculum size, mannitol and FOS showed significant quadratic effects. Other than main quadratic effects, interactions between inoculum size and FOS, and inoculum size and inulin produced strongest influence towards growth, while the other interactions were insignificant.

In this study, patterns of mean doubling time (Y_2) were studied using the response surface (Figure 5.19) that was generated from the equation:

$$Y_2 = 291.21 + 1.53X_1 + 0.32X_2 - 0.28X_3 - 1.31X_4 - 0.97X_1^2 + 0.095X_2^2 - 0.40X_3^2 - 0.60X_4^2 + 0.34X_1X_2 + 0.42X_1X_3 - 0.16X_1X_4 + 0.66X_2X_3 - 0.21X_2X_4 + 0.70X_3X_4$$

Inoculum size, FOS and inulin showed significant quadratic effect, while mannitol did not (Table 5.17). FOS mainly contributed to the interaction effects, with only interaction terms involving FOS showed significant influence on mean doubling time. All these significant interaction terms also showed positive regression coefficients, indicating that either a decrease or increase in both factors will contribute to an increase in mean doubling times.

The SCFA (Y_3) was obtained as a total of individual fatty acids, namely acetic, butyric and propionic acids. A response surface (Figure 5.20) was generated from the second-order equation:

$$Y_3 = 60.03 + 6.67X_1 + 0.62X_2 + 2.30X_3 + 3.29X_4 - 6.08X_1^2 - 9.65X_2^2 - 10.69X_3^2 - 12.34X_4^2 + 0.66X_1X_2 + 3.80X_1X_3 + 4.84X_1X_4 + 1.45X_2X_3 + 1.29X_2X_4 + 3.20X_3X_4$$

All factors produced significant quadratic effects on production of SCFA. Response surfaces produced showed that the production of SCFA appeared to be growth associated.

5.3.4 DISCUSSION

Various factors normally affect the response surfaces that are produced. Thus, screening experiments are needed to segregate important main effects from less important ones (Montgomery, 1996). In this study, first degree order equation was generated and significance of factors was tested using screening experiments. A complete replication of the seven factors using a 2^x factorial design would need 128 experimental runs. However, only seven degree of freedoms would be needed to estimate main effects, and 21 degree of freedoms would estimate two-factor interaction effects, while the remaining 99 degree of freedoms would estimate error or/and three or higher-factor interaction effects (Cox and Reid, 2000). Thus, a partial two-level factorial design (2^{7-2}) was applied in this study. Partial factorial designs are capable of identifying important factors using less number of experimental runs without loss of information on main factor effects and their interactions (Li *et al*. 2002). Following the screening of significant factors, design points were subjected to steepest ascent before subsequent optimization steps. Steepest ascent or steepest descent involved the generation of mathematical movements along an ascending or descending path until no improvement occurred (Montgomery, 1996).

A significant quadratic regression, insignificant lack-of-fit and a small total variation (4.60%) that was not explained by the model, suggested that the model accurately represented data in the experimental region. This also indicated that second-order terms were sufficient and higher-order terms were not necessary (Oh *et al.* 1995). It must also be noted that the *t* value of the quadratic term of inoculum size (X_1^2) was higher than others (Table 5.15), indicating that the quadratic effect of inoculum size had the strongest effect on cholesterol removal, which was also confirmed using the perturbation plot. Validation experiments showed that the predicted value was 58.142 μg/mL while the actual experimental result was 52.941 μg/mL. However, it must be noted that the conditions for both were slightly different. The predicted value was obtained at the predicted 2.64% w/v inoculum size, 4.14% w/v mannitol, 3.28% w/v FOS and 5.82% w/v inulin, while the actual experiments were conducted with 2.60% w/v inoculum size, 4.10% w/v mannitol, 3.30% w/v FOS and 5.80% w/v inulin. Under such dissimilarity, the difference between the prediction and actual data was only 8.95%. The obvious difference of cholesterol removal between the optimum, high-point, low-point and center-point media proved the validity of the model and the reproducibility of the prediction.

From Table 5.15, it must be noted that the coefficient estimates of the interaction terms of (X_2, X_4) and (X_3, X_4) had negative signs (X_{24} = -0.50, X_{34} = -1.01). These negative signs may imply that for an increase of the response, the coded levels of (X_2, X_4) and (X_3, X_4) must have different signs, either one must be higher than zero and the other lower than zero (Oh *et al*. 1995). However, it must be noted that the optimum was achieved at (X_2 = -0.082, X_4 = -0.092) and (X_3 = -0.115, X_4 = -0.092), which would produce a positive sign instead. This may be due to other terms that may dominate this particular interaction term (Oh *et al*. 1995). Considering that the lack-of-fit test was insignificant, other higher terms would not have contributed to this, thus, we postulate that the linear term might have played a role.

The response surface of growth showed similar patterns with the response surface of removal of cholesterol, indicating a strong correlation between removal of cholesterol and growth. Previous studies also showed that cholesterol assimilation by strains of *L. acidophilus* during refrigerated storage of nonfermented milk was associated with bacterial growth and their viability, and was growth dependent (Piston and Gilliland, 1994; Pereira and Gibson, 2002). This has led us to postulate that cholesterol removal *in-vitro* was growth associated. Significant interaction terms of inoculum size with FOS and inulin showed that these two prebiotics strongly encouraged growth of *L. acidophilus* ATCC 4962. Comparing these two, a higher coefficient of regression for X_1X_3 than X_1X_4 indicated that FOS was more preferred than inulin. Studies using bifidobacteria showed that the bifidogenic effects of inulin and FOS are independent of chain lengths or GF_n type. FOS of the GF_2 and GF_3 moiety were also found to be more rapidly consumed compared to GF_4 (Kaplan and Hutkins, 2000). All these may have contributed to the preference of *L. acidophilus* ATCC 4962 on FOS than on inulin, and the fact that linear decrease in concentration of inulin contributed to an increase in growth.

Mean doubling time was used as a measure of the effectiveness of a specific carbon source in modulating bacterial growth rate (Bruno *et al*. 2002). Of all factors, FOS contributed significantly in the interaction patterns of mean doubling time, and higher growth rates (lower mean doubling time) were obtained at lower concentration of FOS (Figure 5.15). It was previously reported that both the uptake and hydrolysis of FOS are induced by higher oligosaccharides but repressed by products of their hydrolysis (Kaplan and Hutkins, 2003). In this experiment, it appeared that at higher concentration of FOS, more product of hydrolysis were produced and repressed bacterial growth rate, producing a higher mean doubling time. It must also be noted that the interaction between FOS and inulin produced lower mean doubling times when one factor was at lower levels and the other at

higher levels. This indicated that when FOS was at its lower level, *L. acidophilus* ATCC 4962 utilized a higher level of inulin for higher growth rate and vice versa. It appeared that although *L. acidophilus* ATCC 4962 preferred FOS over inulin, but under conditions of substrate limitation, inulin was beneficially utilized for the modulation of growth rate.

The major products of metabolism of prebiotics are short chain fatty acids (SCFA), carbon dioxide and hydrogen, and bacterial cell mass (Cummings *et al*. 2001). Although much work has been done on SCFA production and the significance of the individual acids, no particular pattern of SCFA production from prebiotic fermentation has emerged as yet. Hence, in this study, we analysed the SCFA production from fermentation of mannitol, FOS and inulin by *L. acidophilus* ATCC 4962. Production of SCFA appeared to be growth associated and correlated with the patterns of cholesterol removal. Although all factors significantly affected the production of SCFA, mannitol exhibited the strongest effect (Table 5.17). While the utilization of FOS and inulin has been widely reported, the utilization of mannitol to produce high concentration of SCFA was less studied and was also found to be strain dependent. Lactic acid bacteria that produced NADH oxidase would have the alternative NADH-H^+-oxidizing mechanism, resulting in higher ability to grow on substrates more chemically reduced than glucose, such as mannitol (Stanton *et al*. 1999). This may contribute to the better growth of *L. acidophilus* ATCC 4962 in the presence of mannitol and subsequently produced higher amount of SCFA and higher cholesterol removal. Previous study showed that strains of *L. acidophilus* that utilized mannitol also exhibited capability of cholesterol uptake (Gupta *et* al. 1996).

In conclusion, cholesterol removal was optimized after selecting a combination of inoculum size and prebiotic, with the predicted optimum removal of 58.142 μg mL^{-1} obtained at 2.64% w/v inoculum size, 4.14% w/v mannitol, 3.28% w/v FOS and 5.82% w/v inulin. Validation experiment showed that RSM was reliable in developing a model, optimization of factors, and analysis of interaction effects. Analysis of growth, mean doubling time and production of SCFA showed that cholesterol removal and the production of SCFA was growth associated.

Table 5.11 Treatment combinations and response for screening experiments.

Standard order	Factors*							Response
	Inoculum size (% w/v)	Sorbitol (% w/v)	Mannitol (% w/v)	FOS (% w/v)	Hi-maize (% w/v)	Inulin (% w/v)	Maltodextrin (% w/v)	Cholesterol assimilated (μg mL^{-1})
1	-1	-1	-1	-1	-1	1	1	31.36
3	1	-1	-1	-1	-1	-1	-1	33.13
5	-1	1	-1	-1	-1	-1	-1	25.52
7	1	1	-1	-1	-1	1	1	36.09
9	-1	-1	1	-1	-1	-1	1	27.71
11	1	-1	1	-1	-1	1	-1	39.17
13	-1	1	1	-1	-1	1	-1	32.53
15	1	1	1	-1	-1	-1	1	36.15
17	-1	-1	-1	1	-1	-1	-1	27.50
19	1	-1	-1	1	-1	1	1	39.01
21	-1	1	-1	1	-1	1	1	31.51
23	1	1	-1	1	-1	-1	-1	34.90
25	-1	-1	1	1	-1	1	-1	34.58
27	1	-1	1	1	-1	-1	1	36.15
29	-1	1	1	1	-1	-1	1	30.64
31	1	1	1	1	-1	1	-1	39.58
33	-1	-1	-1	-1	1	1	-1	28.70
35	1	-1	-1	-1	1	-1	1	34.22
37	-1	1	-1	-1	1	-1	1	26.30
39	1	1	-1	-1	1	1	-1	36.20
41	-1	-1	1	-1	1	-1	-1	28.49
43	1	-1	1	-1	1	1	1	38.54
45	-1	1	1	-1	1	1	1	31.09
47	1	1	1	-1	1	-1	-1	34.01
49	-1	-1	-1	1	1	-1	1	25.25
51	1	-1	-1	1	1	1	-1	38.23
53	-1	1	-1	1	1	1	-1	30.16
55	1	1	-1	1	1	-1	1	35.73
57	-1	-1	1	1	1	1	1	33.59
59	1	-1	1	1	1	-1	-1	36.82
61	-1	1	1	1	1	-1	-1	31.61
63	1	1	1	1	1	1	1	40.52
65	0	0	0	0	0	0	0	32.81
66	0	0	0	0	0	0	0	31.98
67	0	0	0	0	0	0	0	33.02
68	0	0	0	0	0	0	0	31.88
69	0	0	0	0	0	0	0	33.96

*Inoculum size: 0.10-0.30% w/v; Sorbitol: 0.50-1.50% w/v; Mannitol: 0.50-1.50% w/v; Maltodextrin: 0.50-1.50% w/v; Hi-amylose maize: 0.50-1.50% w/v; FOS: 0.50-1.50% w/v; Inulin: 0.50-1.50% w/v.

Table 5.12 Analysis of variance and coefficient estimates for the evaluation of the first-order model.

Source of variation	Sum of squares	DF*	Mean square	F-value	P-value
Model†	1115.371	4	278.84	148.73	< 0.0001
Curvature	1.41	1	1.41	0.75	0.3890
Residual	118.11	63	1.87		
Lack-of-fit	49.73	27	1.84	0.97	0.5269
Pure error	68.38	36	1.90		
Correlation total	1234.90	68			

Factor:	Coefficient estimate	DF	Standard error	t-value	P-value
Inoculum size (X_1)	3.50	1	0.17	20.43	0.0001‡
Mannitol (X_3)	1.17	1	0.17	6.83	0.0001‡
FOS (X_6)	0.83	1	0.17	4.85	0.0001‡
Inulin (X_7)	1.77	1	0.17	10.36	0.0001‡

*DF: degree of freedom.

†R^2 = 0.9042.

‡Significant at alpha 0.05.

Table 5.13 Coordination path of steepest ascent for all chosen factors in coded and natural levels.

Step	Coded factors*				Natural factors†				Cholesterol removed (μg mL^{-1})
	ξ_1	ξ_3	ξ_6	ξ_7	X_1	X_2	X_3	X_4	
1) Base	0	0	0	0	0.20	1.00	1.00	1.00	16.478
Δ	5	1.67	1.19	2.53	(5)(0.1) = 0.5	(1.67)(0.50) = 0.84	(1.19)(0.50) = 0.60	(2.53)(0.50) = 1.27	
2) Base + Δ	5	1.67	1.19	2.53	0.70	1.84	1.60	2.27	36.563
3) Base + 2Δ	10	3.34	2.38	5.06	1.20	2.68	2.20	3.54	44.375
4) Base + 3Δ	15	5.01	3.57	7.59	1.70	3.52	2.80	4.81	50.781
5) Base + 4Δ	20	6.68	4.76	10.12	2.20	4.36	3.40	6.08	50.938
6) Base + 5Δ	25	8.35	5.95	12.65	2.70	5.20	4.00	7.35	48.813
7) Base + 6Δ	30	10.02	7.14	15.18	3.20	6.04	4.60	8.62	47.497

*ξ_1: inoculum size (% w/v), ξ_3: mannitol (% w/v), ξ_6: FOS (% w/v); ξ_7: inulin (% w/v).

†X_1: inoculum size (% w/v), X_2: mannitol (% w/v), X_3: FOS (% w/v); X_4: inulin (% w/v).

Table 5.14 Combination matrix of the central composite design (CCD) using coded levels for the response of cholesterol removal.

Standard run	Block*	Factors				Cholesterol removal ($\mu g\ mL^{-1}$)†
		Inoculum size (X_1)	Mannitol (X_2)	FOS (X_3)	Inulin (X_4)	
1	1	-1	-1	-1	-1	30.367
2	1	1	-1	-1	-1	46.304
3	1	-1	1	-1	-1	29.586
4	1	1	1	-1	-1	41.461
5	1	-1	-1	1	-1	26.461
6	1	1	-1	1	-1	42.086
7	1	-1	1	1	-1	31.929
8	1	1	1	1	-1	47.086
9	1	-1	-1	-1	1	28.023
10	1	1	-1	-1	1	40.367
11	1	-1	1	-1	1	23.648
12	1	1	1	-1	1	39.117
13	1	-1	-1	1	1	18.023
14	1	1	-1	1	1	38.179
15	1	-1	1	1	1	24.273
16	1	1	1	1	1	34.351
17	1	0	0	0	0	53.179
18	1	0	0	0	0	63.648
19	1	0	0	0	0	56.304
20	1	0	0	0	0	60.054
21	2	-2	0	0	0	15.211
22	2	2	0	0	0	33.414
23	2	0	-2	0	0	32.164
24	2	0	2	0	0	23.804
25	2	0	0	-2	0	34.586
26	2	0	0	2	0	24.976
27	2	0	0	0	-2	25.523
28	2	0	0	0	2	35.836
29	2	0	0	0	0	60.836
30	2	0	0	0	0	50.523

*1, first day of experiment; 2, second day of experiment.

†All factorial and axial points are means of duplicates.

Table 5.15 Analysis of variance of the second-order model* and coefficient estimates for the response Y_0 and factors X_1, X_2, X_3 and X_4.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model†	4302.42	14	307.32	10.78	0.0001
Residual	399.17	14	28.51		
Lack-of-Fit	284.11	10	28.41	0.99	0.5541
Pure error	115.07	4	28.77		
Total	4870.60	29			

Factor‡	Coefficient estimate	DF	Standard error	t-value	P-value
Intercept	$c = 56.58$	1	2.21		
X_1	$c_1 = 6.38$	1	1.09	5.85	0.0001§
X_2	$c_2 = -0.63$	1	1.09	-0.58	0.5735
X_3	$c_3 = -1.49$	1	1.09	-1.36	0.1938
X_4	$C_4 = -1.19$	1	1.09	-1.10	0.2915
X_1^2	$c_{11} = -7.34$	1	1.02	-7.20	0.0001§
X_2^2	$c_{22} = -6.42$	1	1.02	-6.30	0.0001§
X_3^2	$c_{33} = -5.97$	1	1.02	-5.86	0.0001§
X_4^2	$C_{44} = -5.75$	1	1.02	-5.64	0.0001§
X_1X_2	$c_{12} = -0.72$	1	1.33	-0.54	0.5993
X_1X_3	$c_{13} = 0.34$	1	1.33	0.250	0.8044
X_1X_4	$C_{14} = -0.034$	1	1.33	-0.026	0.9799
X_2X_3	$C_{23} = 1.51$	1	1.33	1.13	0.2774
X_2X_4	$C_{24} = -0.50$	1	1.33	-0.38	0.7120
X_3X_4	$C_{34} = -1.01$	1	1.33	-0.76	0.4615

*$Y_0 = 56.58 + 6.38X_1 - 0.63X_2 - 1.49X_3 - 1.19X_4 - 7.34X_1^2 - 6.42X_2^2 - 5.97X_3^2 - 5.75X_4^2 - 0.72X_1X_2 + 0.34X_1X_3 - 0.034X_1X_4 + 1.51X_2X_3 - 0.50X_2X_4 - 1.01X_3X_4$

†$R^2 = 0.9540$.

‡X_1: inoculum size (% w/v), X_2: mannitol (% w/v), X_3: FOS (% w/v), X_4: inulin (% w/v).

§Significant at alpha 0.05.

Table 5.16 Combination matrix of the central composite design (CCD) using coded levels for the factors and five responses.

Standard run	Block*	Factors†				Responses‡		
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	1	-1	-1	-1	-1	39.629	288.677	6.308
2	1	1	-1	-1	-1	35.996	290.797	13.064
3	1	-1	1	-1	-1	38.381	288.303	8.220
4	1	1	1	-1	-1	33.925	290.649	16.503
5	1	-1	-1	1	-1	28.365	284.406	5.992
6	1	1	-1	1	-1	35.774	288.435	16.711
7	1	-1	1	1	-1	30.550	286.989	8.915
8	1	1	1	1	-1	36.249	290.791	15.324
9	1	-1	-1	-1	1	28.398	287.901	5.131
10	1	1	-1	-1	1	32.935	288.418	24.531
11	1	-1	1	-1	1	23.948	285.813	11.966
12	1	1	1	-1	1	32.318	288.530	17.959
13	1	-1	-1	1	1	20.730	286.911	7.239
14	1	1	-1	1	1	32.278	288.579	35.922
15	1	-1	1	1	1	24.742	286.840	7.448
16	1	1	1	1	1	31.398	291.750	62.947
17	1	0	0	0	0	38.706	290.243	67.026
18	1	0	0	0	0	48.981	291.175	53.419
19	1	0	0	0	0	38.739	290.372	46.826
20	1	0	0	0	0	42.216	291.505	67.139
21	2	-2	0	0	0	19.677	284.734	36.543
22	2	2	0	0	0	31.106	292.091	45.701
23	2	0	-2	0	0	24.825	292.169	31.714
24	2	0	2	0	0	24.734	293.195	22.015
25	2	0	0	-2	0	32.519	291.310	23.119
26	2	0	0	2	0	27.326	290.102	22.252
27	2	0	0	0	-2	46.054	290.716	16.866
28	2	0	0	0	2	31.942	289.108	15.285
29	2	0	0	0	0	45.946	290.791	44.787
30	2	0	0	0	0	38.688	291.465	72.814

*1, first day of experiment; 2, second day of experiment.

†X_1 = inoculum size, X_2 = mannitol, X_3 = FOS, X_3 = inulin.

‡Y_1 = growth (%), Y_2 = mean doubling time (min), Y_3 = SCFA (mmol l^{-1}).

Table 5.17 Regression coefficients of the second-order equation* for the five responses†.

Coefficient	Y_1	Y_2	Y_3
c	41.97	291.21	60.03
c_1	2.46‡	1.53‡	6.67‡
c_2	-0.12	0.32	0.62
c_3	-1.49‡	-0.28	2.30
c_4	-3.35‡	-0.31	3.29
c_{11}	-3.90‡	-0.97‡	-6.08‡
c_{22}	-4.05‡	0.095	-9.65‡
c_{33}	-2.77‡	-0.40‡	-10.69‡
c_{44}	-0.50	-0.60‡	-12.34‡
c_{12}	-0.22	0.34	0.66
c_{13}	1.66‡	0.42‡	3.80
c_{14}	1.63‡	-0.16	4.84
c_{23}	0.89	0.66‡	1.45
c_{24}	-0.08	-0.21	1.29
c_{34}	0.53	0.70‡	3.20
R^2	0.9173	0.9377	0.8448
P-value	0.0001	0.0001	0.0016

*$Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3$.

†Y_1 = growth (%), Y_2 = mean doubling time (min), Y_5 = SCFA (mmol l^{-1}).

‡Significant at alpha = 0.05.

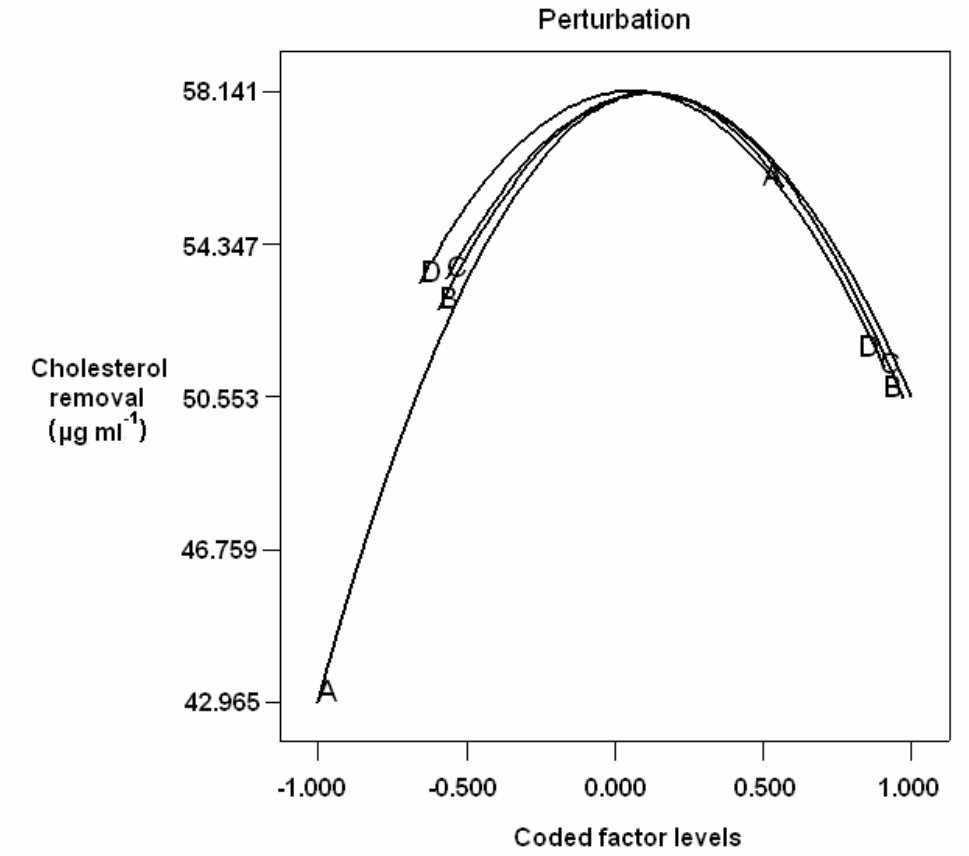
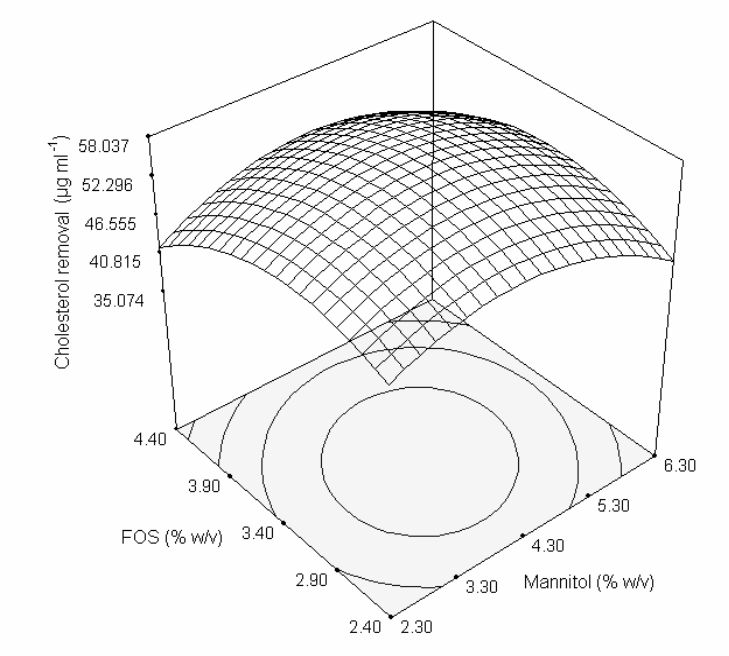
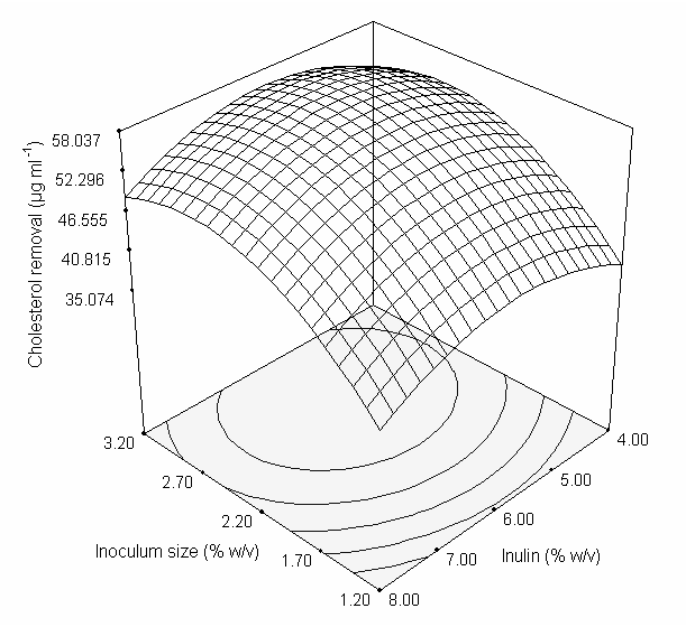


Fig. 5.15 Perturbation plot of inoculum size (A), mannitol (B), FOS (C) and inulin (D).



(A)



(B)

Fig. 5.16 Response surface for cholesterol removal (µg mL^{-1}) from the effects of (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.

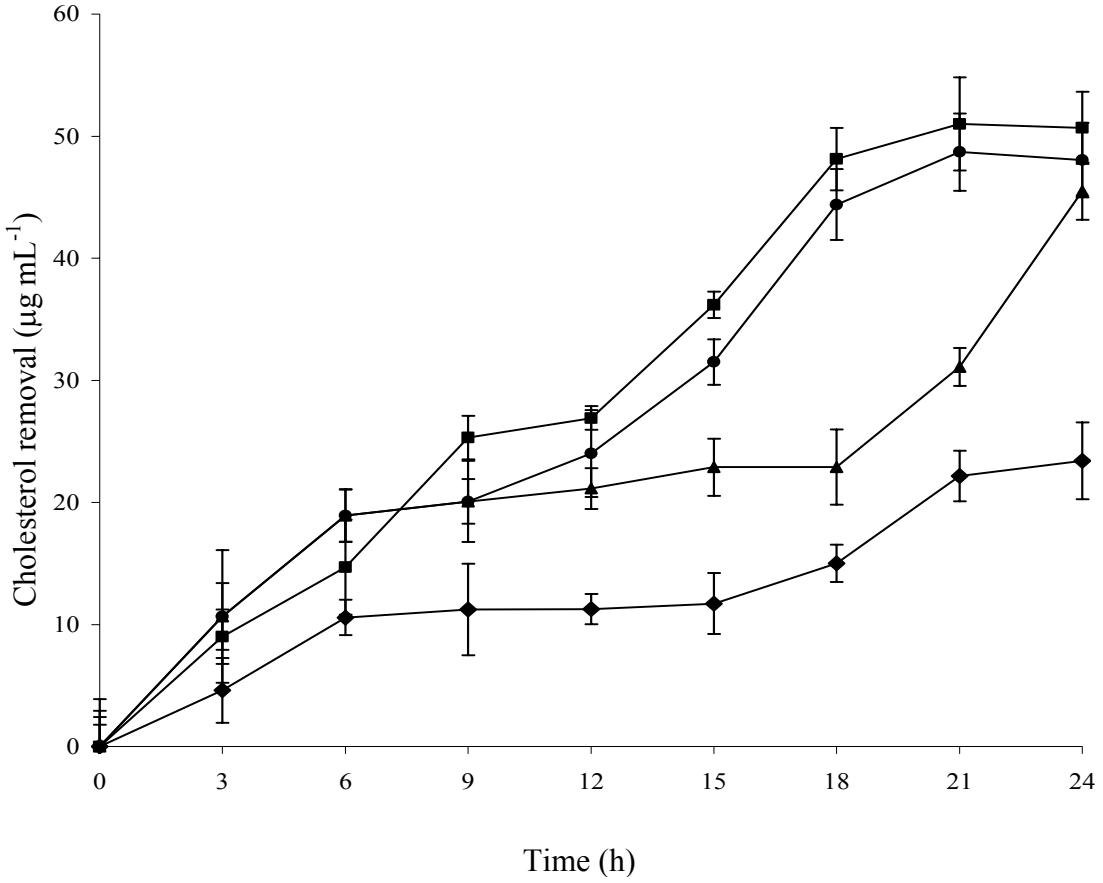
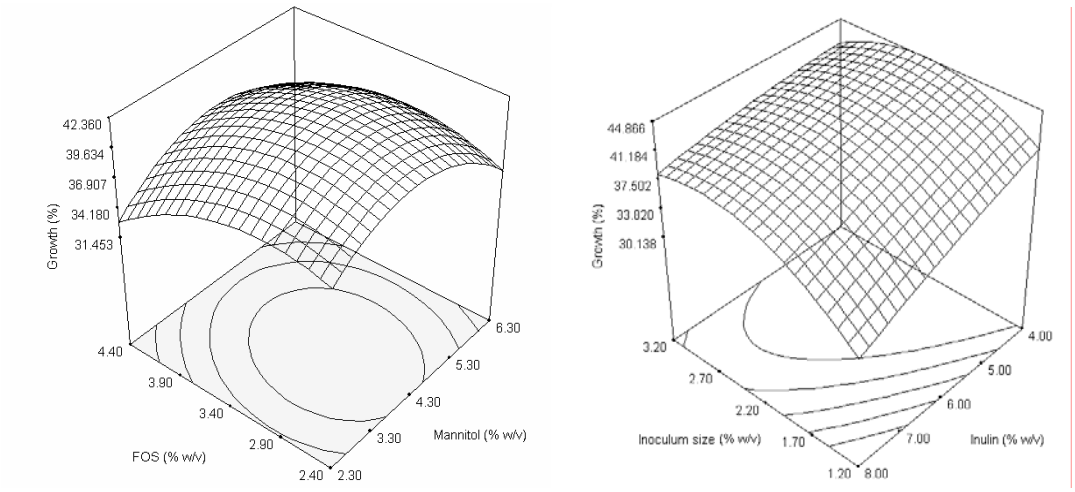
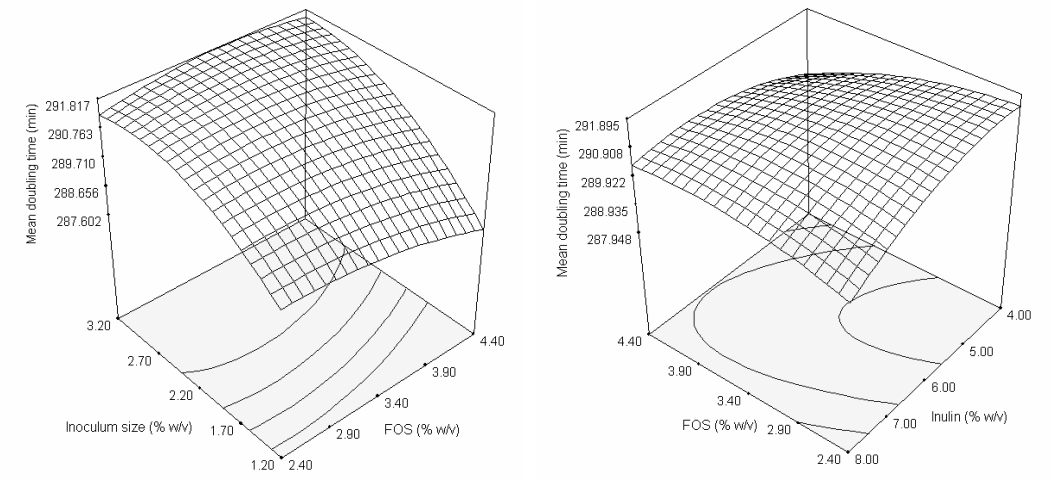


Fig. 5.17 Cholesterol removal by *L. acidophilus* ATCC 4962 in the optimum (■), center-point (●), high-point (▲) and low-point (♦) media, for the validation experiments. Factors combination for optimum medium were: inoculum size 2.60% w/v, mannitol 4.10% w/v, FOS 3.30% w/v and inulin 5.80% w/v. Center-point medium were: inoculum size 2.20% w/v, mannitol 4.30% w/v, FOS 3.400% w/v and inulin 6.00% w/v. High-point medium were: inoculum size 3.20% w/v, mannitol 6.30% w/v, FOS 4.40% w/v and inulin 8.00% w/v, and low-point medium were inoculum size 1.20% w/v, mannitol 2.30% w/v, FOS 2.40% w/v and inulin 4.00% w/v. Error bars represent standard error of means; n = 3.



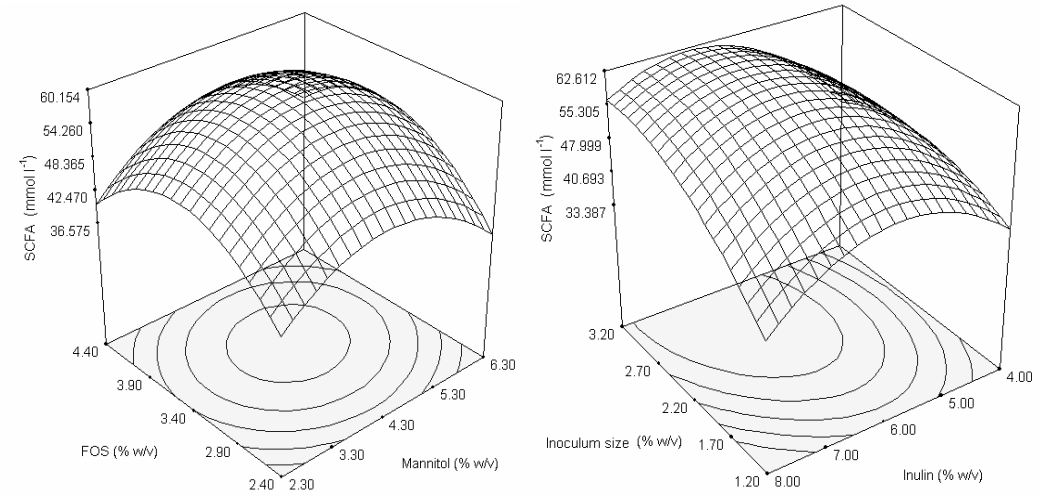
(A) (B)

Fig. 5.18 Response surface for growth (%) from the effects of (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.



(A) (B)

Fig. 5.19 Response surface for mean doubling time (min) from the effects of (A) inoculum size and FOS, and (B) FOS and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.



(A) (B)

Fig. 5.20 Response surface for the production of SCFA (mmol l^{-1}) from the effects of (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.

5.4 Production of organic acids from fermentation of mannitol, FOS and inulin by a cholesterol removing *Lactobacillus acidophilus* strain

5.4.1 INTRODUCTION

Colonic bacteria generally ferment prebiotics into hydrogen, carbon dioxide, and organic acids such as acetate, propionate and butyrate, while lactic acid bacteria such as lactobacilli additionally produce lactic acid. Organic acids contribute to normal large bowel function, prevent pathology through their actions in the lumen and on the colonic musculature and vasculature, and through their metabolism by colonocytes (Topping and Clifton 2001). When rats were fed fermentable carbohydrates such as oligosaccharides, non-starch polysaccharides, resistant starch or fibre, cecal pH was lowered by 1-2 units (Choct *et al*. 1998). Lower pH values prevent the overgrowth of pH-sensitive pathogenic bacteria. Propionate or formate has been shown to kill *Escherichia coli* and Salmonella at pH 5 (Cherrington *et al*. 1991). Acetate can reduce the concentration of serum fatty acids, which is an important factor in lowering glucose utilization by tissues and inducing insulin resistance. Long-term dietary supplementation with propionate was shown to decrease blood glucose in rats and humans, while butyrate possessed antineoplastic properties and is mostly used by colonocytes as an energy source (Gillet *et al*. 1998; Rizkalla *et al*. 2000). Although much attention has been directed to studying organic acids production from the fermentation of prebiotics by lactobacilli, most of these studies involved *in-vivo* models, where emphasis was mainly on the determination of organic acids in faecal and cecal samples. Furthermore, the interaction between probiotics and prebiotics to produce SCFA has been poorly understood with only selective prebiotics have been extensively studied.

We have previously found that *L. acidophilus* ATCC 4962 was capable of removing cholesterol *in-vitro* by various mechanisms, namely assimilation of cholesterol during growth, incorporation of cholesterol into the cellular membrane, binding of cholesterol to cell surface, bile salt deconjugation activity and co-precipitation of cholesterol with deconjugated bile (Chapter 3.0, section 3.1.3 and Chapter 4.0, section 4.1.3). Our recent study developed an optimum combination of synbiotic for the removal of cholesterol by *L. acidophilus* ATCC 4962 in the presence of three prebiotics namely mannitol, fructooligosaccharide (FOS) and inulin.

This section has been published: *Liong, MT and Shah, NP. 2005. Production of organic acids from fermentation of mannitol, FOS and inulin by a cholesterol removing Lactobacillus acidophilus strain.* ***J Appl Microbiol*** *99: 783-793.*

We have also previously reported that the production of short-chain fatty acids (SCFA) within the optimal cholesterol removal region was growth and substrate dependent (section 5.3.3.3). Although it has been shown that these prebiotics affected the production of organic acids (Topping and Clifton 2001), the effects of these prebiotics on the production patterns of individual organic acids have not been reported.

Thus, the aim of this study was to further examine the production patterns of organic acids from fermentation of mannitol, FOS and inulin by *L. acidophilus* ATCC 4962, using the experimental region of the optimal cholesterol removal that we have developed previously. Response surface methodology (RSM) was utilized to study those patterns as a response from the main and/or interaction effects.

5.4.2 MATERIALS AND METHODS

5.4.2.1 Bacteria and media preparation

L. acidophilus ATCC 4962 was used in this study. All working cultures and media were prepared as described in section 5.1.2.1.

5.4.2.2 Determination of organic acids

Organic acids were determined using HPLC as described in section 5.1.2.6.

5.4.2.3 Growth of *L. acidophilus* ATCC 4962 in the presence of prebiotics

Growth was determined using the pour plate method as described in section 5.1.2.3. The growth was expressed as the difference in $\log_{10}$ CFU of the initial count obtained at time = 0 and that at the end of the incubation period.

5.4.2.4 Experimental design and statistical analyses

The response surface methodology was applied with four independent factors namely, inoculum size of *L. acidophilus* ATCC 4962 (X_1), mannitol (X_2), FOS (X_3) and inulin (X_4) to generate a central composite design (CCD) matrix. The treatment combinations, generation of design matrix and statistical analyses of data were described in section 5.1.2.7. The coded factors produced are shown in Table 5.18.

5.4.3 RESULTS

The hydrolysis of prebiotic in the human intestine is affected by a number of bacterial cell-associated hydrolases that can hydrolyse a range of carbohydrates. Fermentation yields metabolizable energy for microbial growth, maintenance and metabolic end-products. In adult humans, the principal end-products are SCFA together with gasses including CO_2, CH_4 and H_2 (Topping and Clifton 2001). Thus, we analysed the production patterns of organic acids as influenced by different inoculum sizes and various concentrations of mannitol, FOS and inulin. The response obtained for acetic, butyric, formic, propionic and lactic acids is presented in Table 5.19, while the statistical analyses with coefficient estimates and the significance of each response model are presented in Table 5.20.

All second-order models that produced the quadratic response surfaces were generated based on the following equation:

$$Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_4X_4 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{44}X_4^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{14}X_1X_4 + c_{23}X_2X_3 + c_{24}X_2X_4 + c_{34}X_3X_4$$

where $c \ldots c_{34}$ are regression coefficients and X_1, X_2, X_3, X_4 are the coded independent factors. Here, the second-order regression model involved four factors, thus producing 4 linear, 4 quadratic and 6 interaction terms.

The response surface of acetic acid (Y_1) and the coefficient estimates of factors showed that all factors had significant second-order regression with inulin having the strongest effect while the inoculum size had least effect on the production of acetic acid (Figure 5.21; Table 5.20). Increasing inoculum size, and concentrations of mannitol, FOS and inulin showed increased production of acetic acid until their concentrations reached 3.09% w/v, 5.33% w/v, 3.54% w/v and 6.99% w/v, respectively. A further increase in concentrations beyond those caused a decrease in the production of acetic acid. Coefficient estimates of all interaction terms showed positive values, indicating that in order to achieve highest production of acetic acid, the interacting factors must both have positive terms or both with negative terms. The highest production of acetic acid was obtained at inoculum size of +0.890 coded value, mannitol of +0.515 coded value, FOS of +0.140 coded value and inulin of +0.495 coded value. Thus, as supported by the coefficient estimates, the highest production of acetic acid was obtained when combination of any two interaction factors had positive terms.

The first-order regression for the production of butyric acid was found to be significant while the higher regression models were non significant ($P > 0.05$). Thus, the response surface of butyric acid (Y_2) was generated based on a first-order equation:

$$Y_2 = 1.60 + 0.87X_1 - 0.16X_2 + 0.19X_3 + 0.022X_4$$

The inoculum size, and concentrations of mannitol and FOS affected the production of butyric acid significantly ($P < 0.05$), while concentrations of inulin did not, as supported by the non-prominent change in the response surface obtained (Figure 5.22). The inoculum size showed the strongest effect as indicated by the highest coefficient estimate (Table 5.20); with increasing inoculum size producing a prominent increase in the production of butyric acid (Figure 5.22).

The response surface of formic acid (Figure 5.23) showed that increasing inoculum size, and the concentrations of mannitol, FOS and inulin produced an increase in the production of formic acid, until their respective concentrations of 3.20% w/v, 5.98% w/v, 3.48% w/v and 4.00% w/v were reached. A further increase in concentrations caused a decrease in the production of formic acid. It must be noted that the coefficient of inulin was negative and the interaction terms of X_1X_4 and X_2X_4 also showed negative coefficient estimates. Thus, in order to produce negative coefficient estimates for both interaction terms, X_1 and X_2 must have a positive coefficient estimate. As shown in Figure 5.23, when the highest amount of formic acid was produced, both X_1 and X_2 were +1.000 and +0.820, respectively while X_4 was -1.000, producing negative values for X_1X_4 and X_2X_4. This also supported the inverse effect of inulin on the production of formic acid that occurred only at higher inoculum size and mannitol. Although production patterns were not entirely alike, the response surface of formic acid showed a similarity with that of acetic acid, indicating a certain degree of correlation. The interaction term of X_2X_3 showed a negative coefficient estimate, indicating that in order to achieve an increase in formic acid production, one of the interacting terms must have a negative value. The response surface produced showed that the highest amount of formic acid was produced when both mannitol and FOS had positive values (+0.820 and +0.080, respectively). This would generate a positive term instead of a negative one as indicated by the coefficient estimate. This may be due to other terms that may dominate this particular interaction term. Considering that the lack-of-fit test was non significant, other higher terms would not have contributed to this, thus, we postulate that the linear term might have played a role.

Production of propionic acid (Y_4) was clearly affected by the quadratic effects of inoculum size (Figure 5.24). Increasing the inoculum size of *L. acidophilus* ATCC 4962 from 1.20% w/v to 3.02% w/v produced increasing amount of propionic acid. At larger

inoculum sizes, the production of propionic acid reached a plateau. An optimum was obtained from the combination of 3.02% w/v of inoculum size and 5.32% w/v of inulin, with both mannitol and FOS at constant values of 6.30% w/v and 2.40% w/v, respectively. The inoculum size-inulin interaction also generated a production pattern that resembled those of acetic and formic acids. It must be noted that the coefficient estimates of X_1X_4 and X_2X_3 showed negative values. This was supported by the negative coded term of FOS (-1.000) and inulin (-0.340) when propionic acid was produced in highest amount, as compared to inoculum size and mannitol with coded values of 0.820 and 1.000, respectively.

Although the response surface of lactic acid (Y_5) showed a significant second-order regression, the inoculum size and inulin exhibited prominent first-order effects (Figure 5.25). A gradual increase in the inoculum size of *L. acidophilus* ATCC 4962 linearly increased the production of lactic acid. At a smaller inoculum size (1.20% w/v), an increase in concentration of inulin produced a minor increase in the production of lactic acid. Conversely, at a larger inoculum size (3.20% w/v), increasing concentration of inulin produced an apparent increase in the production of lactic acid. The concentrations of mannitol and FOS least affected the production of lactic acid, regardless of individual or interaction effects.

The response surface of growth (Figure 5.26) shows that an optimum point was perceptible. Through numerical analysis, the highest growth obtained from the predicted combination of 2.27% w/v of inoculum size, 3.99% w/v of mannitol, 3.02% w/v of FOS and 4.00% w/v of inulin. All factors showed significant quadratic effects on the growth. The response surfaces of acetic and formic acids indicated that the production of these acids were growth associated.

5.4.4 DISCUSSION

We previously reported that the best conditions for cholesterol removal by *L. acidophilus* ATCC 4962 were 2.64% w/v of inoculum, 4.13% w/v of mannitol, 3.29% w/v of FOS and 5.81% w/v of inulin (section 5.2.3.1). In the present study, the same experimental region as reported earlier was applied to further evaluate the production patterns of individual organic acids.

A face-centered central composite design (CCD) was applied. Face-centered CCD is generally created from a middle point, four factorial points and four axial or star points (parallel with axis and located at a distance from the center of the design space of α).

Increment of α will distance axial points towards the exterior of the square region and experimental region will form rotatability (Araujo and Brereton, 1996). Rotatability is a desirable property because in rotatable designs, the points with the same distance from the middle points will have similar estimated error. It also indicates the axial point in the CCD. The alpha could be obtained from:

$$\alpha = 2^{k/4}$$; where k is the number of factor.

Thus, with four factors, an α value of ± 2.00 was used, with an axial point located at 2.00 times the level coded as ±1 (Araujo and Brereton, 1996). The complete design matrix is presented in Table 5.19.

Response surfaces produced indicated that the region for optimum growth (Figure 5.26) was similar to that of the maximum or near-maximum for both acetic acid and formic acid. It appears that the production of these acids, under the influence of mannitol, FOS and inulin may be growth associated. Response surfaces of acetic acid (Figure 5.21) and formic acid (Figure 5.23) also showed some degree of similarity, indicating a good correlation between the productions of both acids. Comparing FOS and inulin, a higher coefficient of regression for X_3^2 than X_4^2 indicated that FOS was more preferred for growth than inulin. Similarly, the production of formic acid had a higher coefficient of regression for X_3^2 than X_4^2. However, both the coefficients of regression were similar for the production of acetic acid. Studies using bifidobacteria showed that the bifidogenic effects of inulin and FOS are independent of chain lengths or GF_n type. FOS of the GF_2 and GF_3 moiety were also found to be more rapidly consumed compared to GF_4 (Kaplan and Hutkins 2003). Increasing inoculum size (Figure 5.26) from 1.20 to 2.27% w/v was observed to increase the growth of *L. acidophilus* ATCC 4962, however, a further increase beyond that showed a decrease in growth. This may be contributed by substrate limitation with increasing cell numbers. Similarly, increasing the concentration of FOS from 2.40 to 3.02% w/v was accompanied by an increase in growth, but further increase beyond these concentrations caused a reduction in the growth. Growth was highest at the lowest concentration of inulin. It was previously reported that the uptake of FOS and its hydrolysis by *L. paracasei* 1195 were repressed by the products of their hydrolysis. Inhibited uptake of FOS, as a result of excess glucose, fructose, or sucrose in the media, indicated that these sugars may be transported by the FOS transport system (Kaplan and Hutkins 2003). We anticipate that an increase in hydrolysis of FOS and inulin beyond 3.02% and 4.00% w/v, respectively, would have generated an appreciable amount of end-products that repressed the growth of *L. acidophilus* ATCC 4962. Due to the similarity of growth repression region and the region of decreased production of

acetic and lactic acids (Figures 5.25 and 5.21), we speculate that the substrate inhibition factor subsequently decreased the production of these acids.

The theoretical balance of mannitol, based on the formation of *x* mole of formate per mole of mannitol has been reported as follows:

mannitol $\rightarrow$ (1 + 0.5*x*) acetate + (1- *x*) lactate + (0.5 + 0.5*x*) ethyl alcohol + *x* formate.

The fermentation balance of mannitol by Gram-negative bacteria was based on the above equation, with capabilities of such conversion aided by mannitol dehydrogenase and fructokinase (De Vries and Stouthamer 1968). In our study, the concentration of mannitol affected the growth, and the production of acetic and formic acids. Using *Lactococcus lactis*, it was previously found that when growth ceased, only 68% of mannitol was consumed, and the major end product from the catabolism of mannitol was formate (Neves *et al*. 2002). It must be noted that in our study, the interaction between inoculum size and mannitol was significant ($P < 0.05$) only on the production of formic acid, indicating that the fermentation of mannitol by *L. acidophilus* ATCC 4962 had the strongest influence on the production of formic acid.

All the factors studied exhibited only linear effects on the production of butyric acid. Only the inoculum size, mannitol and FOS had significant effects, with the strongest effect being of inoculum size, as indicated by the highest coefficient estimate value. An increase in inoculum size produced a prominent increase in the production of butyric acid (Figure 5.22). An optimum production value was not obtained, presumably a continuous production of butyric acid may occur with further increase in the inoculum size. It must be noted that the production of butyric acid was not entirely growth associated. The production of butyric acid proceeded beyond the cessation of growth (Figure 5.26). However, other than the different orders of regression, the production of butyric acid exhibited resemblance to the production of acetic acid. This may be explained by the study of Miller and Wolin (1996), that found a free radioactive acetate being incorporated into butyrate. Asanuma *et al*. (2003) found that the rate of butyrate production increased with an increase in acetate concentration in the medium; acetate stimulated butyrate production by acting as an acceptor in the enzyme reaction.

The general reaction of SCFA production and overall stoichiometry for a hexose has been summarized as: $59C_6H_{12}O_6 + 38H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3\ CH_2COOH + 18\ CH_3\ CH_2\ CH_2COOH + 96\ CO_2 + 268\ H^+$ + heat + additional bacteria. Normal faecal SCFA are found to be in order as predicted from the equation, with acetate > propionate ≥ butyrate (Topping and Clifton 2001). In our study, we have found that all prebiotics studied indicated

a similar production yield. The acetic acid was produced at the highest level compared to propionic acid, while least amount of butyric acid was produced. It must be noted that the production of propionic acid was also not growth associated. An increasing amount of inoculum size, mannitol and FOS produced an increasing level of propionic acid (Figure 5.24). Using slurries of human faecal and radioisotope analyses, Miller and Wolin (1996) found that propionic acid was formed by the CO_2 fixation pathway. However, up to now, there has been no study on such formation pathway from catabolism of mannitol, FOS or inulin by lactic acid bacteria. Results from our study could indicate that higher concentrations of mannitol and FOS may induce the CO_2 fixation pathway and subsequently increased the production of propionic acid. Further studies are needed to confirm this.

The response of lactic acid indicated that a linear increase in the inoculum size increased the production of lactic acid. It appears that the production of lactic acid was not growth associated and its production proceeded at certain regions where growth ceased. However, it must also be noted that its production also appeared to exhibit an inverse effect on growth. Although the reason may not be clear at this point, the response surface indicated that low production of lactic acid occurred in regions with high growth. The regions with highest growth exhibited the lowest production of lactic acid (Figures 5.25 and 5.26). Amrane and Prigent (1998) observed an inhibition effect due to lactic acid addition at different levels ranging from 0 to 50 g l^{-1} on the growth rate of *Lactobacillus helveticus*. Similarly, The inhibition effect of lactic acid was also suggested to be associated with total lactic acid, lactate, or undissociated lactic acid (Adolf *et al*. 2002). As high amount of lactic acid was produced in our study, it may have highly influenced the inhibition of growth at those experimental regions.

In conclusion, the inoculum size highly affected the production of organic acids (acetic, butyric, formic and propionic acids). The interaction of inoculum size and mannitol favoured the production of formic acid, while the production of lactic acid was significantly affected by interaction between the inoculum size and inulin. The production of acetic and formic acids was growth associated and highly influenced by mannitol and FOS, while the production of lactic acid appeared to inhibit the growth of *L. acidophilus* ATCC 4962.

Table 5.18 Individual factors (X_1-X_4) with their respective coded values as obtained from their real values.

Factors	Real values[a]			Coded value[b]
	x_i	X_0	ΔX_i	X_i
X_1	0.2	2.2	1	-2
	1.2			-1
	2.2			0
	3.2			+1
	4.2			+2
X_2	0.3	4.3	2	-2
	2.3			-1
	4.3			0
	6.3			+1
	8.3			+2
X_3	1.4	3.4	1	-2
	2.4			-1
	3.4			0
	4.4			+1
	5.4			+2
X_4	2.0	6	2	-2
	4.0			-1
	6.0			0
	8.0			+1
	10.0			+2

*x_i is the real value of the factor; X_0 is the real value of the factor at center point; and ΔX_i is the step change value.

†Xi is the coded value of an independent factor; Xi = (xi – X0)/ΔXi.

‡X_1: Inoculum size (0.20-4.20 % w/v); X_2: Mannitol (0.30-8.30 % w/v); X_3: FOS (1.40-5.40 % w/v); X_4: Inulin (2.00-10.00 % w/v).

Table 5.19 The combination matrix of the central composite design (CCD) using coded levels for the response of acetic, butyric, formic, propionic, lactic acids and growth of *L. acidophilus* ATCC 4962.

Runs	Block[a]	Factors[b]				Response[c]					
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	1	-1	-1	-1	-1	4.872	0.595	1.455	0.841	33.500	2.883
2	1	1	-1	-1	-1	7.947	2.315	1.373	2.802	101.220	2.515
3	1	-1	1	-1	-1	6.645	0.529	0.854	1.046	43.757	2.558
4	1	1	1	-1	-1	9.784	1.739	9.145	4.980	95.953	2.286
5	1	-1	-1	1	-1	4.239	0.734	0.670	1.019	42.371	2.059
6	1	1	-1	1	-1	9.934	3.027	3.374	3.750	108.197	2.358
7	1	-1	1	1	-1	6.107	1.137	0.668	1.671	49.886	2.032
8	1	1	1	1	-1	9.738	2.233	8.285	3.353	99.047	2.578
9	1	-1	-1	-1	1	3.838	0.502	0.860	0.791	38.082	1.917
10	1	1	-1	-1	1	20.015	2.974	2.385	1.542	146.622	2.027
11	1	-1	1	-1	1	9.520	0.089	1.056	2.357	69.099	1.853
12	1	1	1	-1	1	13.270	1.562	3.230	3.127	205.712	2.425
13	1	-1	-1	1	1	5.541	0.472	0.993	1.226	49.648	1.638
14	1	1	-1	1	1	30.165	2.601	2.844	3.156	171.819	2.121
15	1	-1	1	1	1	5.551	0.647	1.321	1.250	44.630	1.801
16	1	1	1	1	1	57.203	2.364	4.561	3.380	137.747	2.160
17	1	0	0	0	0	61.673	1.495	8.068	3.858	100.953	2.917
18	1	0	0	0	0	47.947	1.653	8.167	3.819	97.546	3.195
19	1	0	0	0	0	41.371	1.444	7.609	4.011	96.143	2.924
20	1	0	0	0	0	62.737	1.329	5.923	3.073	100.540	2.728
21	2	-2	0	0	0	35.411	0.432	0.380	0.700	31.261	1.396
22	2	2	0	0	0	37.893	3.856	3.961	3.952	185.734	2.458
23	2	0	-2	0	0	24.391	1.935	0.952	5.388	149.111	1.875
24	2	0	2	0	0	16.307	1.420	2.470	4.288	187.631	1.617
25	2	0	0	-2	0	15.753	1.588	0.864	5.778	169.581	2.236
26	2	0	0	2	0	13.963	2.444	2.188	5.845	171.312	2.015
27	2	0	0	0	-2	12.831	1.370	4.082	2.665	90.207	2.813
28	2	0	0	0	2	10.111	2.184	6.090	2.990	154.026	2.116
29	2	0	0	0	0	40.111	1.283	5.837	3.393	91.510	3.038
30	2	0	0	0	0	67.798	0.764	7.475	4.252	92.321	2.639

*1, first day of experiment; 2, second day of experiment.

†X_1: Inoculum size (0.20-4.20 % w/v); X_2: Mannitol (0.30-8.30 % w/v); X_3: FOS (1.40-5.40 % w/v); X_4: Inulin (2.00-10.00 % w/v).

‡Y_1: Acetic acid (mmol l^{-1}); Y_2: Butyric acid (mmol l^{-1}); Y_3: Formic acid (mmol l^{-1}); Y_4: Propionic acid (mmol l^{-1}); Y_5: Lactic acid (mmol l^{-1}); Y_6: Growth ($\log_{10}$ CFU ml^{-1}).

Table 5.20 Regression coefficients of the second-order equation[a] for the six responses[b].

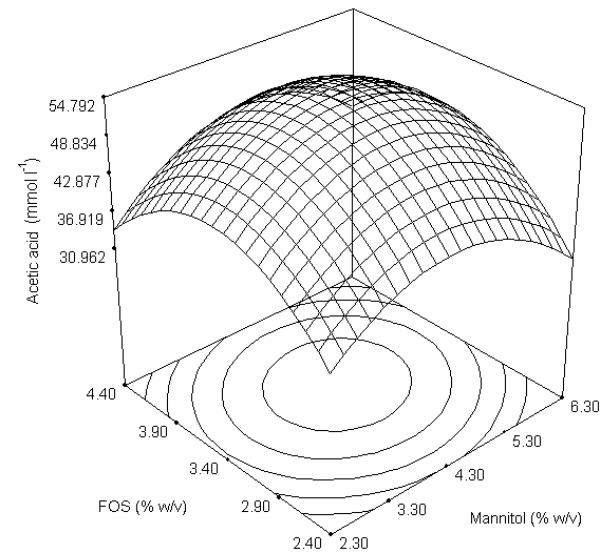
Coefficient	Y_1	Y_2[c]	Y_3	Y_4	Y_5	Y_6
c	54.7	1.60	7.14	3.96	103.28	2.885
c_1	4.86	0.87*	1.44*	0.93*	41.85*	0.161*
c_2	0.63	-0.16*	0.76*	0.16	5.48	-0.014
c_3	2.04	0.19*	0.21	0.061	-1.13	-0.090*
c_4	3.35	0.022	-0.19	-0.083	17.38*	-0.197*
c_{11}	-5.58*	-	-1.24*	-0.63*	-5.76	-0.222*
c_{22}	-9.66*	-	-1.36*	-0.005	9.21*	-0.268*
c_{33}	-11.03*	-	-1.4*	0.24	9.73*	-0.173*
c_{44}	-11.88*	-	-0.51	-0.51*	-2.35	-0.088*
c_{12}	0.79	-	0.96*	0.071	-2.07	0.043
c_{13}	3.72	-	0.22	0.066	-2.17	0.103
c_{14}	5.04	-	-0.61	-0.3	14.1*	0.083
c_{23}	1.64	-	-0.079	-0.31	-8.49	0.039
c_{24}	1.29	-	-0.56	0.048	2.98	0.056
c_{34}	3.19	-	0.13	0.067	-5.05	0.044
R^2	0.8422	0.8499	0.8862	0.8422	0.9165	0.9038
P-value	0.0017	0.0001	0.0002	0.0017	0.0001	0.0001

†$Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_4X_4 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{44}X_4^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{14}X_1X_4 + c_{23}X_2X_3 + c_{24}X_2X_4 + c_{34}X_3X_4$.

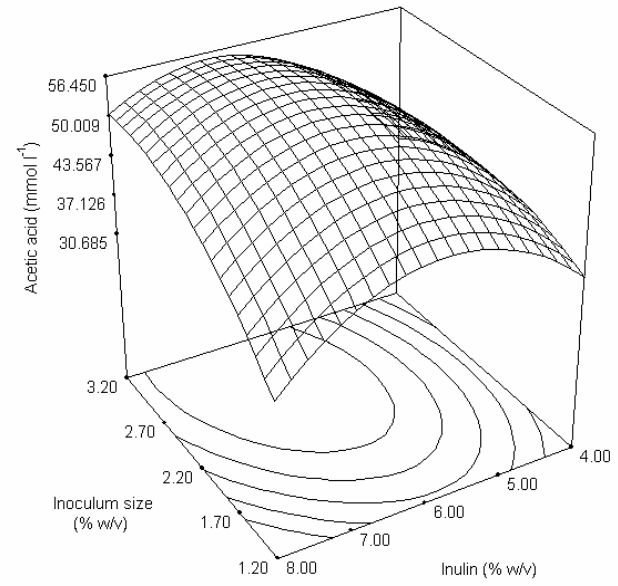
‡Y_1 = Acetic acid (mmol l^{-1}); Y_2: Butyric acid (mmol l^{-1}); Y_3: Formic acid (mmol l^{-1}); Y_4: Propionic acid (mmol l^{-1}); Y_5: Lactic acid (mmol l^{-1}); Y_6: Growth ($\log_{10}$ CFU ml^{-1}).

§Only first order regression was significant ($P < 0.05$).

*Significant at alpha = 0.05 with multivariate analysis of variance (ANOVA).

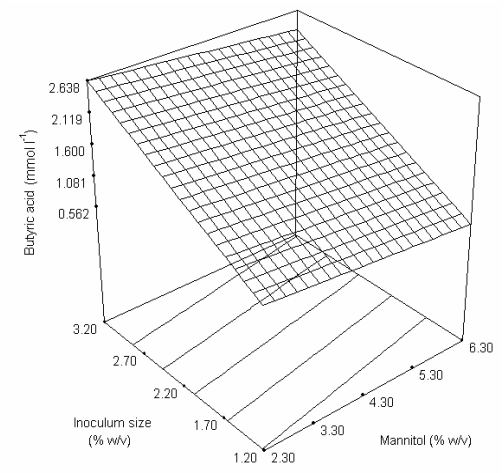


(A)

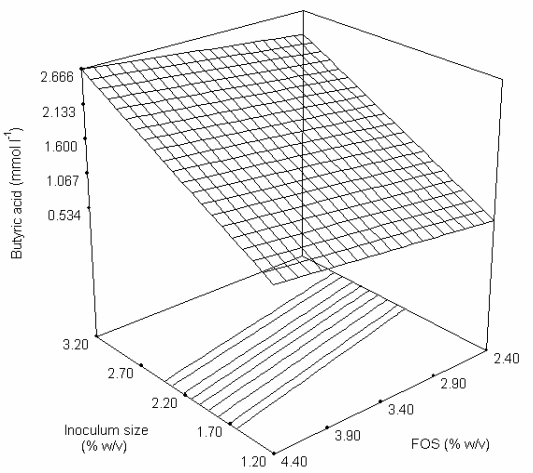


(B)

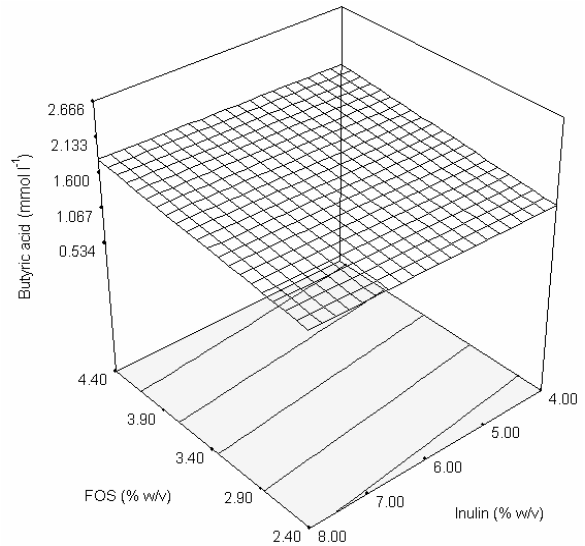
Fig. 5.21 Response surface for the production of acetic acid (mmol l^{-1}) (Y_1) as a function of (A) mannitol and FOS (X_2X_3), and (B) inoculum size and inulin (X_1X_4). $Y_1 = 54.7 + 4.86X_1 + 0.63X_2 + 2.04X_3 + 3.35X_4 - 5.58X_1^2 - 9.66X_2^2 - 11.03X_3^2 - 11.88X_4^2 + 0.79X_1X_2 + 3.72X_1X_3 + 5.04X_1X_4 + 1.64X_2X_3 + 1.29X_2X_4 + 3.19X_3X_4$. Fermentation was conducted at 37 °C for 20 h.



(A)



(B)



(C)

Fig. 5.22 Response surface for the production of butyric acid (mmol l^{-1}) (Y_2) as a function of (A) inoculum size and mannitol (X_1X_2), (B) inoculum size and FOS (X_1X_3) and (C) FOS and inulin (X_3X_4). $Y_2 = 1.60 + 0.87X_1 - 0.16X_2 + 0.19X_3 + 0.022X_4$. Fermentation was conducted at 37 °C for 20 h.

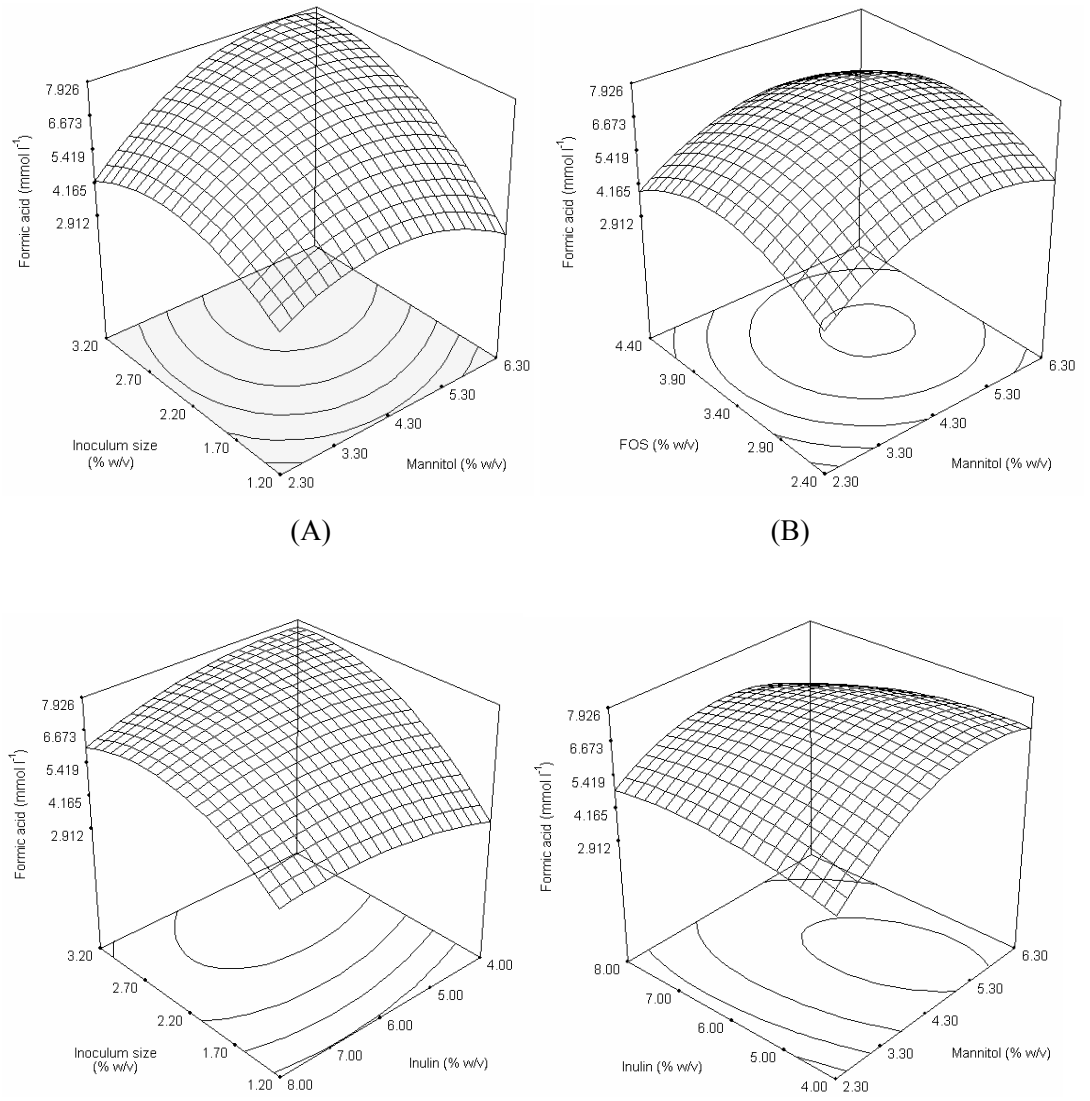
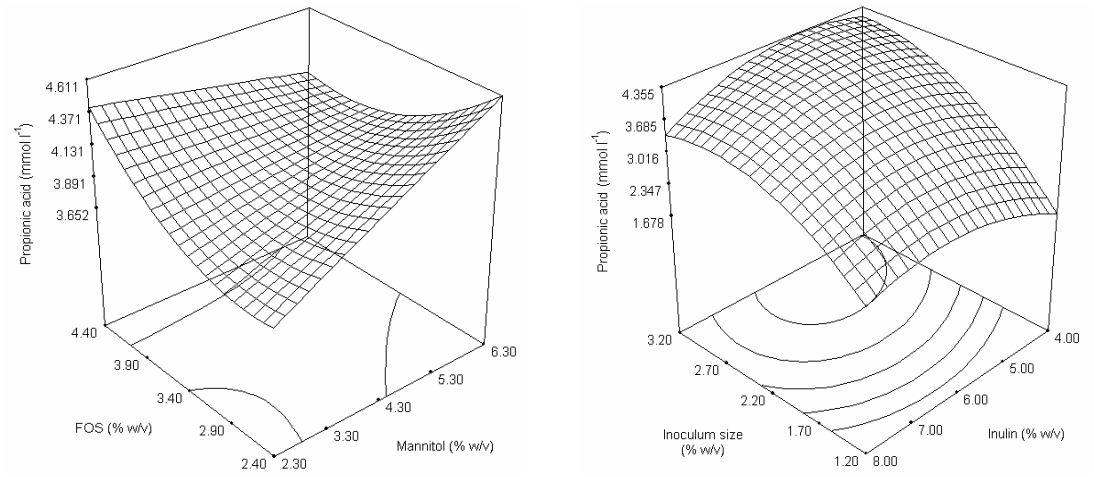
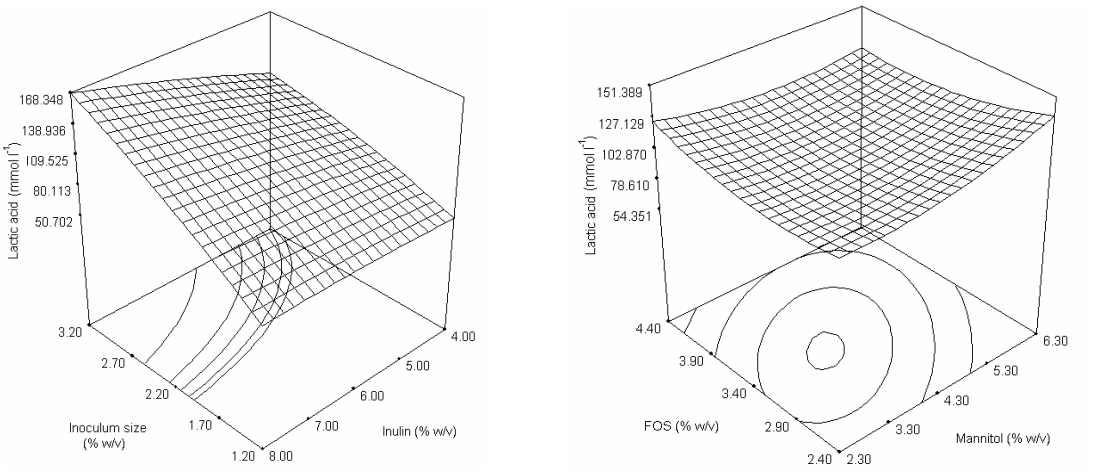


Fig. 5.23 Response surface for the production of formic acid (mmol l^{-1}) (Y_3) as a function of (A) inoculum size and mannitol (X_1X_2), (B) mannitol and FOS (X_2X_3), (C) inoculum size and inulin (X_1X_4), and (D) mannitol and inulin (X_2X_4). $Y_3 = 7.14 + 1.44X_1 + 0.76X_2 + 0.21X_3 - 0.19X_4 - 1.24X_1^2 - 1.36X_2^2 - 1.40X_3^2 - 0.51X_4^2 + 0.96X_1X_2 + 0.22X_1X_3 - 0.61X_1X_4 - 0.079X_2X_3 - 0.56X_2X_4 + 0.13X_3X_4$. Fermentation was conducted at 37 °C for 20 h.



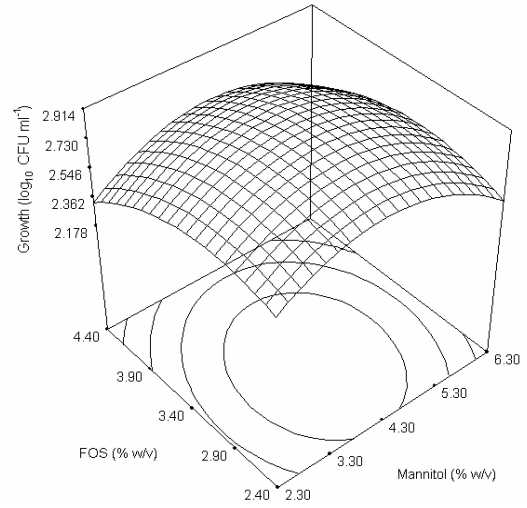
(A) (B)

Fig. 5.24 Response surface for the production of propionic acid (mmol l^{-1}) (Y_4) as a function of (A) mannitol and FOS (X_2X_3), and (B) inoculum size and inulin (X_1X_4). $Y_4 = 3.96 + 0.93X_1 + 0.16X_2 + 0.061X_3 - 0.083X_4 - 0.63X_1^2 - 0.005X_2^2 + 0.24X_3^2 - 0.51X_4^2 + 0.071X_1X_2 + 0.066X_1X_3 - 0.30X_1X_4 - 0.31X_2X_3 + 0.048X_2X_4 + 0.067X_3X_4$. Fermentation was conducted at 37 °C for 20 h.

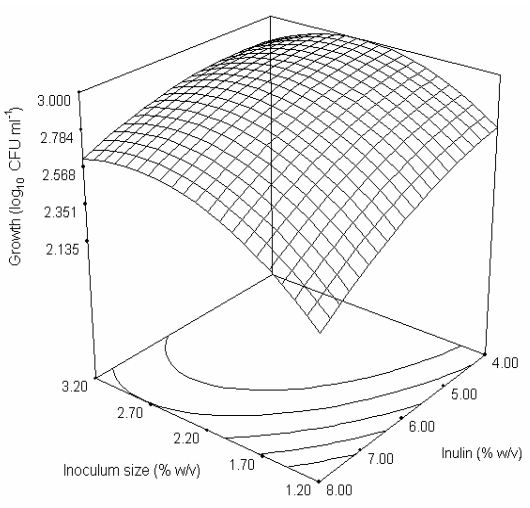


(A) (B)

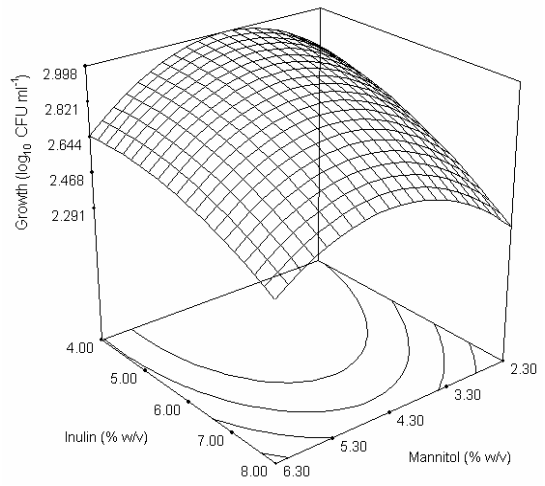
Fig. 5.25 Response surface for the production of lactic acid (mmol l^{-1}) (Y_5) as a function of (A) inoculum size and inulin (X_1X_4), and (B) mannitol and FOS (X_2X_3). $Y_5 = 103.28 + 41.85X_1 + 5.48X_2 - 1.13X_3 + 17.38X_4 - 5.76X_1^2 + 9.21X_2^2 + 9.73X_3^2 - 2.35X_4^2 - 2.07X_1X_2 - 2.17X_1X_3 + 14.10X_1X_4 - 8.49X_2X_3 + 2.98X_2X_4 - 5.05X_3X_4$. Fermentation was conducted at 37 °C for 20 h.



(A)



(B)



(C)

Fig. 5.26 Response surface for growth (log_{10} CFU mL^{-1}) (Y_6) as a function of (A) mannitol and FOS (X_2X_3), (B) inoculum size and inulin (X_1X_4), and (C) mannitol and inulin (X_2X_4). $Y_6 = 2.885 + 0.161X_1 - 0.014X_2 - 0.090X_3 - 0.197X_4 - 0.222X_1^2 - 0.268X_2^2 - 0.173X_3^2 - 0.088X_4^2 + 0.043X_1X_2 + 0.103X_1X_3 + 0.083X_1X_4 + 0.039X_2X_3 + 0.056X_2X_4 + 0.044X_3X_4$. Fermentation was conducted at 37 °C for 20 h.

5.5 THE APPLICATION OF RESPONSE SURFACE METHODOLOGY TO OPTIMIZE REMOVAL OF CHOLESTEROL, AND TO EVALUATE GROWTH CHARACTERISTICS AND PRODUCTION OF ORGANIC ACIDS BY *Bifidobacterium infantis* ATCC 17930 IN THE PRESENCE OF PREBIOTICS

5.5.1 INTRODUCTION

Probiotics are defined as 'cultures of live microorganisms that, applied in animals or humans, benefit the host by improving properties of indigenous microflora' (Arihara and Itoh, 2000). Probiotic-containing products have gained attention by the consumer due to their health promoting applications such as the prevention of gastrointestinal infections, diarrhoea and suppression of tumour (Kirjavainen *et al.* 1999). Studies have shown that a small reduction in the serum cholesterol of 1 per cent could reduce the risk of coronary heart disease by 2 to 3 percent (Manson *et al.* 1992). We have previously shown that cholesterol was removed by *Bifidobacterium* strains in laboratory media (Chapter 3.0, section 3.2.3). Various *in-vivo* studies have reported that some probiotic could lower total- and low-density-lipoprotein (LDL) cholesterol (Anderson and Gilliland, 1999; Kok and Delzenne, 1998). However, the role of probiotic-containing products as a hypocholesterolemic agent in humans is still debated.

A prebiotic is a food ingredient that is neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract, and is selectively used as a substrate for beneficial bacteria in the colon (Collins and Gibson, 1999). Most widely researched prebiotics fall in the group of oligosaccharides, especially oligofructose and inulin. Feeding rats with a diet supplemented with oligosaccharide lowered plasma concentrations of VLDL, triacylglycerol and phospholipids, and reduced postprandial triglyceridemia (Kok and Delzenne, 1998).

Recently, polyols such as mannitol and xylitol have been included as prebiotics due to their indigestible properties. Among them, the sugar alcohol D-sorbitol has been widely used as an alternative to oligosaccharides. The serum cholesterol concentration was reportedly lowered approximately by 25% in veal calves fed a high-fat milk replacer diet supplemented with 0.8% sorbitol (Bauchart *et al.* 1985). Similar to the U.S.Age of probiotics, *in-vivo* and *in-vitro* studies on the role of prebiotics have also failed to reach a firm conclusion.

This section has been published: *Liong, MT and Shah, NP. 2006. The application of response surface methodology to optimize removal of cholesterol, and to evaluate growth characteristics and production of organic acids by Bifidobacterium infantis ATCC 17930 in the presence of prebiotics.* ***Int J Probiotics Prebiotics*** *1: 41-56.*

Advancing from the sole use of probiotics or prebiotics for a healthier intestinal microflora balance is the application of synbiotic, a product that contains both probiotic and prebiotic. The concept of synbiotic has been widely studied, mostly to modulate colonic microbial population and to improve stability or survivability of probiotics both *in-vitro* and *in-vivo* (Cummings *et al.* 2001). However, to our knowledge, there is no information on suitable combinations of probiotics and prebiotics specifically targeting removal of cholesterol, although a limited number of *in-vitro* and *in-vivo* studies addressed the use of prebiotics or synbiotic to remove cholesterol, and to regulate hepatic lipogenesis and lipid metabolism (Suskovic *et al.* 2001). Yet, in most of these studies, the interaction patterns of synbiotics to reduce cholesterol are poorly understood.

There are also growing evidence that supports the role of probiotic bacteria and their production of organic acids in mediating health and disease of the host (Flickinger *et al.* 2002). Organic acids have been reported to contribute to normal large bowel function, and prevent pathology through their actions in the lumen (Topping and Clifton, 2001). Lower pH values contributed by lactic acid also prevented the overgrowth of pH-sensitive pathogenic bacteria. Formate has also been shown to kill *Escherichia coli* and Salmonella at pH 5 (Cherrington *et al.* 1991). Acetate can reduce the concentration of serum fatty acids, which is an important factor in lowering glucose utilization by tissues and inducing insulin resistance (Rizkalla *et al.* 2000). It was previously found that the production of these organic acids by strains of *Bifidobacterium* was highly influenced by types of prebiotics. The production of acetic acid was higher in the presence of lactulose, inulin and hi-maize compared to the control, while higher production of lactic acid was found in the presence of inulin compared to the control (Bruno *et al.* 2002).

The traditional one-factor-at-one-time method used for optimization requires a large number of experiments to describe the effects of individual factors, is time consuming and can not distinguish the interaction effects of multiple factors. Response surface methodology (RSM) is an experimental strategy first described by Box and co-workers to evaluate the optimum conditions for the multivariate systems (Box *et al.* 1978). RSM consists of a group of mathematical and statistical procedures to study the relationships between single or multiple responses and defines the effect of variables on the process. Up till now, there has been no report on the use of RSM in removing or reducing cholesterol *in-vitro* or in animal models. Thus, the aim of this study was to optimize cholesterol removal by *B. infantis* ATCC 17930 in the presence of selected prebiotics, using a response surface approach. This information will provide better understanding of the interactions involved in cholesterol

reduction in a synbiotic system. In addition, growth characteristics and production of organic acids by *B. infantis* ATCC 17930 in the presence of these prebiotics were also studied.

5.5.2 MATERIALS AND METHODS

5.5.2.1 Bacteria and media preparation

Bifidobacterium infantis ATCC 17930 was used in this study. All working cultures and media were prepared as described in section 5.1.2.1.

5.5.2.2 Cholesterol removal

Removal of cholesterol was quantified as described in section 5.1.2.2.

5.5.2.3 Growth of *B. infantis* ATCC 17930

Growth was determined using the pour plate method as described in section 5.1.2.3. The growth was expressed as the difference in $\log_{10}$ CFU of the initial count obtained at time = 0 and that at the end of the incubation period (20 h).

5.5.2.4 Mean doubling time

Mean doubling time was determined as described in section 5.1.2.4.

5.5.2.5 Determination of organic acids

The production of organic acids was determined using HPLC as described in section 5.1.2.6.

5.5.2.6 Experimental design and statistical analyses

The design of experiment and statistical analyses of all data obtained was performed as described in section 5.1.2.7.

5.5.3 RESULTS

5.5.3.1 Screening of factors

In-vitro removal of cholesterol was assessed using seven factors namely inoculum size (X_1), sorbitol (X_2), mannitol (X_3), maltodextrin (X_4), hi-amylose maize (X_5), inulin (X_6) and FOS (X_7). Screening experiments involved a partial two-level factorial design 2^{7-2} resulting in 64 experimental runs (including duplicates) and 5 middle point runs (Table 5.21). In the partial factorial design, the factors were coded according to the following equation:

$$X_i = (x_i - X_0)/\Delta X_i$$

where X_i is the coded value of an independent factor; x_i is the real value of the factor; X_0 is the real value of the factor at center point; and ΔX_i is the step change value (Kong *et al.* 2004). The range and coded values of the factors are shown in Table 5.21.

Analysis of variance (ANOVA) showed that the first-order model was significant with only 9.58% variation unexplained by the model (Table 5.22). Both curvature and lack-of-fit tests were insignificant, indicating that the model produced was suitable for prediction. Of the seven factors studied, only inoculum size (X_1), sorbitol (X_2), maltodextrin (X_4), and inulin (X_6) significantly ($P < 0.05$) influenced cholesterol removal from the media. Other factors were insignificant, hence were excluded from further optimization procedures.

5.5.3.2 Steepest ascent experiments

A first-order model was formed based on the first-order equation for the response of cholesterol removal (Y), and with the significant factors now redefined as inoculum size (X_1), sorbitol (X_2), maltodextrin (X_3) and inulin (X_4):

$$Y = 20.06 + 5.50X_1 + 2.34X_2 + 1.60X_3 + 3.34X_4. \quad (4)$$

Inoculum size produced the most significant effect, as observed by the highest coefficient estimate value (Table 5.22). Thus, it was used as the fundamental scale for the steepest ascent procedures. The steepest ascent design was based on an increase in the concentration of 0.50% (w/v) concentration for X_1. This produced 5 coded factors (0.50/0.10 = 5). Thus, the movement for X_2 was 2.13 design units ((2.34/5.50)(5) = 2.13), and those for X_3 and X_4 were 1.45 design units ((1.60/5.50)(5) = 1.45) and 3.04 design units ((3.34/5.50)(5) = 3.04), respectively. The steepest ascent coordinates were generated as shown in Table 5.23. The removal of cholesterol increased with each step of steepest ascent and the highest was obtained at step 6, with 51.178 μg/mL cholesterol removed. The removal of cholesterol was found to decrease beyond this point. This indicated that the experimental runs approached the optimum region. Thus, the coordinates obtained from step 6 (2.70% (w/v) of probiotic,

6.30% (w/v) of sorbitol, 4.60% (w/v) of maltodextrin and 8.60% (w/v) of inulin) were used as middle points for optimization experiments.

5.5.3.3 Optimization experiments

Optimization experiments were conducted using the central composite design (CCD). It involved an alpha value of 2.00 for four factors resulting in 30 experimental runs and was conducted in two blocks (Table 5.24). ANOVA results (Table 5.25) indicated that the second-order model obtained was significant, with only 3.6% of the total variation unexplained by the model. The insignificant lack-of-fit test and a good coefficient regression indicated that the model used was a good predictor. All factors showed significant quadratic effects on the removal of cholesterol, while only the interactions of inoculum size-sorbitol (X_1X_2) and inoculum size-inulin (X_1X_4) were significant ($p < 0.05$).

Perturbation plots were used to assess the changes in the response as each factor moves from the chosen reference point, with all other factors held constant at reference values (Oh *et al.* 1995). In our study, factors were held at their respective optimum points when one particular factor was being assessed. We defined the response surface model as obtained from Table 5.25 as $\hat{Y} = f(X_1, X_2, X_3, X_4)$. The optimum points of the factors were defined as (X^*_1, X^*_2, X^*_3, X^*_4), which in our experiments would be (1.000, 0.582, -0.090, -1.000). Thus, the perturbation effect of X_1 was defined as:

$$\hat{Y}(X_1) = f(X_1, X^*_2, X^*_3, X^*_4) \quad (5)$$

Similarly, the perturbation effects of X_1 and X_2 would be:

$$\hat{Y}(X_2) = f(X^*_1, X_2, X^*_3, X^*_4) \quad (6)$$

$$\hat{Y}(X_3) = f(X^*_1, X^*_2, X_3, X^*_4) \quad (7)$$

$$\hat{Y}(X_4) = f(X^*_1, X^*_2, X^*_3, X_4) \quad (8)$$

The vertical axis represented $\hat{Y}(X_j)$ and the horizontal axis represented X_j in the perturbation effect curves. By overlying all perturbation curves, we obtain a perturbation plot (Oh *et al.* 1995) as shown in Figure 5.27. Although all factor showed significant quadratic effect, inoculum size produced the most prominent curve, compared to other factors that were fixed at their maximum levels. This indicated that the inoculum size was the most significant factor that contributed to the removal of cholesterol with the most obvious quadratic effect. Although the P-values of both sorbitol and maltodextrin showed similar levels of significance, it could be clearly seen from the perturbation plot that the response curve of maltodextrin was more prominent than that of sorbitol, indicating a strong quadratic

effect. Inulin showed the least prominent curve, indicating the weakest quadratic effect, as supported by the higher P-value obtained.

The best explanatory equation to produce a second-order model and the response surface was expressed as:

$$Y_0 = c + \Sigma c_i X_i + \Sigma c_{ii} X_i^2 + \Sigma c_{ij} X_i X_j \quad (9)$$

where c_i, c_{ii} and c_{ij} are the regression coefficients for linear, quadratic and interaction terms. X_i and X_j are independent factors while intercept c is the estimated response at the center point, with the coded values of X_i at 0. Only two factors could be plotted at a time, with the vertical axis representing cholesterol removal and the other two horizontal axes representing the actual levels of two factors. The factor that was not represented by the two horizontal axes in each plot was fixed at its actual optimum level. In our study, the second-order regression model involved four factors, thus producing four linear, four quadratic and six interaction terms. A response surface was generated (Figure 5.28) based on the second-order equation:

$$Y_0 = 47.60 + 5.70X_1 + 1.16X_2 - 0.039X_3 - 1.28X_4 - 4.16X_1^2 - 2.52X_2^2 - 2.79X_3^2 - 1.81X_4^2 + 1.60X_1X_2 + 0.23X_1X_3 - 2.95X_1X_4 + 0.61X_2X_3 - 0.23X_2X_4 + 1.05X_3X_4 \quad (10)$$

An optimum point was produced with optimum cholesterol removal obtained at 52.180 μg/mL. The combination that produced the optimum point was (X_1, X_2, X_3, X_4) = (0.871, 0.551, 0.006, -0.931). The original levels that correlated with those coded values were found to be the inoculum size at 3.74% (w/v), sorbitol concentration at 7.40% (w/v), maltodextrin at 4.61% (w/v) and inulin at 7.67% (w/v).

The reliability of the model used was evaluated using a validation experiment. The removal of cholesterol was assessed using four media (Figure 5.29) namely the optimum medium (inoculum size, 3.70% (w/v); sorbitol, 7.40% (w/v); maltodextrin, 4.60% (w/v); inulin, 7.70% (w/v)), high-point medium (inoculum size, 3.90% (w/v); sorbitol, 8.30% (w/v); maltodextrin, 6.60% (w/v); inulin, 9.60% (w/v)), low-point medium (inoculum size, 1.50% (w/v); sorbitol, 4.30% (w/v); maltodextrin, 2.60% (w/v); inulin, 7.60% (w/v)) and the center-point medium (inoculum size, 2.70% (w/v); sorbitol, 6.30% (w/v); maltodextrin, 4.60% (w/v); inulin, 8.60% (w/v)). The actual values obtained from the optimum-point, center-point, high-point and the low-point media were 53.678, 49.325, 43.264 and 33.487 μg/mL, respectively. This produced an error of 2.247%, 3.497%, 7.158% and 2.506%, respectively, for the four media as compared to the predicted values. A small error and similar cholesterol removal patterns proved the validity of the model and the reproducibility of the predicted values.

5.5.3.4 Growth, mean doubling time and production of organic acids

Growth, mean doubling time and production of organic acids were studied within the experimental region for optimum cholesterol removal. The responses obtained are shown in Table 5.26 while the coefficient estimates were shown in Table 5.27.

The growth of *B. infantis* ATCC 17930 was significant in a first-order manner while the other regressions were insignificant. Individual factors for the inoculum size, and concentrations of sorbitol, maltodextrin and inulin significantly ($p < 0.05$) influenced the growth of the organism. The response surface of growth (Figure 5.30) was obtained based on the following linear equation:

$$Y_1 = 1.94 + 0.78X_1 – 0.14X_2 + 0.40X_3 – 0.12X_4 \quad (11)$$

Increasing inoculum size from 1.50 to 3.90% (w/v) showed a continuous increase in the growth of the organism. Similarly, increasing concentration of maltodextrin from 2.60 to 6.60% (w/v) contributed to a linear increase in the growth, while concentration of inulin and sorbitol showed least influence on the growth.

Similar to growth, regression analysis also showed that the factors studied influenced the response of mean doubling time linearly. The following first-order equation was used to construct the response surface (Figure 5.31):

$$Y_2 = 216.16 + 3.72X_1 – 0.64X_2 + 0.74X_3 - 0.16X_4 \quad (12)$$

The response surface obtained showed that an increase in the inoculum size produced the most apparent increase in the mean doubling time, although the concentration of maltodextrin also showed a significant coefficient estimate.

As shown in Figure 5.32A, the inoculum size of *B. infantis* ATCC 17930, concentrations of maltodextrin and inulin had significant second-order regression ($p < 0.05$), with inoculum size having the strongest effect while inulin had the least effect on the production of acetic acid (Y_3). Sorbitol affected the production of acetic acid in a linear manner (Figure 5.32B) and this was supported by the significant coefficient of the first-order regression (Table 5.27). However, it must also be noted that increasing production of acetic acid was achieved at decreasing concentration of sorbitol. Increasing levels of inoculum size showed apparent increase in the production of acetic acid, while maltodextrin showed similar effect only at higher level of inoculum size (Figure 5.32A). Although inulin had significant quadratic effect, its effect on increased production of acetic acid was minimal, as indicated by the small change in the response surface produced (Figure 5.32B).

Although sorbitol, maltodextrin and inulin showed a significant second-order regression effect (Figure 5.33), it was the first-order effect of inoculum size that exhibited the most prominent change on the production of lactic acid (Y_4). A gradual increase in the inoculum size of *B. infantis* ATCC 17930 caused a linear increase in the production of lactic acid. As supported by the strong coefficient estimate (Table 5.27), the inoculum size had the strongest influence on the production of lactic acid, and may further increase its production at a larger inoculum size beyond the experimental region.

The inoculum size was the sole factor that exhibited a significant second-order regression ($p < 0.05$) on the production of formic acid (Y_3), while maltodextrin showed a prominent linear effect (Figure 5.34). Increasing the level of maltodextrin contributed to a continuous increase in the production of formic acid that may be further increased beyond the experimental region, as indicated by the response surface (Figure 5.34A). Although inoculum size exhibited a significant quadratic effect, it only contributed to an increase in the production of formic acid at higher concentration of maltodextrin (6.60% w/v). Maltodextrin exhibited a stronger effect than other prebiotics (Table 5.27). Sorbitol and inulin did not contribute to a significant ($p > 0.05$) production of formic acid; this was also supported by the near-plateau response surface produced (Figure 5.34B).

5.5.4 DISCUSSION

Response surfaces produced are often affected by various factors. The initial aim of the optimization processes is to identify important factors using the screening experiments. Factorial designs, a class of experimental design, are useful in identifying the important factors, and interactions between two or more factors (Kong *et al.* 2004). A complete replication of the seven factors using a 2^x factorial design would need 128 experimental runs. However, only seven degree of freedoms (DF) would be needed to estimate main effects, and 21 DF would estimate two-factor interaction effects, while the remaining 99 DF would estimate error or/and three or higher-factor interaction effects (Cox and Reid, 2000). Thus, a partial two-level factorial design (2^{7-2}) was applied in this study. Partial factorial designs are capable of identifying important factors and determining interaction effects between factors using less number of experimental runs as compared to full factorial design without loss of information from main factor effects and their interactions (Li *et al.* 2002).

Following the screening of significant factors, we have found that the optimum condition was not within the domain of our experimental region. Design points were subjected to steepest ascent, which was another set of experiment. Steepest ascent formed the

direction at right angles to show the relative amounts by which the factors had to vary in order to attain a maximum increase in responses (Kong *et al.* 2004). The path begins at the center of the initial experimental region and stretched outside the design space (Myers and Montgomery, 1995). Thus, in our study, the path of steepest ascent was stretched to increase the inoculum size and concentrations of sorbitol, maltodextrin and inulin in order to improve removal of cholesterol.

An alpha value of ± 2.00 was used in the central composite design to produce rotatability of design. Rotatability is a desirable property because in rotatable designs, the points with the same distance from the middle points will have similar estimated error. It also indicates the axial point in the CCD. Thus, with an alpha value of 2.00, then the axial point is located at 2.00 times the level coded as +1. The alpha was obtained from:

$\propto = 2^{k/4}$; where k is the number of factor (Araujo and Brereton, 1996). (14)

A significant quadratic regression and a strong determinant coefficient suggested that the model accurately represented data within the experimental region. It must be noted that the coefficient estimates for the interaction terms (X_1, X_2) and (X_1, X_4) were both significant ($p < 0.05$) with positive (1.60) and negative (-2.95) signs, respectively. The positive sign implies that for an increase in the response, the coded levels of X_1 and X_2 must have similar signs, i.e., both must be larger than zero or both must be lower than zero, while one of the coded levels of X_1 and X_4 must be lower than zero to produce a negative sign. When the optimum point was achieved, X_1 and X_2 were 0.871 and 0.551, respectively, while X_1 and X_4 were 0.871 and –0.931, respectively. These strongly indicated that second-order terms were sufficient and higher-order terms were not necessary (Oh *et al.* 1995), as also supported by the insignificant lack-of-fit test. Validation experiments showed a good correlation between the results and the prediction by the model, proving that the response model was adequate to reflect the conditions for optimal removal of cholesterol.

It must be noted that the regression of cholesterol removal differed from that of the growth, with cholesterol removal showing significant quadratic regression while the growth had a linear regression ($p < 0.05$). We previously found that removal of cholesterol was growth associated via cholesterol assimilation, binding to dead cells and incorporation of cholesterol into the cellular membrane (Chapter 3.0, section 3.2.3). In the present study, the response surfaces produced (Figures 5.28 and 5.30) also showed a strong correlation between growth and the removal of cholesterol, despite different order of regression. Increasing growth supported increasing removal of cholesterol. Only at factorial points of higher inoculum size, that removal of cholesterol ceased despite proceeding growth at those

regions (Figure 5.30). The mean doubling time is a measure of effectiveness of a specific carbon source in modulating bacterial growth rate (Bruno et al. 2002). Thus, considering that the mean doubling time was also higher in those regions (Figure 5.31), we postulate that due to substrate limitation at higher inoculum size, growth may continuously increase but at a slower substrate utilization rate and this may have reduced cholesterol removal within those regions. Nevertheless, the strong similarities between both response surfaces strongly led us to conclude that the removal of cholesterol was growth associated. Although previous studies found that cholesterol was removed by non-growing cells via binding to the surface of cells (Brashears *et al.* 1998; Kimoto *et al.* 2002), this study suggests that it would be beneficial to maintain viability of the organism in *in-vivo* models for cholesterol-lowering effects.

The response surfaces produced (Figures 5.28, 5.30 and 5.31) indicated a strong degree of correlation despite different order of regressions. It must be noted that the response surfaces for growth and mean doubling time exhibited a similar pattern. Larger inoculum size and higher concentrations of prebiotics increased the growth of the organism (Figure 5.30), but substrate utilization rates decreased (increasing mean doubling time) within those regions (Figure 5.31). This may be due to the fact that higher inoculum size reaches its maximum density faster, thus resulting in longer mean doubling time. Superimposition of the response surfaces of cholesterol removal, growth and mean doubling time showed that the removal of cholesterol was increased by higher growth and at higher substrate utilization rates.

The major products of metabolism of prebiotics are organic acids, carbon dioxide and hydrogen, and bacterial cell mass (Cummings *et al.* 2001). Bifidobacteria have been reported to ferment non-digestible carbohydrate into acetic and lactic acids as major end-products (Niness, 1999). Much attention has been directed to the production of organic acids and the various health benefits of individual acids. However, no particular pattern of organic acids production from prebiotic fermentation has emerged as yet. Although the response for the growth of *B. infantis* ATCC 17930 exhibited a first-order regression, response surfaces produced showed a strong correlation with the production of acetic and lactic acids, indicating that these acids were growth associated. Inoculum size of *B. infantis* ATCC 17930 and maltodextrin were the main factors contributing to an increase in growth of the organism and the production of acetic and lactic acids. Sorbitol exerted least effect as the ability of bifidobacteria to ferment sorbitol is strain dependant. Sorbitol fermenting strains of bifidobacteria has been used as a specific indicator for the determination of human faecal pollution (Mara and Oragui, 1983), while Toure *et al.* (2003) also found that infant strains of bifidobacteria were able to ferment both L-sorbose and sorbitol. The study on metabolic

pathways revealed that sorbitol-specific enzymes are needed to transport and phosphorylate D-sorbitol, and were required for growth on D-sorbitol (Yebra and Perez-Martinez, 2002). Considering that *B. infantis* ATCC 17930 was able to grow and produce organic acids on fermentation of sorbitol, it was very likely that the organism has such sorbitol-phosphorylating enzymes as well. However, it must also be noted that the growth of *B. infantis* ATCC 17930 and the production of acetic and lactic acids were repressed by an increased concentration of sorbitol, while a higher production of acetic and lactic acids were achieved at lower concentration of sorbitol. We speculate that subsequent glycolysis of phosphorylated sorbitol would have generated end-product of fructose, which may have inhibited the growth of *B. infantis* ATCC 17930 and the production of acetic and lactic acids.

The major end products of fermentation of glucose by bifidobacteria are 2 molecules of lactate and 3 molecules of acetate, with a ratio of acetate: lactate of 1.5:1. It must be noted that in the present study, the ratio of acetic acid: lactic acid was approximately 3:1. This showed deviation from the theoretical value, and it appeared that the presence of sorbitol, maltodextrin and inulin encouraged the production of acetic acid. Deviations from the theoretical value have also been reported previously, mediated by the addition of hi-maize and raftilose in the media (Bruno *et al.* 2002).

Although inoculum size greatly influenced the production of formic acid, the response surface for growth and mean doubling time showed little resemblance to the response surface of formic acid, indicating its production was not growth dependent, but may be mediated by non-metabolic pathways. This is supported by the fact that the pyruvate-formate-lyase system that produces formic acid from pyruvic acid has not been reported in bifidobacteria. Considering that only maltodextrin affected the production of formic acids positively, we speculate that it may be an important factor in the formate synthesis mechanism. More work is needed to elucidate this.

Optimal amount of cholesterol removed by *B. infantis* ATCC 17930 was 56.314 μg/mL with inoculum size of 3.74% (w/v), sorbitol concentration of 7.40% (w/v), maltodextrin of 4.61% (w/v) and inulin of 7.67% (w/v). Validation experiments showed that the prediction by the model correlated well with the results obtained, indicating that the response model generated reflected the optimized conditions for removal of cholesterol. Analyses of growth and mean doubling time provided experimental support that higher removal of cholesterol by *B. infantis* ATCC 17930 was obtained at higher growth and at higher substrate utilization rates. At higher concentrations, sorbitol exhibited inhibition of

the end product for the production of acetic and lactic acids. Maltodextrin induced the production of acetic, lactic and formic acids linearly.

Table 5.21. Treatment combinations and response for screening experiments[1,2].

Order	Inoculum size (X_1) (% w/v)	Sorbitol (X_2) (% w/v)	Mannitol (X_3) (% w/v)	FOS (X_4) (% w/v)	Hi-maize (X_5) (% w/v)	Maltodextrin (X_6) (% w/v)	Inulin (X_7) (% w/v)	Cholesterol assimilated (Y_0) (μg/mL)
1	-1	-1	-1	-1	-1	1	1	16.77
2	1	-1	-1	-1	-1	-1	-1	18.72
3	-1	1	-1	-1	-1	-1	-1	7.07
4	1	1	-1	-1	-1	1	1	23.81
5	-1	-1	1	-1	-1	1	-1	10.94
6	1	-1	1	-1	-1	-1	1	29.66
7	-1	1	1	-1	-1	-1	1	20.47
8	1	1	1	-1	-1	1	-1	24.12
9	-1	-1	-1	1	-1	-1	-1	10.61
10	1	-1	-1	1	-1	1	1	27.71
11	-1	1	-1	1	-1	1	1	17.50
12	1	1	-1	1	-1	-1	-1	21.67
13	-1	-1	1	1	-1	-1	1	21.47
14	1	-1	1	1	-1	1	-1	24.12
15	-1	1	1	1	-1	1	-1	17.81
16	1	1	1	1	-1	-1	1	33.75
17	-1	-1	-1	-1	1	-1	1	12.97
18	1	-1	-1	-1	1	1	-1	20.38
19	-1	1	-1	-1	1	1	-1	8.56
20	1	1	-1	-1	1	-1	1	23.44
21	-1	-1	1	-1	1	-1	-1	12.19
22	1	-1	1	-1	1	1	1	30.47
23	-1	1	1	-1	1	1	1	16.31
24	1	1	1	-1	1	-1	-1	19.53
25	-1	-1	-1	1	1	1	-1	7.17
26	1	-1	-1	1	1	-1	1	28.49
27	-1	1	-1	1	1	-1	1	15.73
28	1	1	-1	1	1	1	-1	22.97
29	-1	-1	1	1	1	1	1	19.90
30	1	-1	1	1	1	-1	-1	24.22
31	-1	1	1	1	1	-1	-1	17.55
32	1	1	1	1	1	1	1	35.99
33	0	0	0	0	0	0	0	18.54
34	0	0	0	0	0	0	0	17.71
35	0	0	0	0	0	0	0	18.54
36	0	0	0	0	0	0	0	17.60
37	0	0	0	0	0	0	0	19.58

[1] $X_1 = (x_1 - 0.20)/0.10$; $X_2 = (x_2 - 1.00)/0.50$; $X_3 = (x_3 - 1.00)/0.50$; $X_4 = (x_4 - 1.00)/0.50$; $X_5 = (x_5 - 1.00)/0.50$; $X_6 = (x_6 - 1.00)/0.50$; $X_7 = (x_7 - 1.00)/0.50$.

[2] All points are means of duplicate values.

Table 5.22. Analysis of variance and coefficient estimates for the evaluation of the first-order model.

Source of variation	Sum of squares	DF[1]	Mean square	F-value	P-value
Model[2]	3214.13	5	642.83	115.38	0.0001
Curvature	12.88	1	12.88	2.31	0.1334
Residual	345.43	62	5.57		
Lack-of-fit	137.81	26	5.30	0.92	0.5831
Pure error	207.62	36	5.77		
Correlation total	3572.45	68			

Factor:	Coefficient estimate	DF	Standard error	t-value	P-value
Inoculum size (X_1)	5.50	1	0.30	18.64	0.0001*
Sorbitol (X_2)	2.34	1	0.30	7.94	0.0001*
Maltodextrin (X_6)	3.34	1	0.30	11.31	0.0001*
Inulin (X_7)	1.60	1	0.30	5.43	0.0001*

[1]DF: degree of freedom.

[2]$R^2 = 0.9042$.

*Significant at alpha 0.05.

Table 5.23. The coordination path of steepest ascent for all chosen factors in coded and natural levels.

Step	Coded factors[1]				Natural factors[2]				Cholesterol assimilated (μg/mL)
	ξ_1	ξ_3	ξ_6	ξ_7	X_1	X_2	X_3	X_4	
1) Base	0	0	0	0	0.20	1.00	1.00	1.00	
Δ	5	2.13	1.45	3.04	(5)(0.1) = 0.5	(2.13)(0.50) = 1.07	(1.45)(0.50) = 0.73	(3.04)(0.50) = 1.52	22.513
2) Base + Δ	5	2.13	1.45	3.04	0.70	2.07	1.73	2.52	32.344
3) Base + 2Δ	10	4.26	2.90	6.08	1.20	3.14	2.46	4.04	44.063
4) Base + 3Δ	15	6.39	4.35	9.12	1.70	4.21	3.19	5.56	49.531
5) Base + 4Δ	20	8.52	5.80	12.16	2.20	5.28	3.92	7.08	50.313
6) Base + 5Δ	25	10.65	7.25	15.20	2.70	6.35	4.65	8.60	51.178
7) Base + 6Δ	30	12.78	8.70	18.24	3.20	7.42	5.38	10.12	49.659

[1]ξ_1: inoculum size (% w/v), ξ_2: sorbitol (% w/v), ξ_6: maltodextrin (% w/v); ξ_7: inulin (% w/v).

[2]X_1: inoculum size (% w/v), X_2: sorbitol (% w/v), X_3: maltodextrin (% w/v); X_4: inulin (% w/v).

Table 5.24. The combination matrix of the central composite design (CCD) using coded levels for the response of cholesterol removal.

Standard run	Block[1]	Factors				Cholesterol removal (μg/mL)[2]
		Inoculum size (X_1)	Sorbitol (X_2)	Maltodextrin (X_3)	Inulin (X_4)	
1	1	-1	-1	-1	-1	34.579
2	1	1	-1	-1	-1	47.860
3	1	-1	1	-1	-1	32.235
4	1	1	1	-1	-1	51.610
5	1	-1	-1	1	-1	31.298
6	1	1	-1	1	-1	40.829
7	1	-1	1	1	-1	28.642
8	1	1	1	1	-1	53.954
9	1	-1	-1	-1	1	33.017
10	1	1	-1	-1	1	37.235
11	1	-1	1	-1	1	34.423
12	1	1	1	-1	1	39.579
13	1	-1	-1	1	1	34.267
14	1	1	-1	1	1	38.329
15	1	-1	1	1	1	35.048
16	1	1	1	1	1	41.923
17	1	0	0	0	0	50.829
18	1	0	0	0	0	51.298
19	1	0	0	0	0	50.360
20	1	0	0	0	0	49.579
21	2	-2	0	0	0	16.767
22	2	2	0	0	0	41.298
23	2	0	-2	0	0	33.642
24	2	0	2	0	0	37.548
25	2	0	0	-2	0	33.173
26	2	0	0	2	0	35.829
27	2	0	0	0	-2	39.267
28	2	0	0	0	2	37.548
29	2	0	0	0	0	42.704
30	2	0	0	0	0	45.360

[1]1, first day of experiment; 2, second day of experiment.

[2]All points are means of duplicates.

Table 5.25. Analysis of variance of the second-order model[1] and coefficient estimates for the response Y_0 and factors X_1, X_2, X_3 and X_4.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model[2]	1760.51	14	125.75	26.75	0.0001
Residual	65.82	14	4.70		
Lack-of-fit	60.68	10	6.07	4.72	0.0740
Pure error	5.14	4	1.28		
Corr total	1963.21	29			

Factor[3]	Coefficient estimate	DF	Standard error	t-value	P-value
Intercept	$c = 47.60$	1	0.90		
X_1	$c_1 = 5.70$	1	0.44	12.89	0.0001*
X_2	$c_2 = 1.16$	1	0.44	2.62	0.0202*
X_3	$c_3 = -0.039$	1	0.44	-0.088	0.9310
X_4	$c_4 = -1.28$	1	0.44	-2.88	0.0120*
X_1^2	$c_{11} = -4.16$	1	0.41	-10.04	0.0001*
X_2^2	$c_{22} = -2.52$	1	0.41	-6.08	0.0001*
X_3^2	$c_{33} = -2.79$	1	0.41	-6.74	0.0001*
X_4^2	$c_{44} = -1.81$	1	0.41	-4.38	0.0006*
X_1X_2	$c_{12} = 1.60$	1	0.54	2.95	0.0104*
X_1X_3	$c_{13} = 0.23$	1	0.54	0.43	0.6720
X_1X_4	$c_{14} = -2.95$	1	0.54	-5.44	0.0001*
X_2X_3	$c_{23} = 0.61$	1	0.54	1.12	0.2828
X_2X_4	$c_{24} = -0.23$	1	0.54	-0.43	0.6720
X_3X_4	$c_{34} = 1.05$	1	0.54	1.95	0.0721

[1] $R^2 = 0.9640$.

[2] $Y_0 = 47.60 + 5.70X_1 + 1.16X_2 - 0.039X_3 - 1.28X_4 - 4.16X_1^2 - 2.52X_2^2 - 2.79X_3^2 - 1.81X_4^2 + 1.60X_1X_2 + 0.23X_1X_3 - 2.95X_1X_4 + 0.61X_2X_3 - 0.23X_2X_4 + 1.05X_3X_4$

[3] X_1: inoculum size (% w/v), X_2: sorbitol (% w/v), X_3: maltodextrin (% w/v), X_4: inulin (% w/v).

*Significant at alpha 0.05.

Table 5.26. The combination matrix of the central composite design (CCD) using coded levels for the factors and five responses.

Run	Block[1]	Factors[2]				Responses[3]				
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3	Y_4	Y_5
1	1	-1	-1	-1	-1	0.758	215.050	54.963	155.909	3.239
2	1	1	-1	-1	-1	3.182	215.214	55.137	188.283	2.520
3	1	-1	1	-1	-1	0.728	212.239	48.869	154.923	3.110
4	1	1	1	-1	-1	2.100	215.653	52.068	181.405	2.527
5	1	-1	-1	1	-1	1.936	210.699	56.799	159.624	6.320
6	1	1	-1	1	-1	3.188	216.556	66.421	219.751	5.546
7	1	-1	1	1	-1	1.576	212.913	43.102	144.815	12.309
8	1	1	1	1	-1	3.089	218.710	82.225	193.987	4.748
9	1	-1	-1	-1	1	0.654	210.894	53.367	157.613	3.212
10	1	1	-1	-1	1	2.385	216.432	72.873	209.816	2.997
11	1	-1	1	-1	1	0.103	213.899	52.189	167.933	2.828
12	1	1	1	-1	1	1.953	217.978	60.999	205.393	2.870
13	1	-1	-1	1	1	1.502	220.471	44.342	144.605	6.929
14	1	1	-1	1	1	2.842	217.861	67.863	178.083	6.013
15	1	-1	1	1	1	1.050	219.765	54.672	136.636	5.885
16	1	1	1	1	1	2.940	219.743	59.363	164.096	6.234
17	1	0	0	0	0	2.027	213.928	40.563	131.520	5.124
18	1	0	0	0	0	1.994	214.156	44.234	130.645	4.095
19	1	0	0	0	0	1.930	214.592	42.716	129.874	5.669
20	1	0	0	0	0	1.842	212.971	41.005	130.549	5.798
21	2	-2	0	0	0	0.586	213.820	54.476	72.179	13.475
22	2	2	0	0	0	3.213	193.014	75.429	192.106	7.895
23	2	0	-2	0	0	2.105	219.350	60.663	160.825	4.905
24	2	0	2	0	0	1.899	219.804	30.856	137.681	4.621
25	2	0	0	-2	0	1.352	219.818	52.447	139.230	1.599
26	2	0	0	2	0	2.984	219.734	64.491	171.052	7.964
27	2	0	0	0	-2	1.899	218.401	59.164	156.886	5.061
28	2	0	0	0	2	2.072	207.824	51.503	168.624	3.104
29	2	0	0	0	0	1.889	219.892	40.730	129.098	5.081
30	2	0	0	0	0	1.831	213.920	41.056	131.566	5.562

[1]1, first day of experiment; 2, second day of experiment.

[2]X_1 = inoculum size, X_2 = sorbitol, X_3 = maltodextrin, X_4 = inulin.

[3]Y_1 = Growth ($\log_{10}$ CFU/mL), Y_2 = mean doubling time (min), Y_3 = acetic acid (mM), Y_4 = lactic acid (mM), Y_5 = formic acid (mM).

Table 5.27. Regression coefficients of the second-order equation[1] for the five responses[2].

Coefficient[3]	Y_1	Y_2	Y_3	Y_4	Y_5
c	1.94	216.16	41.45	127.48	5.39
c_1	0.776*	3.27*	6.273*	23.275*	-0.897*
c_2	-0.138*	-0.64	-3.246*	-4.616	0.132
c_3	0.397*	0.74*	2.017	-0.668	1.809*
c_4	-0.116*	-0.16	-0.385	-0.460	-0.303
c_{11}	-	-	6.092*	4.210	1.158*
c_{22}	-	-	1.294	8.487*	-0.323
c_{33}	-	-	4.471*	9.959*	-0.318
c_{44}	-	-	3.687*	11.863*	-0.493
c_{12}	-	-	0.187	-2.350	-0.321
c_{13}	-	-	2.829	1.357	-0.464
c_{14}	-	-	0.276	-1.097	0.556
c_{23}	-	-	1.634	-3.785	0.312
c_{24}	-	-	-0.260	2.024	-0.400
c_{34}	-	-	-3.169	-9.687*	-0.273
R^2	0.9335	0.8142	0.8109	0.8973	0.8842
P-value	0.0001	0.0001	0.0051	0.0001	0.0003

[1] $Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{23}X_2X_3$.

[2] Y_1 = ($\log_{10}$ CFU/mL), Y_2 = mean doubling time (min), Y_3 = acetic acid (mM), Y_4 = lactic acid (mM), Y_5 = formic acid (mM).

[3] c_1: inoculum size (% w/v), c_2: sorbitol (% w/v), c_3: maltodextrin (% w/v), c_4: inulin (% w/v).

*Significant at alpha = 0.05.

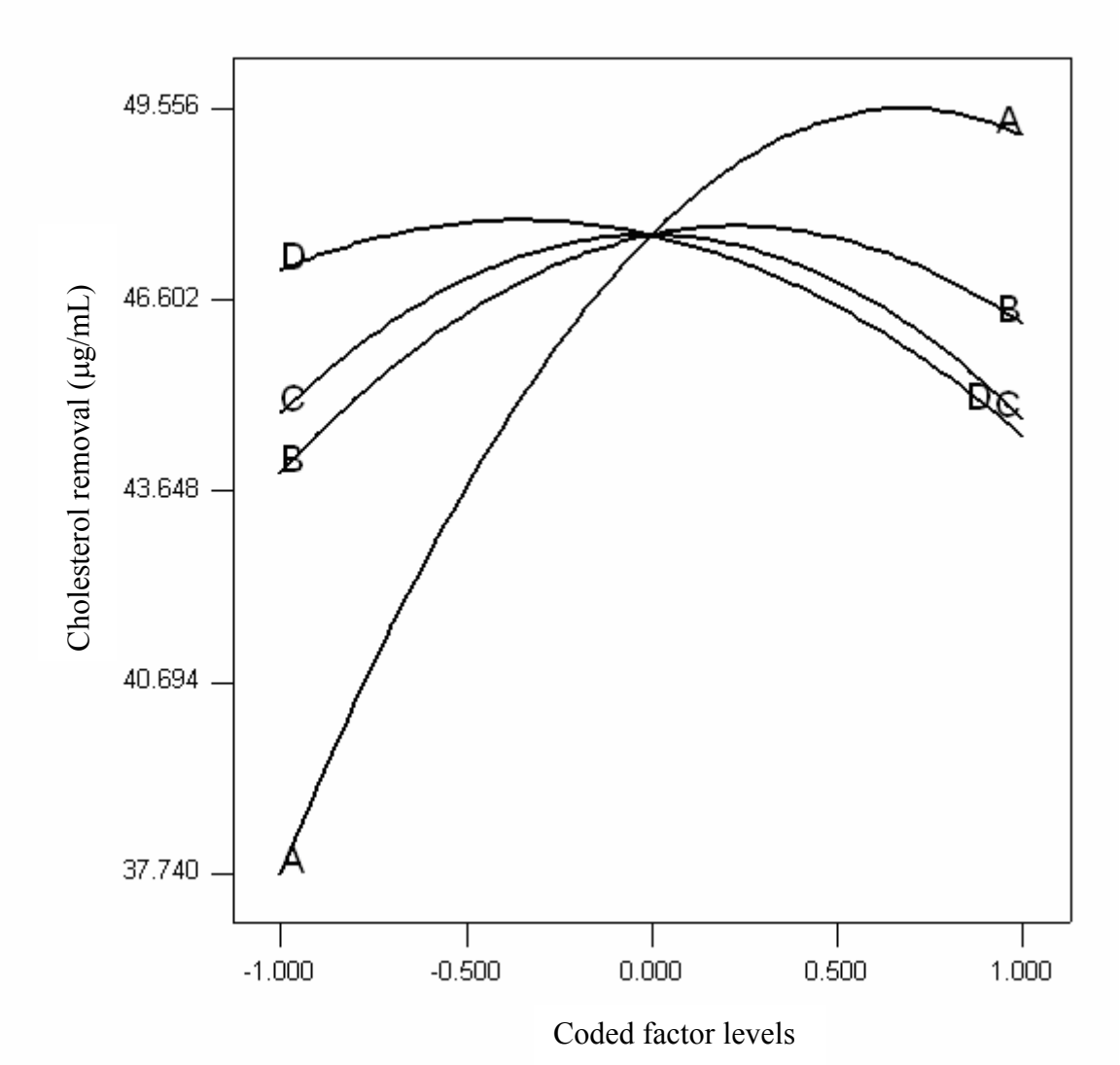


FIG. 5.27. Perturbation plot of inoculum size (A), sorbitol (B), maltodextrin (C) and inulin (D).

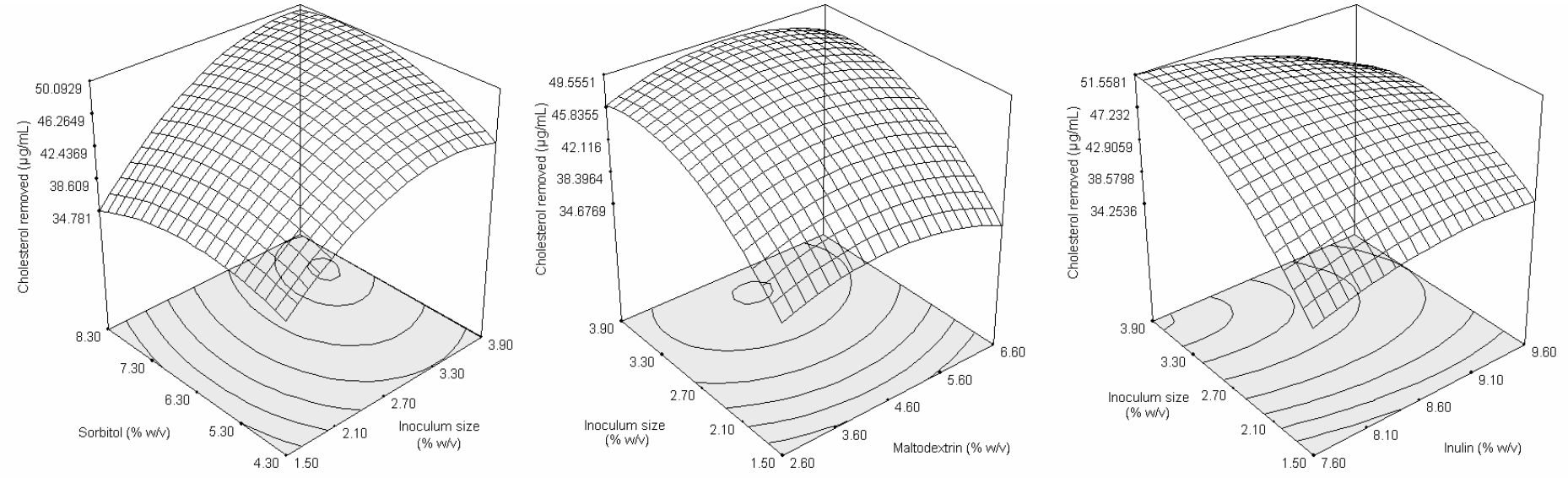


FIG. 5.28. Response surface for cholesterol removal (µg/mL) from the effects of (A) inoculum size and sorbitol, inoculum size and maltodextrin (B) and (C) inoculum size and inulin.

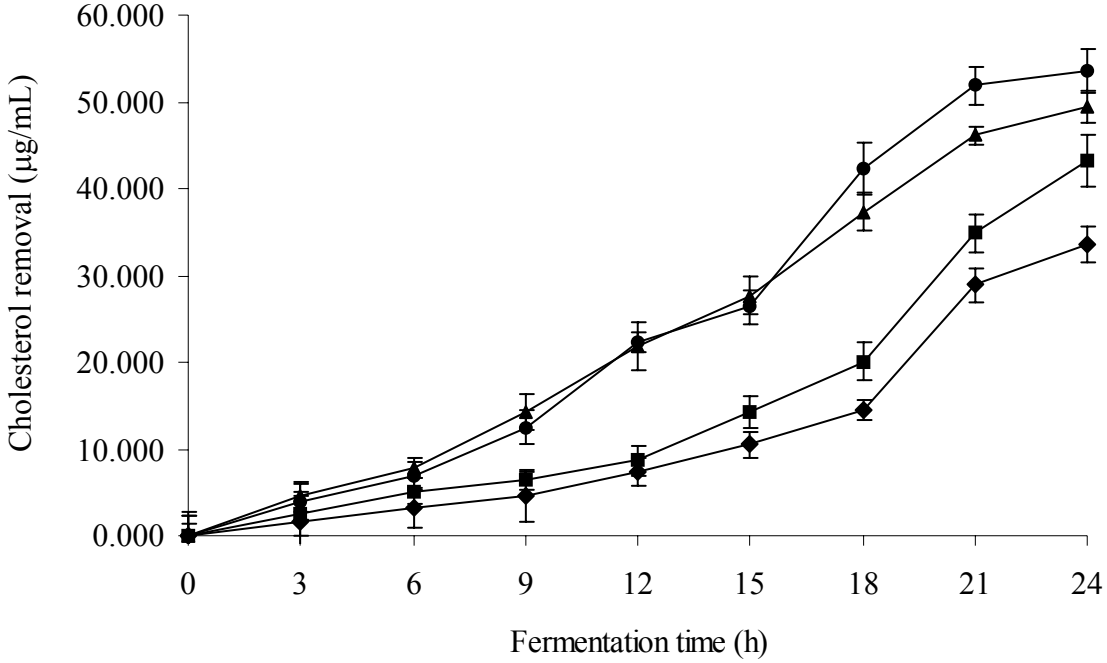
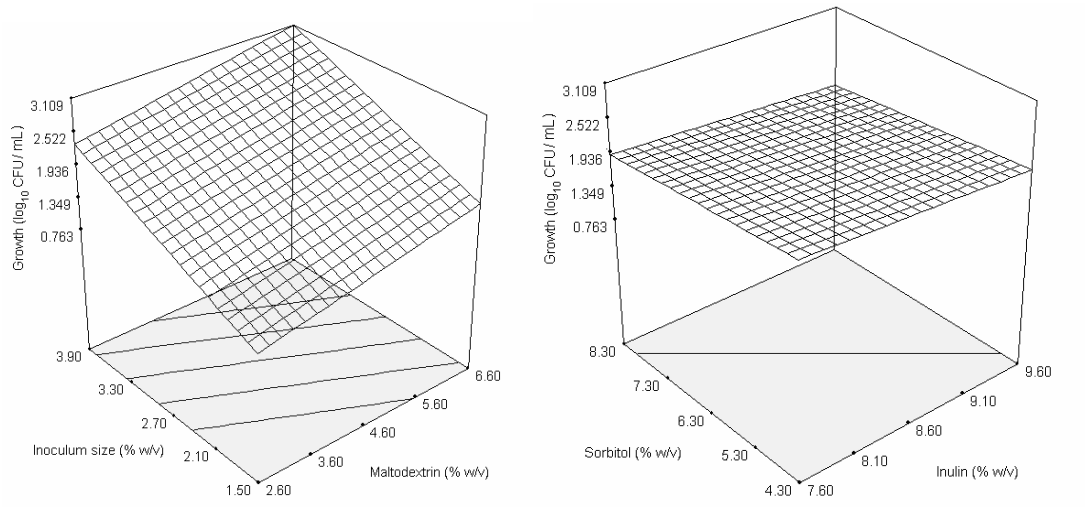


FIG. 5.29. Cholesterol removal by *B. infantis* ATCC 17930 in the optimum (●), center-point (▲), high-point (■) and low-point (♦) media, for the validation experiments. Factors combination for optimum medium were: inoculum size 3.90% (w/v), sorbitol 7.50% (w/v), maltodextrin 4.40% (w/v) and inulin 7.60% (w/v). Center-point medium were: inoculum size 2.70% (w/v), sorbitol 6.30% (w/v), maltodextrin 4.60% (w/v) and inulin 8.60% (w/v). High-point medium were: inoculum size 3.90% (w/v), sorbitol 8.30% (w/v), maltodextrin 6.60% (w/v) and inulin 9.60% (w/v), and low-point medium were inoculum size 1.50% (w/v), sorbitol 4.30% (w/v), maltodextrin 2.60% (w/v) and inulin 7.60% (w/v).



(A) (B)

FIG. 5.30. Response surface for growth ($\log_{10}$ CFU/mL) from the effects of (A) inoculum size and maltodextrin, and (B) sorbitol and inulin.

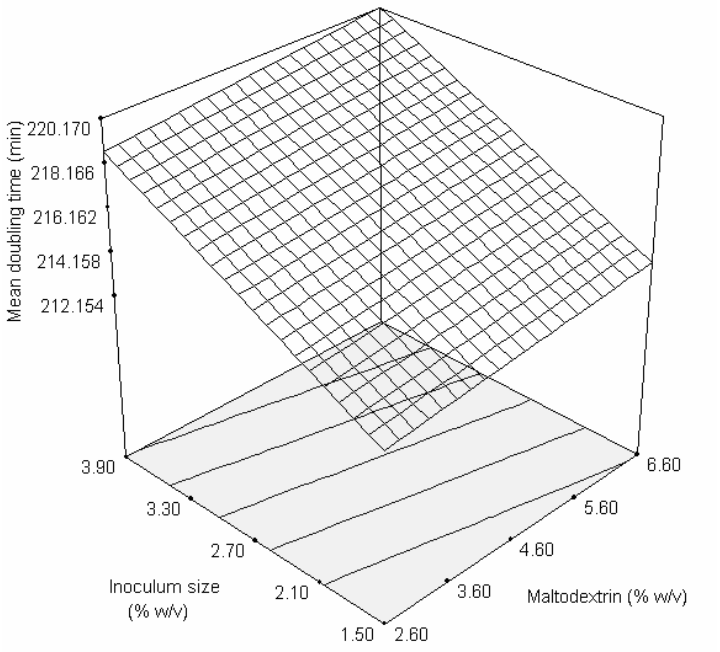
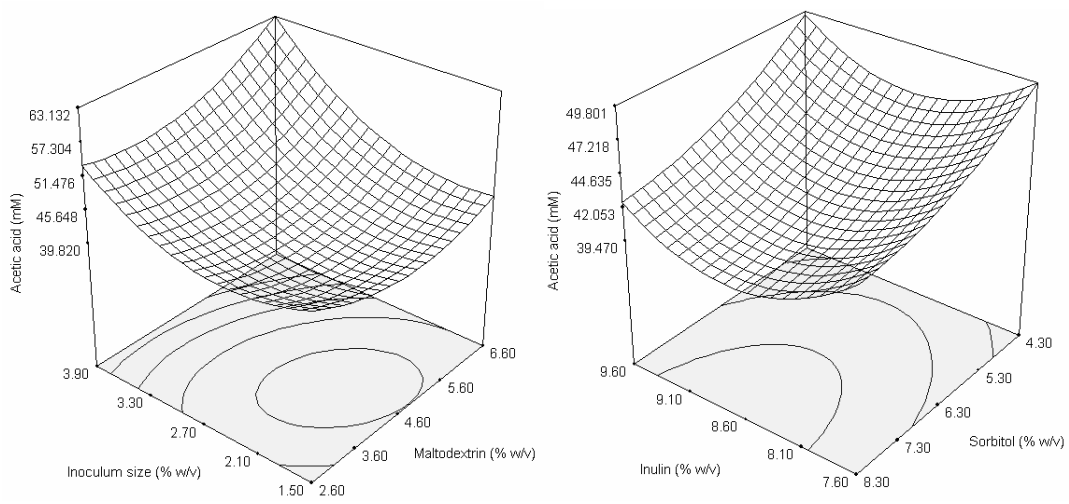
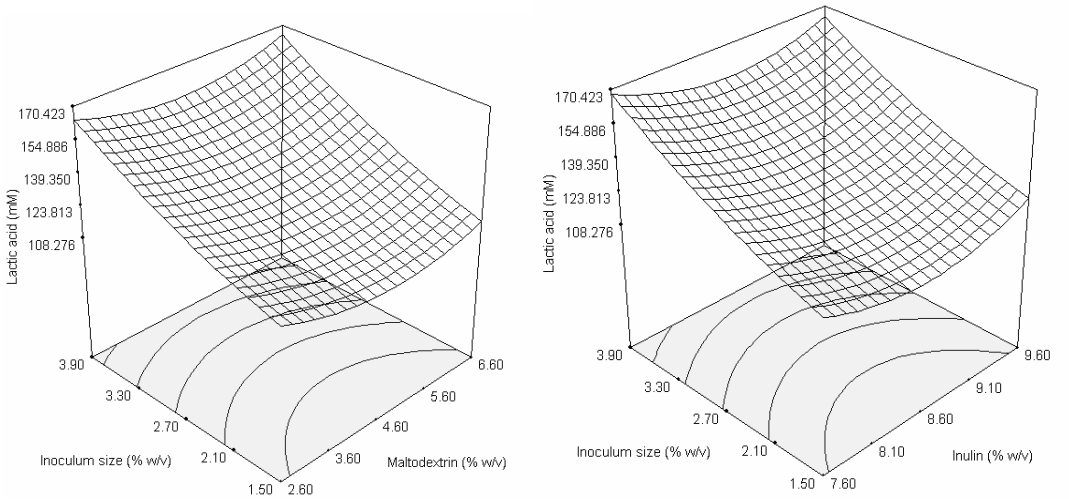


FIG. 5.31. Response surface for mean doubling time (min) from the effects of inoculum size and maltodextrin.



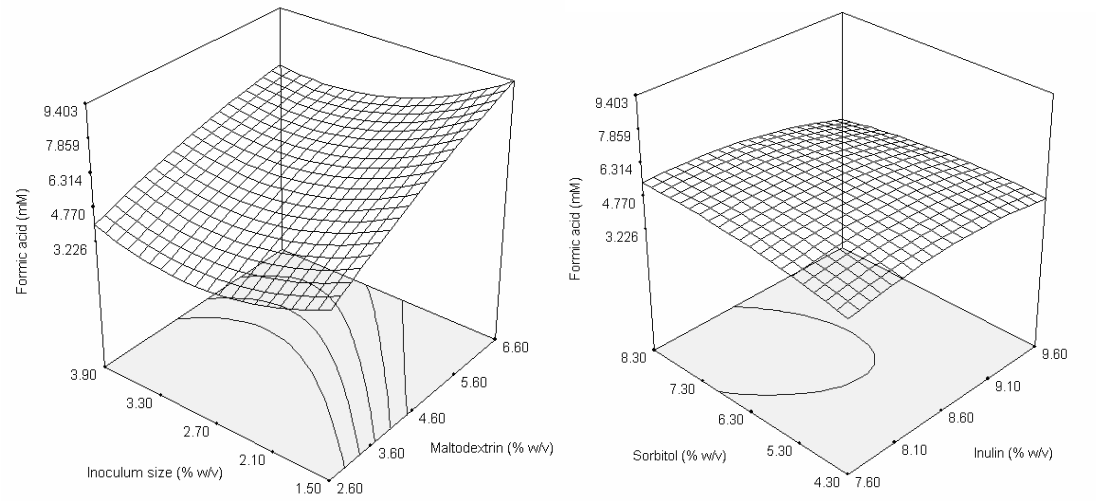
(A) (B)

FIG. 5.32. Response surface for the production of acetic acid (mM) from the effects of (A) inoculum size and maltodextrin, and (B) inulin and sorbitol.



(A) (B)

FIG. 5.33. Response surface for the production of lactic acid (mM) from the effects of (A) inoculum size and maltodextrin, and (B) inoculum size and inulin.



(A) (B)

FIG. 5.34. Response surface for the production of formic acid (mM) from the effects of (A) inoculum size and maltodextrin, and (B) sorbitol and inulin.

6.0 Effects of synbiotics on serum lipid profiles, intestinal microbiology and concentration of organic acids in rats

6.1 Effects of *Lactobacillus casei* ASCC 292, Fructooligosaccharide and Maltodextrin on Serum Lipid Profiles, Intestinal Microflora and Organic Acids Concentration in Rats

6.1.1 INTRODUCTION

Epidemiological studies have shown that higher than normal serum total cholesterol or low-density lipoprotein (LDL) cholesterol increased the risk of coronary heart disease (Usman and Hosono, 2000). Lactic acid bacteria especially lactobacilli are probiotics that have been considered potentially useful in their role to reduce serum cholesterol. Grunewald (1982) found that rats fed milk fermented with *L. acidophilus* for 4 weeks showed lowered serum cholesterol levels compared to the control group that was fed only milk. Several mechanisms are believed to be involved in the reduction of serum cholesterol. Our previous studies reported that lactobacilli are capable of removing cholesterol *in-vitro* via various mechanisms namely assimilation, binding to surface of cells, incorporation into cellular membrane and co-precipitation with deconjugated bile (Chapter 3.0, section 3.1.3). Probiotic numbers are enhanced by prebiotics that are defined as "nondigestable food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacterial species already resident in the colon, and thus improving host health" (Gibson and Roberfroid, 1995). Several classes of resistant starch, fibre, oligosaccharides and sugar alcohols are classified as prebiotics. Fibre sources such as oat bran reportedly lowered plasma cholesterol in rats through enhancing steroid excretion that was accelerated by increased production of propionate (Chen et al., 1984).

The use of both probiotics and prebiotics (known as synbiotic) as a natural mean to counter increased cholesterol levels has generated much interest recently. Using *in-vitro* experiments, we have previously screened and developed a synbiotic product consisting of a *L. casei* strain and prebiotics namely fructooligosaccharide (FOS) and maltodextrin that specifically targeted removal of cholesterol in laboratory media (Chapter 5.0, section 5.1.3). Further studies are needed to evaluate its effect in *in-vivo* models. Although reports have cited positive effect on the use of probiotics, prebiotics or synbiotics in reducing serum cholesterol levels, they have also raised controversial results. Grunewald and Mitchell (1983) previously reported that rats consuming milk fermented by *L. acidophilus* exerted no hypocholesterolemic effect, while Thompson et al. (1982) reported that *acidophilus* milk did not reduce serum cholesterol levels in humans. Resistant starch has showed cholesterol-lowering properties in rats but did not affect plasma cholesterol in humans (Jenkins et al., 1987).

This section has been published: *Liong, MT and Shah, NP. 2006. Effects of Lactobacillus casei ASCC 292, fructooligosaccharide and maltodextrin on serum lipid profiles, intestinal microflora and organic acids concentration in rats.* ***J Dairy Sci*** *89: 1390-1399.*

Probiotic bacteria have achieved GRAS status before being regarded as foodstuffs or drugs. Until now, reports of a harmful effect of these microbes towards a host are rare and their safety has not been questioned. However, there have been reports of probiotic strains such as *L. casei, L. lactis* and *L. plantarum* being isolated from bacterial enterocarditis and *B. adolescentis* being isolated from blood stream infections (Gasser, 1994). These incidences indicated that these bacteria are capable of translocating from the intestine to other organs. Although it is difficult to induce such translocation in healthy animals, there are possibilities of such incidence if the gut environment has been altered by intestinal mucosa injury, immunodeficiency in the host or abnormal intestinal bacterial flora (Ishibashi and Yamazaki, 2001). Although the pH of the stomach may reach as low as 1.5, the lower intestine has a pH of near neutral. High concentrations of organic acids, arising from rapid fermentation of prebiotics by probiotics that inhibit the colonization of acid-sensitive pathogens, could also induce injury to the intestinal mucosa and hence impair its barrier function (Argenzio and Meuten, 1991; Remesy et al., 1993). Thus, it is of utmost importance to ensure that the synbiotics developed are potentially safe and has no indication of harmful translocation.

Other than exerting cholesterol lowering effects, lactobacilli and prebiotics are also associated with the alteration of the intestinal flora population and have been used to suppress growth of pathogens through competitive inhibition, production of short-chain fatty acids (SCFA) and antagonistic activity against pathogens (Buddington et al., 2002; Isolauri et al., 2002). Our previous study (Chapter 5.0, section 5.2.3.2) reported that *L. casei* ASCC 292 was not only capable to remove cholesterol *in-vitro* but also produced SCFA in the presence of FOS and maltodextrin. Thus, the aim of this study was to evaluate the effectiveness of such synbiotics on reducing serum cholesterol using rat model. In addition, the effect of the synbiotics on intestinal microflora, concentration of organic acids and the capability to translocate was also investigated.

6.1.2 MATERIALS AND METHODS

6.1.2.1 Source of culture and prebiotics

L. acidophilus ASCC 292 in freeze-dried form was used in this study. Working cultures were prepared as described in Chapter 5.0, section 5.1.2.1. Two commercially available prebiotics were used including FOS (Orafti Pty. Ltd., Tienen, Belgium) and maltodextrin (Grain Processing Corp., Muscatine, U.S.A.). The FOS used was Raftilose P95 that was extracted from chicory with a purity of 95%, and the remaining 5% contained glucose, fructose and sucrose. The degree of polymerization (DP) of oligofructose ranged

from 2 to 7, with an average DP of 4. The maltodextrin used was Maltrin M100, a glucose polymer with dextrose equivalent (DE) ranging from 9 to 12 and an average DP of 11.

6.1.2.2 Rats and diets

Conventional male Wistar rats (n = 24; Monash University Animal Services, Clayton, Australia) at the age of 8 week were used. The rats were housed and bred as approved by the Animal Ethics Committee of Victoria University (Werribee, Australia). Upon arrival, the animals were kept on rodent chow for a week. After this wash-out period, rats were divided into four groups of six rats in each group (n = 6). Rats were kept separately in metal cages in a room with controlled temperature (20-22°C) and humidity (50-55%) and maintained in a cycle of light for 12 hours (6:00 to 18:00) and dark for 12 hours (18:00 to 6:00). The composition of the high-cholesterol diet (SF00-245) that contained 16% (w/w) fat, 1% (w/w) cholesterol and 0.5% (w/w) cholate (Specialty Feeds; Glen Forrest, Australia) is shown in Table 6.1. Rodent chow was incorporated into the diet to maintain the similar amount of SF00-245 in all groups that had varying amount of synbiotics. Solidified coconut oil (Copha®; Unilever Australasia, Epping, Australia) were added to aid the pelletizing process of feed production. All groups were on diets containing 75% (w/w) of the high-cholesterol diet and 5% (w/w) of copha. Group 1 was the control and was on a diet containing just SF00-245, copha and rodent chow. Group 2 received cholesterol-enriched diet plus *L. casei* ASCC 292 and FOS (LF), group 3 received cholesterol-enriched diet plus *L. casei* ASCC 292 and maltodextrin (LM) and Group 4 was fed the cholesterol enriched diet with *L. casei* ASCC 292, FOS and maltodextrin (LFM). The composition of all four diets is shown in Table 6.2. The composition was in accordance with our previous *in-vitro* optimization study; inoculum size of *L. acidophilus* ATCC 4962, concentrations of FOS, mannitol and inulin were the significant factors for optimum removal of cholesterol, and was achieved from the ratio of 1.7:4.8:6.8 (Chapter 5.0, section 5.3.3.2). Rats were allowed to consume their respective diets and water *ad libitum* for 6 weeks. Body weight and feed intake were recorded weekly.

6.1.2.3 Sampling and analytical procedures

At the end of the 6 weeks feeding trial, rats were fasted overnight and sacrificed by carbon dioxide inhalation. The cecum was tied off immediately after dissection to avoid leakage of cecal content into the colon due to relaxed gut muscle upon death. Blood samples were collected immediately in sterile tubes by heart puncture, and left to stand for 30 min at room temperature (~20°C) for coagulation before being centrifuged for 20 min at 2714 x g

(Sorvall RT7; Newtown, U.S.A.). The serum samples were analysed for total-, high density lipoprotein (HDL)-, and low density lipoprotein (LDL) cholesterol and triglycerides using commercial and reagent kits (Thermo Electron Corp., Melbourne, Australia).

Faecal samples were collected weekly in separate sterile tubes for microbial and organic acids analyses. Samples for microbial analyses were placed into anaerobic jars (Becton Dickinson Microbiology Systems®, Sparks, U.S.A.) with gas generating kits® (Oxoid Ltd., Hampshire, UK) and analyses were carried out within one hour of collection. Each sample was homogenized with a stomacher (John Morris Scientific Pty. Ltd., Melbourne, Australia) using a sterile peptone and water diluents. Subsequent 10-fold serial dilutions of each sample were plated in triplicates. All enumeration medium were obtained from Amyl Media Pty. Ltd. (Dandenong, Australia). Nutrient agar was used for total aerobes while Wilkins-Chalgren agar for total anaerobes (McBain et al., 2003). Eosin methylene blue agar was used for *E. coli* (Swanson et al., 2002a), esculin bile salt agar for bacteroides (Tannock et al., 2000), tryptose sulphite cycloserine agar for clostridia (Wise and Siragusa, 2005), mannitol salt agar for staphylococci (Knowles et al., 2005), violet red bile agar for coliform (Cotton and White, 1992), and de Mann, Rogosa and Sharpe agar (Merck KGaA, Darmstadt, Germany) for total lactobacilli (Swanson et al., 2002a). Plates of total anaerobes, bacteroides, clostridia and lactobacilli were incubated anaerobically at 37°C for 48 h in anaerobic jars with gas generating kits. Plates for the enumeration of total aerobes, *E. coli*, staphylococci and coliforms were incubated at 37°C for 48 h in controlled aerobic incubators (New Brunswick Scientific, Edison, U.S.A.).

Upon dissection, the contents of colon and cecum were collected, stored anaerobically and analysed for microbial analyses in a similar manner as faecal samples. The spleen, liver and kidneys were removed, blotted on a filter paper and immediately kept in separate sterile tubes. All spleens, livers and kidneys were analysed for the presence of lactobacilli to ascertain translocation.

All collected faecal, cecal and colon samples were stored at -40°C until analysed for organic acid contents. The concentration of organic acids was determined using high performance liquid chromatography (HPLC; Varian Australia Pty. Ltd., Mulgrave, Australia). Samples were prepared for HPLC analysis as described previously[29]. Acetic, propionic, butyric, formic and lactic acids were identified using respective standards, and their concentrations determined. The HPLC system was equipped with a UV/Vis detector (Varian, Walnut Creek, U.S.A.) set at 220 nm. An Aminex HPX-87H column (Bio-Rad

Laboratories, Richmond, U.S.A.) was maintained at 65°C, while the degassed mobile phase (0.009 M H_2SO_4) was used at a flow rate of 0.6 mL/min.

The moisture in faeces, and in cecal and colon contents was determined as the difference between the wet mass and the dry mass of the samples after drying at 80°C (Memmert GmbH Ltd., Schwabach, Germany) until a constant weight was achieved. The pH of the samples was measured with a pH meter (Hanna Instruments Pty. Ltd., Kallang Way, Singapore).

6.1.2.4 Experimental design and statistical analysis

The data analysis was carried out with SPSS Inc. software (version 10.0). One-way analysis of variance (ANOVA) was used to study a significant difference between means of the dietary groups at a given intestinal site, and sampling sites within the same dietary group, with a significance level at $P < 0.05$. Tukey's-test was used to perform multiple comparisons between means. All data are presented as mean ± standard error of means; n = 6.

6.1.3 RESULTS

6.1.3.1 Weight and feed intake

All the rats were in general healthy throughout the feeding trial period. The body weight gain, feed intake and also the ratio of body weight to feed intake showed no significant difference ($P > 0.05$) between synbiotic treatment groups and the control (Table 6.3).

6.1.3.2 pH and moisture content of cecal, colon and faecal contents

All groups showed a gradient increase in pH values from cecal content to faecal samples (Table 6.4). The pH of cecal and colon content was not significantly different ($P > 0.05$) among all treatment groups and control. However, the pH of the faecal samples of rats fed diet LM was lower compared to that with the control diet.

All rats showed a significant difference ($P < 0.05$) in moisture content from cecal to faecal samples (Table 6.5). Rats fed the LM and LFM diets had higher moisture content in the faecal samples compared to those on the control diet.

6.1.3.3 Translocation of lactobacilli

Samples from spleen, liver and kidney of each rat on the control, LF, LM and LFM diets were plated for the presence of total lactobacilli. No growth of lactobacilli was detected from the samples indicating the absence of translocation (data not shown).

6.1.3.4 Lipid profiles

Serum total cholesterol, triglycerides, HDL- and LDL-cholesterol levels of rats fed the control, LF, LM and LFM diets are shown in Figure 6.1. Rats fed LFM diet had significantly ($P < 0.05$) lower total cholesterol and triglycerides levels compared to the control, while rats that were fed diets containing only FOS or maltodextrin did not show any difference. HDL-cholesterol level increased in serum of rats that were fed with diet containing only maltodextrin compared to the control diet. However, diets containing FOS and a mixture of both FOS and maltodextrin did not increase the serum HDL-cholesterol level. Although rats fed LF and LFM diets showed a comparatively lower serum LDL-cholesterol level, all synbiotic diets had similar effect on LDL-cholesterol level compared to the control diet.

6.1.3.5 Microbial populations

Total aerobes ranged between 7.156 to 9.929 CFU $\log_{10}$/g dry weight of cecal, colonic and faecal samples of rats across all treatment diets (Table 6.6). A gradient decrease in total aerobes was observed from cecal to faecal samples, with the latter from all diets showing comparatively lower total aerobes count, compared to the samples from other intestinal sites. Rats supplemented with LFM diet showed a comparatively lower concentration of total aerobes in the colonic and faecal samples compared to the control. Staphylococci averaged between 6.184 to 9.149 CFU $\log_{10}$/g dry weight of all samples. The LF and LM diets showed a significant decrease of staphylococci in after the cecal region compared to the control, which showed a similar staphylococci count along the intestinal gradients. Rats supplemented with LFM diet showed a lower concentration of staphylococci in the colonic and faecal samples, as compared to the control. A gradient decrease of *E. coli* was also observed from cecal samples to faecal samples across all diets. Rats supplemented with LF diet showed lower count of *E. coli* in the faecal samples compared to the control, while the LFM diet lowered total count of *E. coli* in the cecal and colon regions. Total coliforms ranged between 6.757 to 9.215 CFU $\log_{10}$/g dry weight of all samples studied. Faecal samples of all diets showed a lower concentration of coliforms compared to other

intestinal sites. Total coliforms were reduced from the upper intestinal sites of rats supplemented with the LFM diet compared to those with the control.

The concentration of total anaerobes of rats fed the control, LF, LM and LFM diets is shown in Table 6.7. Only rats supplemented with LF and LFM diets showed a significant decrease in total anaerobes after the colonic region, compared to the control which had similar total anaerobic counts across all intestinal sites. Concentrations of clostridia averaged from 3.618 to 5.102 CFU $\log_{10}$/g dry weight across all samples studied. Most diets showed a consistent population of clostridia along all intestinal sites. However, the LFM diet produced a lower concentration of clostridia in the cecum and colonic regions compared to the control. All diets showed decreasing counts of bacteroides along the intestinal gradients. LFM diet contributed to a decrease of bacteroides after the cecal region while control only reduced the concentration of bacteroides in the lower colonic regions. Most of the synbiotic diets reduced the concentration of bacteroides in all intestinal gradients studied compared to the control. Rats fed with LF and LFM diets maintained a consistent concentration of lactobacilli across all intestinal sites studied, while the control diet showed a gradient decrease after the colonic region. Rats fed the LF diet also showed higher concentration of lactobacilli in the faecal samples compared to the control, while the LM diet contributed to a comparatively higher count of lactobacilli in most of the intestinal samples compared to the control.

6.1.3.6 Concentration of organic acids

The concentration of organic acids in different intestinal sites of rats fed control and all treatment diets is shown in Table 6.8. The concentration of acetic acid was consistent in the cecal and colon regions in rats fed the LF and LM diets, while the control and LFM diets produced a decrease in concentration after the region. Rats supplemented with the LM and LFM diets also showed a comparatively lower concentration of acetic acid in most intestinal contents compared to the control. All synbiotic diets and the control showed a gradient decrease in the concentration of butyric acid, from samples obtained from cecum to faeces. Rats supplemented with LFM diet showed a significantly lower concentration of butyric acid compared to the control in all intestinal sites studied. Similar to the control diet, a gradient decrease in the concentration of formic acid was observed across intestinal regions from rats fed all synbiotic diets. LF diet contributed to a higher concentration of formic acid in the cecal region compared to the control, while LFM diet caused a decrease in the concentration of formic acid in most intestinal sites compared to the control. Concentration of propionic acid was least affected by all synbiotic diets, with insignificant changes across most intestinal gradients compared to that of the control. The concentration of lactic acid was

higher in the cecal region of rats fed the LM and LFM diets, and also in both the cecal and colonic regions of rats on the LF diet, compared to the control. However, the LM diet contributed to a drastic decrease in the concentration of lactic acid across all intestinal gradients, with lowest amount detected from the faecal samples compared to the other diets.

6.1.4 DISCUSSION

The experiments were conducted to investigate the combined effect of *L. casei* ASCC 292 with FOS (LF), *L. casei* ASCC 292 with maltodextrin (LM), and *L. casei* ASCC 292 with FOS and maltodextrin (LFM) on serum cholesterol, intestinal microflora, concentration of organic acids and the translocation of lactobacilli using rats as a model. The body mass gain was in tandem with feed intake for all treatment diets, indicating a similar feed efficiency across all diets. Our results showed that the LM and LFM diets contributed to higher faecal moisture compared to control. A major factor in determining the water content of the lumen is the osmolality of the contents; osmolality would be increased substantially by the presence of the unfermented carbohydrate used in this study. This is the basis for the use of nondigestible sugar alcohols and oligosaccharides as inexpensive bowel regulators and treatments for constipation in humans. Our results here indicated that the LM and LFM diets had higher laxative potential compared to the control.

All synbiotic diets did not exhibit harmful translocation of lactobacilli in the organs such as spleen, liver and kidney. Results from this study clearly indicated that lactobacilli from all synbiotic diets were safe and did not translocate to other internal organs.

Overall, the combination of *L. casei* ASCC 292, fructooligosaccharide and maltodextrin (diet LFM) produced lower total cholesterol and triglycerides levels. This supports our previous study on *in-vitro* optimization, which reported optimum removal of cholesterol when *L. casei* ASCC 292 was used in the presence of FOS and maltodextrin (Chapter 5.0, section 5.1.3.2). The combination of *L. casei* ASCC 292 and maltodextrin (diet LM) beneficially increased the level of HDL-cholesterol, while all synbiotics used did not alter the level of LDL-cholesterol compared to the control. The hypocholesterolemic effect of the LFM diet may be contributed by various factors. A possible mechanism involves the alteration of lipid metabolism by short-chain fatty acids (SCFA). Propionate was reported to inhibit fatty acid synthesis *in-vitro* while acetate is a lipogenic substrate (Delzenne and Kok, 2001). It was also found that hepatocytes isolated from rats fed an oligofructose diet had a 40% lower capacity to synthesize triacylglycerol from [^{14}C]acetate than the control rats (Kok et al., 1996). In our study, results showed that the concentration of propionic acid in cecum

of rats fed LFM was higher than the control, while the concentration of acetic acid in the faecal samples was comparatively lower. Results from our study indicated that the microbial fermentation of FOS and maltodextrin supplemented in LFM diet induced the concentration of propionic acid that may have altered the cholesterol synthesis pathways, and/or decreased the concentration of acetic acid that may lead to a decreased lipogenesis.

Rats that were supplemented with diets containing FOS (LF and LFM) showed a constant lactobacilli count across cecal, colonic and faecal samples, while the control group showed a decline in lactobacilli counts after the colonic region. Our previous *in-vitro* study (Chapter 5.0, section 5.1.3.3) found that FOS was a good specific carbon source in modulating growth rate of *L. casei* ASCC 292, and thus may have exerted similar effects in this *in-vivo* trial. In addition, the LFM diets had also reduced total population of clostridia. It was previously reported that an increase in bifidobacteria was often accompanied by a decrease in concentration of clostridia (Gibson et al., 1995). This study shows that a constant population of lactobacilli may possess such a similar effect. Although the FOS diets maintained a consistent lactobacilli count along the intestinal gradients, it was only the LM diet that contributed to increased population of lactobacilli compared to the control. It was previously reported that maltodextrin-like oligosaccharide had a lower rate of fermentation than FOS and was more fermentable in the distal part of the large intestine (Flickinger et al., 2000). Diet LFM also contributed to a decreased count of staphylococci and total coliforms in most intestinal samples while the control group exhibited a comparatively higher count. However, the LF diet increased the population of staphylococci compared to the control. This is supported by a previous study which reported better utilization of chicory oligofructose by staphylococci compared to synthetic oligofructose or even glucose (Roberfroid et al., 1998). The LFM diet also reduced population of *E. coli* in all intestinal samples compared to the control. Lactobacilli have been reported to inhibit enteropathogenic *E. coli* from binding to intestinal cells (Bernet et al., 1994). Although most inhibition of pathogenic bacteria has been contributed by a decrease in intestinal pH (Swanson et al., 2002b), our current study did not indicate such an association due to insignificant changes of pH along intestinal sites. In this study, all these positive influence on pathogen populations may be supported by the higher concentration of lactic acid in cecum of rats fed the synbiotic diets compared to the control. Lactic acid is the major end product of lactate-producing lactobacilli (Swanson et al., 2002a), and our results showed that the production of organic acids was dominated by lactic acid. Thus, this could indicate the possible antimicrobial capability on pathogenic microorganisms.

It must be noted that the concentration of acetic acid by rats supplemented with LM was comparatively lower than control and the other treatment diets. Our *in-vitro* study (Chapter 5.0, section 5.2.3.2) reported that FOS was a better substrate for *L. casei* ASCC 292 for the production of acetic acid than maltodextrin, and was clearly reflected in this current *in-vivo* trial. Although LM diet had a higher concentration of maltodextrin than FOS in LF diet, the short-chain FOS used (average DP of 4) would be more extensively fermented by the colonic bacteria than the longer chain maltodextrin (average DP of 11), and thus contributing to higher concentration of acetic acid. It must also be noted that rats fed LFM diet showed a much lower concentration of acetic acid in the lower bowel regions as compared to the cecal region. We postulate that FOS in LFM diet was quickly fermented by bacteria in the cecal region thus resulting in a lower availability of FOS in the colonic regions thereafter, contributing to a drastic decrease in acetic acid concentration after the cecal region. Results from this study also showed that a significantly lower concentration of butyric acid was detected in all intestinal sites studied from rats fed the LFM diet compared to control. It has been reported that short-chain fatty acids (SCFA) especially butyrate is the major intestinal fuel even in the presence of competing glucose (Windmueller and Spaeth, 1978). This led us to believe that the combination of FOS and maltodextrin may have synergistically accelerated the absorption of butyrate by colonocytes. Although the overall production of butyric acid may have been alternatively decreased, more work is needed to elucidate the absorption phenomenon, because if this postulation is true, the synergistic combination of *L. casei* ASCC 292, FOS and maltodextrin could beneficially reduce the risk of colon cancer which is often associated with decreased butyrate absorption in the bowel (Topping and Clifton, 2001).

Lactobacilli are lactate-producing bacteria and this could be observed from the higher concentration of lactic acid across most intestinal sites studied in all synbiotic diets compared to the control. However, the concentration of lactic acid was higher in rats fed diets containing FOS than maltodextrin, and this agrees with our previous *in-vitro* study (Chapter 5.0, section 5.2.3.2) which reported that FOS was a better substrate for *L. casei* ASCC 292 to produce lactic acid as compared to maltodextrin. In general, acetate is mostly produced predominantly, followed by propionate (Roberfroid et al., 1998). Results from this study did not indicate such orientation. As reported from our previous finding (Chapter 5.0, section 5.2.3.2), acetate was inhibited by the end-product of maltodextrin fermentation. We postulate that the fermentation of maltodextrin in the cecum and colon may have contributed to such end-product inhibition. In addition, we also previously reported that FOS encouraged the production of propionic acid, and this may have generated higher concentration of propionic acid in this study.

6.1.5 CONCLUSIONS

The synbiotic diets used (LM and LFM) produced higher moisture content in the faeces of rats compared to the control, indicating better laxative effects. Diet LFM decreased serum total cholesterol and triglycerides content, while diet LM increased serum HDL-cholesterol level. Diet LFM played a major role in decreasing population of staphylococci, *E. coli,* coliforms, bacteroides and clostridia. The increased population of lactobacilli contributed to a decreased population of clostridia and increased concentration of lactic acid. Diet containing FOS contributed to increased concentration of acetic acid. A much lower concentration of butyric acid was detected in the intestinal sites of rats fed the LFM diet compared to the control and other treatment diets, suggesting the LFM diet accelerated butyrate absorption by colonocytes. This study showed that the combination of *L. casei* ASCC 292, FOS and maltodextrin is beneficial for pathological cholesterol levels and healthier bowel microbial population without exhibiting harmful lactobacilli translocation.

Table 6.1. The composition of the high-cholesterol diet (SF00-245) that contained 16% fat, 1% cholesterol and 0.5% cholate (Specialty Feeds, Glen Forrest, Australia).

Ingredients	g/kg
*Casei*n	200
Methionine	3
Sucrose	520
Cellulose	51
Canola oil	10
Cocoa butter	150
Calcium carbonate	13.1
Sodium chloride	2.6
Potassium citrate	2.5
Potassium dihydrogen phosphate	6.9
Potassium sulphate	1.6
AIN93G trace minerals	1.4
Choline chloride (65%)	10
Sodium cholate	5
Cholesterol (USP)	10
AIN93G vitamins	10
Alpha tocopherol acetate (50% active)	2.6

Table 6.2. The composition of control, diets LF, LM and LFM.

Ingredients (g/kg)	Diets			
	Control	LF	LM	LFM
SF00-245[1]	750.0	750.0	750.0	750.0
Copha[2]	50.0	50.0	50.0	50.0
L. casei ASCC 292	-	8.5	8.5	8.5
FOS[3]	-	24.0	-	24.0
Maltodextrin[3]	-	-	34.0	34.0
Rodent chow[4]	200.0	167.5	157.5	133.5

[1]High-cholesterol diet (Specialty Feeds, Glen Forrest, Australia).

[2]Copha® (Unilever Australasia, Epping, Australia), hardened coconut oil 99%, soya bean lecithin 1%.

[3]Fructooligosaccharide (Raftilose P95; Orafti Pty. Ltd., Tienen, Belgium), purity of 95%, average degree of polymerization (DP) of 4; Maltodextrin (Maltrin M100; Grain Processing Corp., Muscatine, U.S.A.), dextrose equivalent (DE) of 9 to 12, average DP of 11.

[4]Rodent chow (Specialty Feeds, Glen Forrest, Australia), total protein 19.6%, total fat 4.6%, crude fibre 4.8%, trace minerals 2.9%, total vitamins 0.3%.

Table 6.3. Body weight gain, feed intake and feed efficiency of rats fed control, diets LF, LM and LFM.

Component	Treatments[1,2]			
	Control	LF	LM	LFM
Body weight gain (g/rat/week)	34.001 ± 2.404[ab]	33.692 ± 1.013[ab]	39.244 ± 2.188[a]	30.701 ± 3.127[b]
Feed intake (g/week)	140.532 ± 4.011[a]	136.785 ± 4.081[a]	148.064 ± 4.071[a]	131.066 ± 4.071[a]
Ratio body weight: feed intake	0.245 ± 0.041[a]	0.254 ± 0.051[a]	0.272 ± 0.050[a]	0.239 ± 0.014[a]

[1]Treatments include: Control, contained no *L. casei* ASCC 292, FOS or maltodextrin; LF, contained 0.9% (w/w) *L. casei* ASCC 292 and 2.5% (w/w) FOS; LM, contained 0.9% (w/w) *L. casei* ASCC 292 and 3.3% (w/w) maltodextrin; LFM, contained 0.9% (w/w) *L. casei* ASCC 292, 2.5% (w/w) FOS and 3.3% (w/w) maltodextrin.

[2]Results are expressed as mean ± standard error of means, n = 6. [ab]Means in the same row followed by different lowercase letters are significantly different ($P < 0.05$).

Table 6.4. pH values of contents obtained from the cecum, colon and faeces of rats fed control, diets LF, LM and LFM.

pH	Treatments[1,2]			
	Control	LF	LM	LFM
Cecum	$6.43 \pm 0.06^{b,A}$	$6.28 \pm 0.05^{b,A}$	$6.49 \pm 0.06^{a,A}$	$6.36 \pm 0.04^{b,A}$
Colon	$6.79 \pm 0.04^{b,A}$	$6.37 \pm 0.18^{b,A}$	$6.51 \pm 0.02^{a,A}$	$6.64 \pm 0.04^{b,A}$
Faeces	$7.80 \pm 0.08^{a,A}$	$8.13 \pm 0.13^{a,A}$	$7.04 \pm 0.02^{a,B}$	$7.79 \pm 0.01^{a,A}$

[1]Treatments include: Control, contained no *L. casei* ASCC 292, FOS or maltodextrin; LF, contained 0.85% (w/w) *L. casei* ASCC 292 and 2.40% (w/w) FOS; LM, contained 0.85% (w/w) *L. casei* ASCC 292 and 3.40% (w/w) maltodextrin; LFM, contained 0.85% (w/w) *L. casei* ASCC 292, 2.40% (w/w) FOS and 3.40 (w/w) maltodextrin.

[2]Results are expressed as mean ± standard error of means, n = 6. [ab]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [AB]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.5. Moisture content of cecal, colon and faecal content of rats supplemented with diet control, LF, LM and LFM.

Moisture (%)	Treatments[1,2]			
	Control	LF	LM	LFM
Cecum	$77.574 \pm 1.632^{a,A}$	$87.836 \pm 2.144^{a,A}$	$82.184 \pm 3.570^{a,A}$	$88.256 \pm 4.054^{a,A}$
Colon	$58.053 \pm 5.044^{b,B}$	$71.554 \pm 0.543^{a,A}$	$72.804 \pm 3.971^{a,A}$	$69.577 \pm 8.757^{a,A}$
Faeces	$18.427 \pm 1.522^{c,B}$	$24.458 \pm 3.947^{b,B}$	$35.198 \pm 3.270^{b,A}$	$35.186 \pm 4.478^{b,A}$

[1]Treatments include: Control, contained no *L. casei* ASCC 292, FOS or maltodextrin; LF, contained 0.85% (w/w) *L. casei* ASCC 292 and 2.40% (w/w) FOS; LM, contained 0.85% (w/w) *L. casei* ASCC 292 and 3.40% (w/w) maltodextrin; LFM, contained 0.85% (w/w) *L. casei* ASCC 292, 2.40% (w/w) FOS and 3.40% (w/w) maltodextrin.

[2]Results are expressed as mean ± standard error of means, n = 6. [ab]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [AB]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.6. Population of total aerobes, staphylococci, *Escherichia coli*, and total coliforms from cecal, colon and faecal contents of rats fed diets control, LF, LM and LFM.

CFU/g dry sample	Treatments[1,2]			
	Control	LF	LM	LFM
Total aerobes				
Cecum	$9.393 \pm 0.047^{a,A}$	$9.929 \pm 0.014^{a,A}$	$9.225 \pm 0.068^{a,A}$	$9.229 \pm 0.083^{a,A}$
Colon	$9.413 \pm 0.032^{a,A}$	$9.763 \pm 0.094^{a,A}$	$9.032 \pm 0.011^{ab,AB}$	$8.548 \pm 0.063^{a,B}$
Faeces	$7.672 \pm 0.060^{b,AB}$	$7.841 \pm 0.054^{b,AB}$	$8.383 \pm 0.056^{b,A}$	$7.156 \pm 0.053^{b,B}$
Staphylococci				
Cecum	$7.502 \pm 0.031^{a,B}$	$8.860 \pm 0.045^{a,A}$	$9.149 \pm 0.089^{a,A}$	$6.747 \pm 0.015^{a,B}$
Colon	$7.580 \pm 0.037^{a,B}$	$8.484 \pm 0.074^{a,A}$	$7.038 \pm 0.058^{b,BC}$	$6.581 \pm 0.054^{a,C}$
Faeces	$7.280 \pm 0.027^{a,A}$	$7.658 \pm 0.180^{b,A}$	$6.184 \pm 0.045^{c,B}$	$6.257 \pm 0.064^{a,B}$
Escherichia coli				
Cecum	$9.010 \pm 0.047^{a,A}$	$8.448 \pm 0.064^{a,AB}$	$8.540 \pm 0.021^{a,AB}$	$7.948 \pm 0.013^{a,B}$
Colon	$9.027 \pm 0.062^{a,A}$	$8.385 \pm 0.047^{ab,AB}$	$8.330 \pm 0.034^{ab,AB}$	$7.721 \pm 0.086^{ab,B}$
Faeces	$7.415 \pm 0.062^{b,A}$	$6.709 \pm 0.043^{b,B}$	$7.688 \pm 0.082^{b,A}$	$7.182 \pm 0.045^{b,AB}$
Coliforms				
Cecum	$9.057 \pm 0.113^{a,A}$	$9.215 \pm 0.017^{a,A}$	$8.946 \pm 0.015^{a,A}$	$8.054 \pm 0.111^{a,B}$
Colon	$9.050 \pm 0.108^{a,A}$	$8.989 \pm 0.086^{a,A}$	$8.688 \pm 0.071^{a,A}$	$7.812 \pm 0.034^{a,B}$
Faeces	$7.431 \pm 0.033^{b,AB}$	$6.757 \pm 0.085^{b,B}$	$7.641 \pm 0.054^{b,A}$	$6.875 \pm 0.053^{b,B}$

[1]Treatments include: Control, contained no *L. casei* ASCC 292, FOS or maltodextrin; LF, contained 0.85% (w/w) *L. casei* ASCC 292 and 2.40 (w/w) FOS; LM, contained 0.85% (w/w) *L. casei* ASCC 292 and 3.40% (w/w) maltodextrin; LFM, contained 0.85% (w/w) *L. casei* ASCC 292, 2.40% (w/w) FOS and 3.40% (w/w) maltodextrin.

[2]Results are expressed as mean ± standard error of means, n = 6. [abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [ABC]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.7. Population of total anaerobes, clostridia, bacteroides and lactobacilli from cecal, colon and faecal contents of rats fed diets control, LF, LM and LFM.

CFU/g dry sample	Treatments[1,2]			
	Control	LF	LM	LFM
Total anaerobes				
Cecum	$9.102 \pm 0.074^{a,A}$	$9.111 \pm 0.071^{a,A}$	$8.805 \pm 0.138^{a,A}$	$9.102 \pm 0.072^{a,A}$
Colon	$8.867 \pm 0.027^{a,A}$	$8.828 \pm 0.075^{ab,A}$	$8.338 \pm 0.031^{a,A}$	$8.302 \pm 0.085^{ab,A}$
Faeces	$8.725 \pm 0.050^{a,A}$	$8.232 \pm 0.021^{b,AB}$	$8.361 \pm 0.071^{a,AB}$	$7.988 \pm 0.264^{b,B}$
Clostridia				
Cecum	$5.101 \pm 0.115^{a,A}$	$4.402 \pm 0.111^{a,AB}$	$4.530 \pm 0.107^{a,AB}$	$4.055 \pm 0.288^{a,B}$
Colon	$4.540 \pm 0.022^{ab,A}$	$3.921 \pm 0.097^{a,AB}$	$4.068 \pm 0.167^{ab,AB}$	$3.718 \pm 0.031^{a,B}$
Faeces	$3.902 \pm 0.017^{b,A}$	$3.648 \pm 0.059^{a,A}$	$3.621 \pm 0.064^{b,A}$	$3.618 \pm 0.105^{a,A}$
Bacteroides				
Cecum	$8.828 \pm 0.222^{a,A}$	$7.562 \pm 0.045^{a,B}$	$7.186 \pm 0.020^{a,B}$	$7.820 \pm 0.025^{a,B}$
Colon	$8.307 \pm 0.104^{a,A}$	$7.328 \pm 0.022^{a,B}$	$7.057 \pm 0.021^{a,B}$	$7.008 \pm 0.051^{b,B}$
Faeces	$7.333 \pm 0.047^{b,A}$	$6.561 \pm 0.021^{b,B}$	$6.298 \pm 0.031^{b,B}$	$6.898 \pm 0.075^{b,AB}$
Lactobacilli				
Cecum	$7.118 \pm 0.070^{a,B}$	$7.588 \pm 0.011^{a,AB}$	$8.148 \pm 0.031^{a,A}$	$7.057 \pm 0.085^{a,B}$
Colon	$6.700 \pm 0.067^{ab,A}$	$7.238 \pm 0.048^{a,A}$	$7.314 \pm 0.071^{b,A}$	$7.031 \pm 0.064^{a,A}$
Faeces	$6.128 \pm 0.012^{b,B}$	$7.224 \pm 0.021^{a,A}$	$7.036 \pm 0.014^{b,A}$	$6.542 \pm 0.113^{a,AB}$

[1]Treatments include: Control, contained no *L. casei* ASCC 292, FOS or maltodextrin; LF, contained 0.85% (w/w) *L. casei* ASCC 292 and 2.40% (w/w) FOS; LM, contained 0.85% (w/w) *L. casei* ASCC 292 and 3.40% (w/w) maltodextrin; LFM, contained 0.85% (w/w) *L. casei* ASCC 292, 2.40% (w/w) FOS and 3.40% (w/w) maltodextrin.

[2]Results are expressed as mean ± standard error of means, n = 6. [ab]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [AB]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.8. Concentrations organic acids from cecal, colon and faecal content of rats fed diets control, LF, LM and LFM.

mM/g dry sample	Treatments[1,2]			
	Control	LF	LM	LFM
Acetic acid				
Cecum	$14.40 \pm 0.94^{a,A}$	$13.00 \pm 0.45^{a,A}$	$1.20 \pm 0.02^{a,B}$	$10.42 \pm 0.75^{a,A}$
Colon	$5.66 \pm 0.06^{b,B}$	$16.75 \pm 0.06^{a,A}$	$1.09 \pm 0.00^{a,B}$	$2.39 \pm 0.04^{b,B}$
Faeces	$2.25 \pm 0.49^{b,A}$	$6.23 \pm 0.05^{b,A}$	$0.12 \pm 0.01^{b,B}$	$0.33 \pm 0.04^{b,B}$
Butyric acid				
Cecum	$19.51 \pm 1.93^{a,A}$	$11.45 \pm 1.77^{a,B}$	$15.77 \pm 0.99^{a,AB}$	$2.97 \pm 0.12^{a,C}$
Colon	$16.16 \pm 3.41^{a,A}$	$10.87 \pm 2.11^{a,B}$	$5.87 \pm 0.28^{b,BC}$	$1.22 \pm 0.23^{a,C}$
Faeces	$6.01 \pm 2.37^{b,A}$	$5.25 \pm 0.23^{,A}$	$5.78 \pm 0.25^{b,A}$	$0.32 \pm 0.00^{a,B}$
Formic acid				
Cecum	$36.42 \pm 0.95^{a,B}$	$50.78 \pm 0.01^{a,A}$	$41.87 \pm 0.17^{a,AB}$	$34.46 \pm 0.64^{a,B}$
Colon	$24.78 \pm 1.71^{ab,A}$	$21.94 \pm 2.76^{b,A}$	$22.45 \pm 1.56^{b,A}$	$7.89 \pm 0.22^{b,B}$
Faeces	$14.52 \pm 0.03^{b,A}$	$8.03 \pm 0.28^{b,A}$	$8.20 \pm 0.26^{c,A}$	$1.12 \pm 0.13^{b,B}$
Propionic				
Cecum	$26.80 \pm 3.06^{a,B}$	$20.08 \pm 1.74^{a,B}$	$24.21 \pm 1.83^{a,B}$	$37.97 \pm 0.66^{a,A}$
Colon	$13.36 \pm 2.00^{b,A}$	$11.94 \pm 0.58^{ab,A}$	$14.01 \pm 0.43^{a,A}$	$17.44 \pm 2.53^{b,A}$
Faeces	$3.83 \pm 1.10^{b,A}$	$4.30 \pm 0.13^{b,A}$	$1.34 \pm 0.22^{b,A}$	$4.75 \pm 0.38^{c,A}$
Lactic acid				
Cecum	$130.00 \pm 1.41^{a,C}$	$188.02 \pm 0.80^{a,AB}$	$170.41 \pm 2.49^{a,B}$	$207.32 \pm 0.77^{a,A}$
Colon	$74.13 \pm 1.31^{b,B}$	$125.89 \pm 2.01^{b,A}$	$87.85 \pm 0.93^{b,B}$	$92.91 \pm 0.89^{b,B}$
Faeces	$32.78 \pm 0.49^{c,AB}$	$19.38 \pm 0.08^{c,B}$	$12.08 \pm 0.57^{c,C}$	$41.12 \pm 2.44^{c,A}$

[1]Treatments include: Control, contained no *L. casei* ASCC 292, FOS or maltodextrin; LF, contained 0.85% (w/w) *L. casei* ASCC 292 and 2.40% (w/w) FOS; LM, contained 0.85% (w/w) *L. casei* ASCC 292 and 3.40 (w/w) maltodextrin; LFM, contained 0.85% (w/w) *L. casei* ASCC 292, 2.40% (w/w) FOS and 3.40% (w/w) maltodextrin.

[2]Results are expressed as mean ± standard error of means, n = 6. [abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [ABC]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

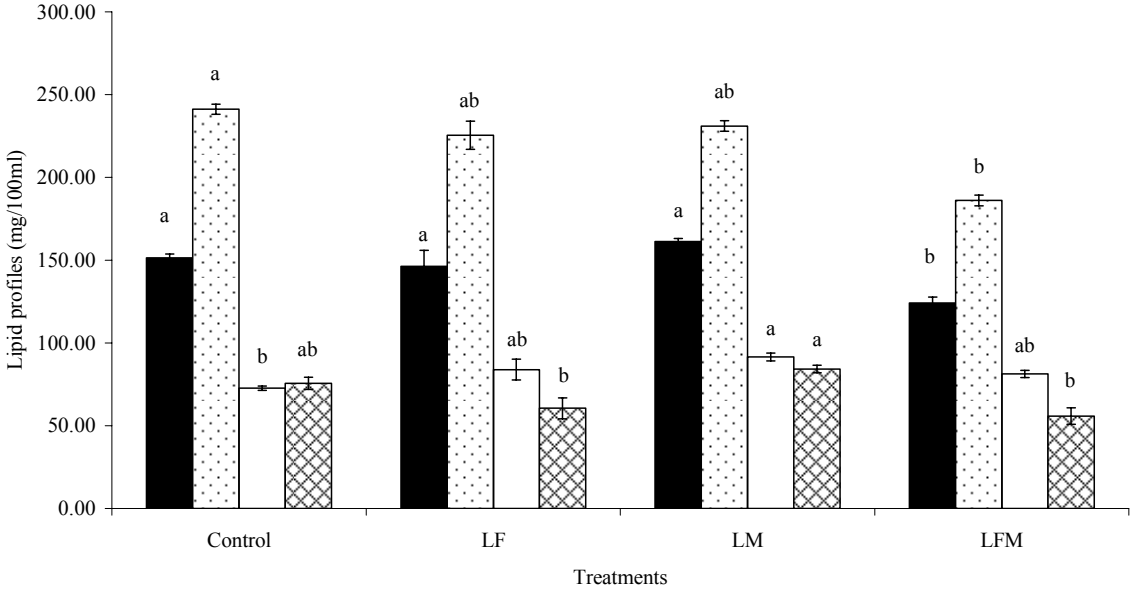


Figure 6.1. Total cholesterol (■), triglycerides (⊡), HDL-cholesterol (□) and LDL-cholesterol (⊠) levels of rats fed with diets control, LF, LM and LFM. Control contained no *L. casei* ASCC 292, FOS or maltodextrin; LF contained 0.85% (w/w) *L. casei* ASCC 292 and 2.40% (w/w) FOS; LM contained 0.85 (w/w) *L. casei* ASCC 292 and 3.40% (w/w) maltodextrin; LFM contained 0.85% (w/w) *L. casei* ASCC 292, 2.40% (w/w) FOS and 3.40% (w/w) maltodextrin. Results are expressed as mean ± standard error of means, n = 6. [ab]Means within the same series followed by different lowercase letters are significantly different ($P < 0.05$).

6.2 Synbiotic effects of *Lactobacillus acidophilus* ATCC 4962, fructooligosaccharide, mannitol and inulin on serum lipid profiles, intestinal microflora population and intestinal organic acids concentration in rats

6.2.1 Introduction

Recent studies have illustrated that higher than normal serum total cholesterol or low-density lipoprotein (LDL) cholesterol could increase the risk of coronary death (Usman and Hosono, 2000). A small reduction of 1% in serum total cholesterol could beneficially reduce the risk of coronary heart disease by 2 to 3% (Manson et al., 1992). Lactic acid bacteria especially *Lactobacillus* are probiotics that are considered potentially useful in their role to reduce serum cholesterol. Grunewald (1982) found that rats given milk fermented with *L. acidophilus* for 4 weeks showed lowered serum cholesterol levels compared to the control group containing only milk. Also, diets containing probiotic organisms lowered serum cholesterol in birds and pigs (Jin, Ho, Abdullah and Jalaludin, 1998; Gilliland, Nelson and Maxwell, 1985). Probiotic bacteria are enhanced by prebiotics that are defined as "nondigestable food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacterial species already resident in the colon, and thus improving host health" (Gibson and Roberfroid, 1995). Several classes of food ingredients that are classified as prebiotics include resistant starch, fibre, oligosaccharides and sugar alcohols. Fibre sources such as oat bran reportedly lowered plasma cholesterol in rats through enhancing steroid excretion (Chen et al., 1984).

The use of both probiotics and prebiotics (known as synbiotic) as a natural mean to counter hypercholesterolemia has generated much interest recently. In the presence of prebiotics, probiotics produce mainly carbon dioxide and short chain fatty acids (SCFA). SCFA such as propionic acid has been reported to inhibit fatty acid synthesis *in-vitro* and lower serum total cholesterol level. Propionate also increased HDL concentration in human trials by affecting the factor that stimulates the synthesis of apolipoproteins, which form the structure of the HDL molecule (Schoonjans, Staels and Auwerx, 1996). Conversely, acetate was reported as a lipogenic substrate acting as a precursor for cholesterol synthesis. High ratio of propionate to acetate after supplementation with fructooligosaccharides (FOS) decreased total serum cholesterol concentration (Wang and Gibson, 1993).

This section has been submitted for publication: *Liong, MT and Shah, NP. 2006. Synbiotic effects of Lactobacillus acidophilus ATCC 4962, fructooligosaccharide, mannitol and inulin on serum lipid profiles, intestinal microflora population and intestinal organic acids production in rats.* ***LWT-Food Sci Technol*** *(In review).*

We have previously screened and developed a synbiotic product consisting of *L. acidophilus* ATCC 4962, mannitol, FOS and inulin that specifically targeted removal of cholesterol in laboratory media containing cholesterol (Chapter 5.0, section 5.3.3.2). However, the beneficial effect of such synbiotic on serum lipid profiles in *in-vivo* models has not yet been studied, considering that the use of probiotics, prebiotics or synbiotics in reducing serum cholesterol levels has shown controversial results. Greany et al. (2004) reported that soy milk containing *L. acidophilus* exerted no hypocholesterolemic effect in postmenopausal women. Similarly, *Lactobacillus* showed no effect on human serum cholesterol in a double-blind, placebo-controlled and crossover trial (Lin, Ayres, Winkler and Sandine, 1989). Diets containing oligofructose lowered serum triacylglycerol and phospholipid concentrations in rats (Fiordaliso et al., 1995), but then did not affect the serum triacylglycerol, total and HDL cholesterol levels in type-II diabetic patients (Luo, Yperselle, Rizkalla, Rossi, Bornet and Slama, 2000).

Probiotics are also considered as indicator organisms for a good gastrointestinal function of the host. *Lactobacillus* has been reported to suppress the growth of putrefactive proteolytic bacteria via competitive inhibition, antagonistic activity and/or the production of SCFA especially lactic acid (Buddington et al., 2002; Isolauri et al., 2002). *Lactobacillus* spp. or *Bifidobacterium* spp. use prebiotics such as inulin and FOS more efficiently than other groups of intestinal bacteria, contributed by their more efficient transmembrane transporters (Yusrizal and Chen, 2003). These oligofructose prebiotics decreased colonization of *Salmonella* in chicken intestine, bacteroides and clostridia counts in human volunteers, while the combination of FOS and mannanoligosaccharide reduced faecal population of *Escherichia coli* in dogs (Bailey, 1991; Gibson, Beatty, Wang and Cummings, 1995; Swanson et al., 2002b). Our previous study reported the capability of *L. acidophilus* ATCC 4962 to produce organic acids in the presence of mannitol, FOS and inulin (Chapter 5.0, section 5.4.3); however the effect of this on gut microbial population has not been determined in *in-vivo* model.

In the U.S., Title 21 of the Code of General Regulations and the FDA Office of Premarket Approval listed several lactic acid microorganisms as approved food additives with generally recognized as safe (GRAS) status. Such lactic acid microorganisms include strains of *Lactobacillus*, *Bifidobacterium* and *Lactococcus* (Chatel, Langella, Adel-Patient, Commissaire, Wal and Corthier, 2001). Although the safety of traditional lactic acid bacteria has never been questioned, there are reports of probiotic strains such as *L. casei, Lactococcus lactis* and *L. plantarum* being isolated from patients with bacterial enterocarditis and *B. adolescentis* from septicemic cases (Gasser, 1994), indicating translocation of probiotic

organisms. Translocation of bacteria occurs when the ecological balance of the normal endogenous microflora is disrupted, when host immune defence is impaired or during physical loss of the mucosal barrier. Although the pH of the stomach may reach as low as 1.5, the lower intestine has a pH of near neutral. Thus, high concentrations of organic acids, arising from rapid fermentation of prebiotics by probiotics inhibiting the colonization of acid-sensitive pathogens, could also induce injury to the intestinal mucosa and hence impair its barrier function (Argenzio and Meuten, 1991; Remesy, Levrat, Gamet and Demigne, 1993). Hence, bacterial translocation is possible even in healthy animals. It is of utmost importance to ensure that bacterial strains used in probiotic or synbiotic applications are safe.

Thus, the aim of this study was to evaluate the effectiveness of *L. acidophilus* ATCC 4962 in the presence of mannitol, FOS and inulin, alone or in a combination, to reduce the serum cholesterol using a rat model. In addition, the effect of the synbiotic on intestinal microflora, intestinal concentration of organic acids and translocation of probiotic bacteria were also investigated.

6.2.2. Methods and Materials

6.2.2.1 Source of probiotic culture and prebiotics

L. acidophilus ATCC 4962 in freeze-dried form was used in this study. Working cultures were prepared as described in Chapter 5.0, section 5.1.2.1. Three commercially available prebiotics were used including mannitol (Mannogem; SPI Polyols Inc., New Castle, USA), FOS (Raftilose P95; Orafti Pty. Ltd., Tienen, Belgium) and inulin (Raftilene ST; Orafti). The FOS had a purity of 95% and the remaining 5% contained glucose, fructose and sucrose. The degree of polymerization (DP) of FOS ranged from 2 to 7, with an average DP of 4. The inulin had a purity of 92%, and an average DP of 10. The mannitol used had DP ranging from 1-2 and was affirmed GRAS under 21CFR: 180.25.

6.2.2.2 Animals and diets

Male Wistar rats were used in this study. The handling and housing conditions of the animals are described in section 6.1.2.2. The allocation of diets is shown in Table 6.9.

6.2.2.3 Sampling and analytical procedures

Samples were collected and analysed as described in section 6.1.2.3.

6.2.2.4 Experimental design and statistical analysis

Data were analysed as described in section 6.1.2.4.

6.2.3 Results

6.2.3.1 Weight and feed intake

All rats were generally healthy throughout the experimental period. Body weight gain, feed intake and feed efficiency of rats on the control, LF, LM, LI and LFMI diets are shown in Table 6.10. There was no significant difference in the feed efficiency among rats with each synbiotic treatment, but was significant ($P < 0.05$) compared to the control.

6.2.3.2 pH and moisture content of cecal, colon and faecal contents

The control group showed similar pH values across the intestine, except in faecal samples (Table 6.11). The diet containing *L. acidophilus* ATCC 4962 and inulin (LI) and mannitol (LM) showed increasing pH values across the intestine, with lowest pH detected in the cecum, while the control group showed increasing pH values only after the colon. Rats on diets LI and LFMI had lower pH values in the cecum than that on the control diet.

The moisture content decreased from the cecal to faecal samples of rats on the control diet (Table 6.12), while those on the LF, LM and LI diets only showed such a decrease only after the colon. The LFMI diet contributed to a decrease in moisture after the cecum. Rats on all the synbiotic diets showed higher moisture levels in the colon contents and faeces compared to those on the control diet.

6.2.3.3 Lipid profiles

Rats on the LF, LI and LFMI diets exhibited lower total serum cholesterol levels by 22.327%, 31.714%, and 32.404%, respectively compared to those on the control (Figure 6.2), while those on the LM diet did not show a positive effect. The serum triglycerides level was lower when rats were on LM (25.138%), LI (24.037%) and LFMI (32.514%) diets compared to those on the control diet, while only the LI (46.900%) and LFMI (42.952%) diets contributed to a decrease in LDL cholesterol compared to those on the control diet. Only rats on the LF diet showed an increase in the HDL cholesterol level (31.610%), while other diets showed no difference ($P < 0.05$).

6.2.3.4 Microbial populations

Total aerobe counts decreased after the colon of all rats except those on diet LI which showed decreasing counts from cecum to faeces (Table 6.13). Only rats on the LI diet showed lower counts of total aerobes in all intestinal samples compared to the control. Diets LM and LFMI showed a reduction in staphylococci after the colon, while there was a similar population of staphylococci across the intestine in the control group. Similar to total aerobes, only the LI diet contributed to lower counts of staphylococci than the control across the intestine. Rats on diets LF and LM had a decreasing population of *E. coli* and coliforms, respectively across the intestine, compared to the control group which only showed a decrease after the colon. Only rats on the LI diet had lower population of *E. coli* and total coliforms than the control across the intestine.

The population of total anaerobes was similar across the intestine in rats on the control diet (Table 6.14). Rats fed the LF, LI and LFMI diets showed a decreasing population of anaerobes after the colon, while LM contributed to a continuous decrease in total anaerobes across the intestine. Total anaerobe counts increased in the cecum and colon of rats fed the LF diet, but was lower when rats were on the LI diet compared to the control. There was a lower population of total clostridia after the cecum of rats fed the LI and LFMI diets compared to the control. A lower population of bacteroides was observed than the control across the intestine of rats on the LM and LI diets. The control group showed decreasing population of total *Lactobacillus* after the colon, while rats on all the synbiotic diets maintained a similar population in the cecum, colon and faeces. The LF diet contributed to higher population of total *Lactobacillus* than the control diet and other synbiotic diets.

6.2.3.5 Concentrations of organic acids

There was a similar trend of acetic acid concentration in rats on all diets, with the highest amount in the cecum and the lowest in the faeces (Table 6.15). Only rats on LF, LM and LFMI diets had a higher concentration of acetic acid in the cecal and colonic contents than those on the control diet. Rats on all synbiotic diets had lower concentration of butyric acid across the intestine than those on the control diet. Butyric acid was not detected after the colon when rats were on the synbiotic diets compared to the control diet. The LM diet contributed to the lowest concentration of formic acid across the intestine than the control diet. The LI diet contributed to a higher concentration of propionic acid across the intestine than the control diet, while rats on the LM diet had undetectable level of propionic acid in

the faeces. However, concentration of lactic acid decreased after the colon in rats on the LM diet while those on the control showed a drastic decrease after the cecum. The LF diet contributed to a higher amount of lactic acid across the intestine, while the LFMI diets showed an increased concentration in the cecum and colon compared to those on the control diet. Rats on the LI and LM diets showed a higher and a lower concentration of lactic acid, respectively in the cecum than those on the control.

6.2.3.6 Translocation of *Lactobacillus*

No growth of *Lactobacillus* was detected from the spleen, liver and kidney samples of rats on the control, LF, LM, LI and LFMI diets. This indicated the absence of *Lactobacillus* translocation.

6.2.4 Discussion

The experiments were conducted to investigate the combined effect of *L. acidophilus* ATCC 4962 and FOS (LF), *L. acidophilus* ATCC 4962 and mannitol (LM), *L. acidophilus* ATCC 4962 and inulin (LI) and *L. acidophilus* ATCC 4962 with FOS, mannitol and inulin (LFMI) on serum cholesterol, intestinal microflora, concentration of organic acids and the translocation of *Lactobacillus* using rats as a model. The feed intake between treatment groups of rats and those on the control diet was not significantly different ($P > 0.05$). It appeared that the differing feed efficiency was contributed by the difference in body weight gain. Despite that, only the LFMI diet produced significantly different weight gain compared to the control. Only the addition of rodent chow, *L. acidophilus* and the prebiotics varied between the control and the treatment groups. Considering that the fat and fibre content of rodent chow was low (< 5%), it should not have altered the outcome of this study.

The synbiotic diets contributed to higher moisture content in samples collected after the cecum compared to those on the control diet, indicating a stronger laxative effect as reflected in the softer faecal pellets (personal observation). It was previously reported that chemically modified tapioca starch exhibited laxative effect with increasing levels of amylase-resistance (Kishida, Nakai and Ebihara, 2000). Results from this study indicated that the LI and LFMI diets retained highest moisture from the colon and faecal contents compared to those on the control diet and other synbiotic diets. Inulin would have higher resistance to digestion with higher number of fructosyl moities than FOS and higher DP than mannitol. *In-vitro* fermentation experiments have shown that molecules with DP > 10 are fermented on an average half as quickly as molecules with a DP of <10 (Roberfroid, Van

Loo and Gibson, 1998). This explains the higher resistant of the LI diet and its ability to reach the colon to exhibit a stronger laxative effect compared to the LF and LM diets, while the LFMI diet showed similar laxative property contributed by its higher non-digestible carbohydrate bulk. Contrary to most dietary fibres, which act mainly as bulking agents, long-chain oligosaccharides are osmotic laxative agents due to the fact that they enter the colon virtually unchanged. The breakdown of these oligosaccharides by bacterial fermentation was found to promote peristalsis of the colon, which enhanced the laxative effects (Olesen and Gudmand-Hoyer, 2000).

Rats that were on the LFMI diet showed reduced total cholesterol, triglycerides and LDL cholesterol levels. This supports our previous findings, which reported optimum removal of cholesterol with *L. acidophilus* ATCC 4962 in the presence of FOS, mannitol and inulin (Chapter 5.0, section 5.3.3.2). Considering that the LI diet had similar effect on serum lipoprotein profiles while the other two synbiotics (LF and LM) did not, it appeared that inulin was the main contributor to decreased levels of cholesterol. A possible explanation could be the alteration of lipid metabolism by short-chain fatty acids (SCFA). Recent studies in rats showed that resistant rice starch in the diet increased serum concentrations of propionate, leading to a reduction in the concentration of total- and LDL cholesterol (Cheng and Lai, 2000). Propionate was found to inhibit fatty acid synthesis *in-vitro* (Delzenne and Kok, 2001) and in this study, rats that were on LI diet had a higher concentration of propionic acid in the cecal, colon and faecal contents than those on the control.

Results from this study showed that an increase in the HDL-cholesterol was not observed with the LM and LI diets, but then suggested that FOS was the main contributor to an increase in the beneficial cholesterol level. Although FOS has been found to increase serum HDL level (Gibson and Roberfroid, 1995), its actual role on the catabolism of HDL remains unknown. It is noteworthy that the LF diet not only increased the serum HDL level but also decreased total cholesterol level. Considering this, FOS may have enhanced the regulation of HDL possibly by an increased apolipoprotein-cell interaction as supported by a previous study that found an alteration in the apolipoprotein-cell interaction could inhibit the generation of HDL coupled with induced cholesterol accumulation in a mouse model (Tomimoto et al., 2001). More work is needed to elucidate this.

In our study, diet LI exhibited positive effects on gut microbiology, with lower population of total aerobes, staphylococci, *E. coli*, coliforms, clostridia and bacteroides across the intestine than the rats on the control diet. Oligosaccharides were reported to possess receptor sequences that inhibit adhesion of pathogens to mucosal cells in the host.

Binding of the pathogens to specific receptor sites in oligosaccharides was found to increase host resistance to infection, reducing the likelihood of pathogen establishment and subsequent elaboration of virulence (Gibson *et al.*, 2000). Although FOS and inulin are oligosaccharides, the better antimicrobial activity of the LI diet than the LF diet indicated that inulin was present in a higher amount in the colon than FOS. Both oligosaccharides have high persistence through the colon, but the longer oligosaccharides may penetrate the more distal colonic regions. This has also been supported by higher the osmolality of the LI diet than the LF diet. In addition, samples of intestinal content from rats on LI had lower pH values compared to those on the control diet. A reduction in pH of the intestinal tract correlated with a decreased growth of intestinal pathogenic bacteria in chickens (Garrido, Skjervheim, Oppegaard and Sorum, 2004). Rats on the LI diet also had a higher concentration of propionic acid across the intestine and also higher concentration of lactic acid in the cecum than those on the control diet. Thus, it appeared that *L. acidophilus* ATCC 4962 in the presence of inulin produced substantial amount of organic acids that reduced the pH values and may have subsequently inhibited the growth of pathogenic bacteria. The suppression of these pathogenic microflora is beneficial considering that staphylococcal food poisoning caused by enterotoxigenic staphylococci is one of the main food-borne diseases (Becker et al., 2001). *E. coli* is reported to cause diarrhoea and haemolytic-uremic syndrome (Friedrich, Borell, Bielaszewska, Fruth, Tschape and Karch, 2003) while strains of clostridia colonized the large bowel of patients undergoing antibiotic therapy and is the leading cause of hospital-acquired diarrhoea (Voth and Ballard, 2005).

Rats on the LF diet were the only group that exhibited higher population of *Lactobacillus* across the intestine than those on the control diet and other synbiotic diets. In addition, rats on the LF diet also had higher concentration of acetic and lactic acids across the intestine compared to those on the control. Considering that *Lactobacillus* is a lactic acid producing bacteria, FOS may have contributed to the higher population of total *Lactobacillus* across the intestine, and subsequently accelerated the concentration of acetic and lactic acids in those regions. Also, lactate and acetate producers in the intestines could utilize FOS as a substrate. This is supported by a study using anaerobic batch culture fermenters and human gut microflora, that showed preference on FOS over oligodextran for the production of acetic and lactic acid (Olano-Martin et al., 2002).

The concentration of butyric acid across the intestine was lower in rats on all synbiotic diets than those on the control diet. Butyrate is the primary respiratory fuel in the distal colonic mucosa, providing 60-70% of the energy needed (Scheppach et al., 1995). In addition, we found that *L. acidophilus* ATCC 4962 produced butyric acid in the presence of

mannitol, FOS and inulin (Chapter 5.0, section 5.4.3). Considering these, the butyric acid that was produced in rats on the synbiotic diets may have been utilized by colonocytes and was undetected in the faeces. Although the reason behind the higher concentration of butyric acid in rats on the control diet remained unclear, results from the present study indicated that the presence of *L. acidophilus* ATCC 4962 have accelerated colonic butyrate utilization. Butyrate has been found to prolong the doubling time and reduced the growth rate of human large-bowel cancel cells (Gamet et al., 1992). Although research on the biological properties of butyrate from the utilization of the synbiotic diets are beyond the objectives of our present study, further investigation would be beneficial, considering that colon is the second most common site for cancer in developed nations (Olano-Martin et al., 2002).

The LM diet contributed to the lowest concentration of formic acid across the intestine than the control diet. The fermentation of mannitol has been reported as follows:

mannitol → (1 + 0.5*x*) acetate + (1-*x*) lactate + (0.5 + 0.5*x*) ethyl alcohol + *x* formate (De Vries and Stouthamer, 1968).

Using *Lactococcus lactis*, it was previously found that when mannitol was consumed, the major end product from the catabolism of mannitol was formate (Neves et al. 2002). In addition, we previously reported that the fermentation of mannitol by *L. acidophilus* ATCC 4962 had the strongest influence on the production of formic acid (Chapter 4.0, section 4.3.4.2) Considering this, the lower concentration of formic acid may not be an indicator of reduced production of formic acid by fermentation on the LM diet. Rather, the formate produced may have acted as and additional substrate and was utilized by the intestinal microflora.

The LI diet contributed to a higher concentration of propionic acid across the intestine than the control diet, while rats on the LM diet had undetectable level of propionic acid in the faeces. The concentration of propionic acid as affected by synbiotic treatments seemed to be in the order of LI > LF > LFMI and LM. *In-vitro* experiments using human gut microflora showed that the utilization of prebiotics was higher from substrates with lower molecular mass than those with higher molecular mass, with the exception of FOS, which was only 50% utilized after 48 hours (Olano-Martin et al., 2002). This may explain the lower concentration of propionic acid contributed by FOS, despite having a lower DP than inulin. Also, the lower concentration of propionic acid from the LFMI diet would most probably be contributed by its high molecular mass. However, the effect of mannitol on the production of propionic acid remained unclear.

The synbiotic diets containing *L. acidophilus* ATCC 4962 did not exhibit harmful lactobacilli translocation to other internal organs such as spleen, liver and kidney. This indicated that the synbiotics studied was safe for consumption.

6.2.5 Conclusion

The LFMI and LI diets contributed to lower serum total cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol levels compared to the control diet. The LI diet decreased the population of total aerobes, staphylococci, *E. coli*, coliforms and bacteroides, possibly by decreasing intestinal pH values. The LFMI diet showed a constant population of total *Lactobacillus* across the intestine followed by a decreased count of clostridia in the colon and faeces. This study showed that the combination of *L. acidophilus* ATCC 4962, FOS, mannitol and inulin (LFMI) was ideal for serum lipoprotein profiles and healthier bowel microbial population without exhibiting harmful translocation of *Lactobacillus*.

Table 6.9 Composition of control, LF, LM, LI and LFMI diets.

Ingredients (g kg^{-1})	Diets[a]				
	Control	LF	LM	LI	LFMI
SF00-245[b]	750.0	750.0	750.0	750.0	750.0
Copha[c]	50.0	50.0	50.0	50.0	50.0
L. acidophilus ATCC 4962	-	13.2	13.2	13.2	13.2
FOS[d]	-	16.4	-	-	16.4
Mannitol[d]	-	-	20.7	-	20.7
Inulin[d]		-	-	29.1	29.1
Rodent chow[e]	200.0	170.4	166.1	157.7	120.6

[a]Diets include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin.

[b]High-cholesterol diet (Specialty Feeds, Glen Forrest, Australia).

[c]Copha® (Unilever Australasia, Epping, Australia), hardened coconut oil 99%, soya bean lecithin 1%.

[d]Fructooligosaccharide (Raftilose P95; Orafti Pty. Ltd., Tienen, Belgium), purity of 95%, average degree of polymerization (DP) of 4; Mannitol (Sigma Chemical Co., St. Louis, U.S.A.); Inulin (Raftiline ST; Orafti Pty. Ltd., Tienen, Belgium), purity of 92%, average DP of 10.

[e]Rodent chow (Specialty Feeds, Glen Forrest, Australia), total protein 19.6%, total fat 4.6%, crude fibre 4.8%, trace minerals 2.9%, total vitamins 0.3%.

Table 6.10 Body weight gain, feed intake and feed efficiency of rats on control, LF, LM, LI and LFMI diets.

Component	Treatments				
	Control	LF	LM	LI	LFMI
Body weight gain (g rat^{-1} $week^{-1}$)	34.0 ± 2.5^{a}	32.4 ± 5.3^{ab}	26.6 ± 1.0^{ab}	27.6 ± 2.7^{ab}	24.6 ± 1.0^{b}
Feed intake (g $week^{-1}$)	140.5 ± 4.0^{a}	178.1 ± 3.6^{a}	129.8 ± 8.0^{a}	133.8 ± 16.7^{a}	142.9 ± 7.1^{a}
Ratio body weight: feed intake	0.24 ± 0.01^{a}	0.18 ± 0.01^{b}	0.21 ± 0.01^{b}	0.21 ± 0.02^{b}	0.17 ± 0.01^{b}

Treatments include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means.

[ab]Means in the same row followed by different lowercase letters are significantly different ($P < 0.05$).

Table 6.11 pH values of contents obtained from the cecum, colon and faeces of rats on control, diets LF, LM, LI and LFMI.

pH	Treatments				
	Control	LF	LM	LI	LFMI
Cecum	$6.4 \pm 0.1^{b,C}$	$6.7 \pm 0.1^{b,B}$	$7.1 \pm 0.1^{c,A}$	$5.8 \pm 0.0^{c,E}$	$6.1 \pm 0.0^{b,D}$
Colon	$6.8 \pm 0.0^{b,BC}$	$6.9 \pm 0.1^{b,B}$	$7.5 \pm 0.0^{bc,A}$	$6.5 \pm 0.0^{b,C}$	$6.4 \pm 0.0^{b,C}$
Faeces	$7.8 \pm 0.1^{a,A}$	$7.8 \pm 0.2^{a,A}$	$7.8 \pm 0.0^{a,A}$	$7.2 \pm 0.0^{a,B}$	$7.3 \pm 0.0^{a,AB}$

Treatments include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means.

[abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

[ABCDE]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.12 Moisture content of cecal, colon and faecal content of rats on diet control, LF, LM, LI and LFMI.

Moisture (%)	Treatments				
	Control	LF	LM	LI	LFMI
Cecum	77.57 ± 1.63^{a,A}	78.71 ± 0.60^{a,A}	78.41 ± 1.97^{a,A}	81.47 ± 1.79^{a,A}	82.50 ± 1.44^{a,A}
Colon	58.05 ± 5.04^{b,C}	63.84 ± 0.79^{a,B}	66.34 ± 2.81^{a,B}	74.39 ± 1.18^{a,A}	74.16 ± 0.47^{b,A}
Faeces	18.43 ± 1.52^{c,D}	46.28 ± 0.16^{b,B}	35.71 ± 0.87^{b,C}	41.49 ± 1.45^{b,B}	56.87 ± 1.46^{b,A}

Treatments include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means.

^{abc}Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

^{ABCD}Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.13 Population of total aerobes, staphylococci, *Escherichia coli*, and total coliforms from cecal, colon and faecal contents of rats on diets control, LF, LM, LI and LFMI.

$Log_{10}CFU\ g^{-1}$ dry mass	Treatments				
	Control	**LF**	**LM**	**LI**	**LFMI**
Total aerobes					
Cecum	$9.393 \pm 0.047^{a,AB}$	$9.963 \pm 0.031^{a,A}$	$9.150 \pm 0.042^{a,B}$	$8.323 \pm 0.034^{a,C}$	$8.931 \pm 0.057^{a,BC}$
Colon	$9.413 \pm 0.032^{a,A}$	$9.600 \pm 0.075^{a,A}$	$9.49 \pm 0.121^{a,A}$	$7.783 \pm 0.023^{b,C}$	$8.557 \pm 0.057^{a,B}$
Faeces	$7.672 \pm 0.06^{b,BC}$	$8.217 \pm 0.081^{b,AB}$	$8.825 \pm 0.378^{b,A}$	$6.747 \pm 0.137^{c,D}$	$7.402 \pm 0.048^{b,C}$
Staphylococci					
Cecum	$7.502 \pm 0.031^{a,AB}$	$8.016 \pm 0.035^{a,A}$	$7.224 \pm 0.072^{a,B}$	$6.628 \pm 0.072^{a,B}$	$7.634 \pm 0.023^{a,A}$
Colon	$7.580 \pm 0.037^{a,AB}$	$7.773 \pm 0.052^{a,A}$	$7.293 \pm 0.058^{a,B}$	$6.307 \pm 0.031^{a,C}$	$7.250 \pm 0.050^{ab,B}$
Faeces	$7.280 \pm 0.027^{a,AB}$	$7.785 \pm 0.057^{a,A}$	$6.862 \pm 0.015^{b,B}$	$6.171 \pm 0.042^{a,C}$	$6.864 \pm 0.032^{b,B}$
***Escherichia* coli**					
Cecum	$9.010 \pm 0.047^{a,B}$	$9.552 \pm 0.067^{a,AB}$	$9.022 \pm 0.047^{a,B}$	$8.182 \pm 0.057^{a,C}$	$9.672 \pm 0.037^{a,A}$
Colon	$9.027 \pm 0.062^{a,A}$	$8.950 \pm 0.087^{b,AB}$	$8.557 \pm 0.062^{a,B}$	$7.630 \pm 0.043^{a,C}$	$9.273 \pm 0.024^{a,A}$
Faeces	$7.415 \pm 0.062^{b,B}$	$7.827 \pm 0.076^{c,B}$	$7.609 \pm 0.060^{b,C}$	$6.721 \pm 0.102^{b,D}$	$8.477 \pm 0.052^{b,A}$
Coliforms					
Cecum	$9.057 \pm 0.113^{a,BC}$	$9.847 \pm 0.052^{a,A}$	$8.887 \pm 0.112^{a,C}$	$8.082 \pm 0.060^{a,D}$	$9.645 \pm 0.042^{a,AB}$
Colon	$9.050 \pm 0.108^{a,B}$	$9.643 \pm 0.012^{a,A}$	$8.283 \pm 0.044^{b,C}$	$7.537 \pm 0.074^{a,D}$	$9.087 \pm 0.040^{b,AB}$
Faeces	$7.431 \pm 0.033^{b,B}$	$8.331 \pm 0.227^{b,A}$	$7.465 \pm 0.086^{c,B}$	$6.395 \pm 0.146^{b,C}$	$7.857 \pm 0.080^{c,AB}$

Treatments include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means. ^{abc}Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$). ^{ABCD}Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.14 Population of total anaerobes, clostridia, bacteroides and *Lactobacillus* from cecal, colon and faecal contents of rats on diets control, LF, LM, LI and LFMI.

$Log_{10}CFU\ g^{-1}$ dry mass	Treatments				
	Control	**LF**	**LM**	**LI**	**LFMI**
Total anaerobes					
Cecum	$9.102 \pm 0.074^{a,B}$	$10.505 \pm 0.064^{a,A}$	$8.835 \pm 0.137^{a,B}$	$8.163 \pm 0.057^{a,C}$	$8.602 \pm 0.030^{a,BC}$
Colon	$8.867 \pm 0.027^{a,B}$	$9.960 \pm 0.033^{a,A}$	$8.153 \pm 0.027^{b,C}$	$7.533 \pm 0.026^{a,D}$	$8.437 \pm 0.045^{a,BC}$
Faeces	$8.725 \pm 0.050^{a,A}$	$9.161 \pm 0.075^{b,A}$	$7.267 \pm 0.073^{c,BC}$	$6.968 \pm 0.071^{b,C}$	$7.785 \pm 0.072^{b,B}$
Clostridia					
Cecum	$5.101 \pm 0.115^{a,AB}$	$5.572 \pm 0.057^{a,A}$	$4.672 \pm 0.147^{a,B}$	$4.622 \pm 0.107^{a,B}$	$4.588 \pm 0.022^{a,B}$
Colon	$4.540 \pm 0.022^{b,AB}$	$4.953 \pm 0.158^{b,A}$	$4.713 \pm 0.243^{a,B}$	$3.877 \pm 0.155^{b,C}$	$3.727 \pm 0.052^{b,C}$
Faeces	$3.902 \pm 0.017^{c,AB}$	$4.272 \pm 0.026^{c,A}$	$3.925 \pm 0.137^{b,AB}$	$3.493 \pm 0.113^{b,BC}$	$3.307 \pm 0.095^{b,C}$
Bacteroides					
Cecum	$8.828 \pm 0.222^{a,A}$	$7.827 \pm 0.092^{a,B}$	$7.945 \pm 0.047^{a,B}$	$6.963 \pm 0.077^{a,C}$	$7.732 \pm 0.050^{a,B}$
Colon	$8.307 \pm 0.104^{a,A}$	$7.890 \pm 0.050^{a,B}$	$6.997 \pm 0.147^{b,C}$	$6.753 \pm 0.025^{ab,C}$	$7.653 \pm 0.082^{ab,B}$
Faeces	$7.333 \pm 0.047^{b,A}$	$7.692 \pm 0.055^{a,A}$	$6.382 \pm 0.030^{b,B}$	$6.391 \pm 0.077^{b,B}$	$7.280 \pm 0.055^{b,A}$
Lactobacillus					
Cecum	$7.118 \pm 0.070^{a,B}$	$8.127 \pm 0.035^{a,A}$	$7.186 \pm 0.034^{a,B}$	$5.462 \pm 0.117^{a,C}$	$6.768 \pm 0.022^{a,B}$
Colon	$6.700 \pm 0.067^{a,B}$	$8.310 \pm 0.085^{a,A}$	$7.110 \pm 0.228^{a,B}$	$5.323 \pm 0.140^{a,C}$	$6.847 \pm 0.141^{a,B}$
Faeces	$6.128 \pm 0.012^{b,C}$	$8.607 \pm 0.045^{a,A}$	$6.807 \pm 0.085^{a,B}$	$5.088 \pm 0.151^{a,D}$	$6.407 \pm 0.042^{a,B}$

Treatments include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means. ^{abc}Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$). ^{ABCD}Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.15 Concentrations of organic acids from cecal, colon and faecal content of rats on diets control, LF, LM, LI and LFMI.

mM g^{-1} dry mass	Treatments				
	Control	**LF**	**LM**	**LI**	**LFMI**
Acetic acid					
Cecum	$14.40 \pm 0.94^{a,C}$	$26.16 \pm 0.17^{a,B}$	$21.71 \pm 0.03^{a,B}$	$13.41 \pm 0.16^{a,C}$	$48.82 \pm 0.80^{a,A}$
Colon	$5.66 \pm 0.06^{b,C}$	$17.27 \pm 0.38^{b,A}$	$11.37 \pm 0.06^{b,B}$	$7.01 \pm 0.48^{b,C}$	$11.02 \pm 0.68^{b,B}$
Faeces	$2.25 \pm 0.49^{c,B}$	$8.90 \pm 0.41^{c,A}$	$4.91 \pm 0.03^{c,B}$	$3.29 \pm 0.76^{c,B}$	$3.49 \pm 0.02^{c,B}$
Butyric acid					
Cecum	$19.51 \pm 1.93^{a,A}$	$7.08 \pm 0.03^{a,B}$	$3.18 \pm 0.85^{a,C}$	$4.18 \pm 0.09^{a,C}$	$3.70 \pm 0.32^{a,C}$
Colon	$16.16 \pm 3.41^{a,A}$	$0.70 \pm 0.00^{b,B}$	$0.80 \pm 0.03^{a,B}$	$1.20 \pm 0.07^{b,B}$	$1.63 \pm 0.19^{a,B}$
Faeces	$6.01 \pm 2.37^{c,A}$	ND	ND	ND	ND
Formic acid					
Cecum	$36.42 \pm 0.95^{a,B}$	$36.23 \pm 0.96^{a,B}$	$18.36 \pm 0.23^{a,C}$	$65.17 \pm 0.35^{a,A}$	$34.66 \pm 0.40^{a,B}$
Colon	$24.78 \pm 1.71^{b,A}$	$13.0 \pm 0.22^{b,BC}$	$9.64 \pm 0.20^{b,C}$	$17.76 \pm 0.64^{b,B}$	$12.37 \pm 0.14^{b,C}$
Faeces	$14.52 \pm 0.03^{c,A}$	$6.60 \pm 0.08^{c,B}$	$4.95 \pm 0.10^{c,BC}$	$2.41 \pm 0.03^{c,C}$	$11.60 \pm 0.09^{b,A}$
Propionic acid					
Cecum	$26.80 \pm 3.06^{a,B}$	$16.96 \pm 0.36^{a,BC}$	$12.61 \pm 0.16^{a,C}$	$75.46 \pm 1.42^{a,A}$	$14.34 \pm 0.12^{a,C}$
Colon	$13.36 \pm 2.00^{b,B}$	$9.54 \pm 0.26^{b,BC}$	$4.41 \pm 0.05^{b,D}$	$27.75 \pm 0.18^{b,A}$	$6.46 \pm 0.04^{b,CD}$
Faeces	$3.83 \pm 1.10^{c,B}$	$2.44 \pm 0.19^{c,B}$	ND	$8.68 \pm 0.09^{c,A}$	$1.61 \pm 0.00^{c,B}$
Lactic acid					
Cecum	$130.00 \pm 1.41^{a,B}$	$200.70 \pm 3.21^{a,A}$	$96.12 \pm 14.70^{a,C}$	$208.55 \pm 0.36^{a,A}$	$228.24 \pm 2.43^{a,A}$
Colon	$74.13 \pm 1.31^{b,C}$	$149.55 \pm 2.82^{b,A}$	$76.64 \pm 2.85^{a,C}$	$83.29 \pm 5.24^{b,BC}$	$117.88 \pm 1.61^{b,AB}$
Faeces	$32.78 \pm 0.49^{c,B}$	$128.62 \pm 0.16^{b,A}$	$30.31 \pm 0.32^{b,B}$	$17.99 \pm 0.06^{c,B}$	$20.28 \pm 0.01^{c,B}$

Treatments include: Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means.

[abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$).

[ABCD]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

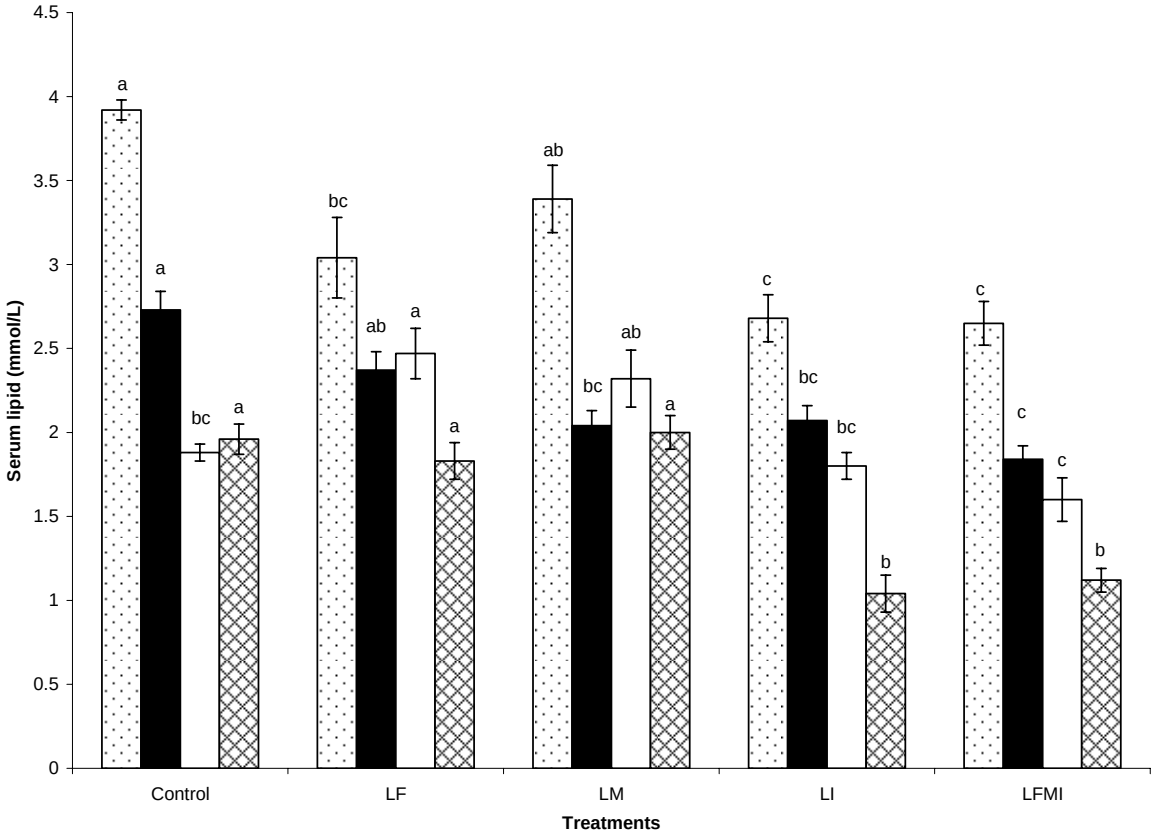


Figure 6.2 Total serum cholesterol (⊡), triglycerides (■), HDL-cholesterol (□) and LDL-cholesterol (⊠) levels of rats on with diets control, LF, LM, LI and LFMI diets. Control, contained no *L. acidophilus* ATCC 4962, FOS, mannitol or inulin; LF, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 1.64% (w/w) FOS; LM, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.07% (w/w) mannitol; LI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962 and 2.91% (w/w) inulin; LFMI, contained 1.32% (w/w) *L. acidophilus* ATCC 4962, 1.64% (w/w) FOS, 2.07% (w/w) mannitol and 2.91% (w/w) inulin. Results are expressed as mean ± standard error of means. [abc]Means within the same series followed by different lowercase letters are significantly different ($P < 0.05$).

6.3 SORBITOL, MALTODEXTRIN, INULIN AND *Bifidobacterium infantis* MODIFY SERUM LIPID PROFILES, INTESTINAL MICROBIAL POPULATION AND ORGANIC ACIDS CONCENTRATION IN RATS

6.3.1 INTRODUCTION

Elevated serum cholesterol levels in humans are associated with various negative health effects including increased mortality rates (Bloomgarden, 2002). Ever since diets were found to contribute to changes in serum lipid profiles, numerous diet-based strategies have been used to decrease serum cholesterol level including substitution of saturated fatty acids with monounsaturated- and polyunsaturated fatty acids, minimization of high-fat diets and more recently, the usage of probiotics and/or prebiotics. We have previously studied the *in-vitro* cholesterol removal capability of *Bifidobacterium infantis* ATCC 17930 in the presence of several prebiotics and reported that the combination of *B. infantis* ATCC 17930 and sorbitol, maltodextrin and inulin was optimal for cholesterol removal (Chapter 5.0, section 5.5.3.3). However, the performance of such a synbiotic has yet to be examined in an *in-vivo* model.

Bifidobacterium has also been associated with the alteration of the intestinal microflora population and has been used to suppress growth of pathogens through competitive inhibition and antagonistic activity (Isolauri *et al.*, 2002), while prebiotics such as fructooligosaccharide and mannanoligosaccharide are reported to decrease the population of *Escherichia coli* in dogs possibly contributed by the production of short-chain fatty acids (SCFA) (Swanson *et al*., 2002b). Our previous study (Chapter 5.0, section 5.5.3.4) reported that *B. infantis* ATCC 17930 was not only capable of removing cholesterol *in-vitro* but also produced SCFA in the presence of sorbitol, maltodextrin and inulin. Thus, the aims of this study were to evaluate the effectiveness of such synbiotics on reducing serum cholesterol, and to modify the intestinal microflora and the concentration of organic acids using rats as a model.

This section has been accepted for publication and is currently in press. Galley proofs have been corrected and returned: *Liong, MT and Shah, NP. 2006. Sorbitol, maltodextrin, inulin and Bifidobacterium infantis modify serum lipid profiles, intestinal microbial population and organic acids concentration in rats.* ***Int J Probiotics Prebiotics.***

Bifidobacterium is a probiotic organism that are generally regarded as safe (GRAS) for consumption. However, reports of isolation of probiotic organisms from patients with bacterial enterocarditis and isolation of *B. adolescentis* from patients with blood stream infections (Gasser, 1994) have raised the issue of safety and the risk of bacterial translocation. In a healthy host, bacterial translocation is a highly regulated event which occurs continuously at a low rate. Indigenous bacteria from the gastrointestinal tract translocate via the bloodstream to the mesenteric lymph nodes and other extraintestinal organs when the integrity of the intestinal barrier is altered (Paven *et al.*, 2003). Although the pH of the stomach may reach as low as 1.5, the lower intestine has a pH of near neutral. High concentrations of organic acids, arising from rapid fermentation of prebiotics by probiotics that inhibit the colonization of acid-sensitive pathogens, could also induce injury to the intestinal mucosa and hence impair its barrier function (Argenzio and Meuten, 1991; Remesy *et al.*, 1993). Thus, it is of utmost importance to ensure that the synbiotics meant for consumption are potentially safe from translocation, and do not impose threats on the mucosal barrier, otherwise their benefits will be outweighed by their harmful effects.

6.3.2 MATERIALS AND METHODS

6.3.2.1 Source of probiotic culture and prebiotics

Bifidobacterium infantis ATCC 17930 in freeze-dried form was used in this study. Working culture were prepared as described in Chapter 5.0, section 5.1.2.1. Three commercially available prebiotics were used including sorbitol (Sorbogem; SPI Polyol Inc., New Castle, USA), maltodextrin (Maltrin M100; Grain Processing Corp., Muscatine, U.S.A.), and inulin (Orafti Pty. Ltd., Tienen, Belgium). The maltodextrin used had a dextrose equivalent (DE) of 9 to 12 with an average degree of polymerization (DP) of 11 while the inulin had an average DP of 10.

6.3.2.2 Animals and diets

Male Wistar rats were used in this study. The handling and housing conditions of the animals are described in section 6.1.2.2. The allocation of diets is shown in Table 6.16.

6.3.2.3 Sampling and analytical procedures

Samples were collected and analysed as described in section 6.1.2.3.

6.3.2.4 Experimental design and statistical analysis

Data were analysed as described in section 6.1.2.4.

6.3.3 RESULTS

6.3.3.1 Weight and feed intake

All the rats were in general healthy throughout the feeding trial period. The body weight gain, feed intake and feed efficiency of rats on the control, BS, BM, BI and BSMI diets are shown in Table 6.17. Rats on the BS, BM and BSMI diets did not show a significant difference ($P < 0.05$) in the feed efficiency compared to the control ($P < 0.05$), except for rats supplemented with the BI diet.

6.3.3.2 pH and moisture content of faecal, cecal and colon contents

pH values increased across the intestine for rats supplemented with the control, BS, BM and BSMI diets, while they remained similar for rats on the BI diet (Table 6.18). The BS diet contributed to a higher pH level in all intestinal segments studied compared to the control diet, while rats on the BM and BI diets showed lower pH value in the faecal samples compared to those on the control.

The moisture content decreased from the cecal to faecal samples of rats on the control diet (Table 6.19), while those on the BS and BI diets showed such a decrease only after the colon. The BS and BI diets also contributed to higher moisture content in the colonic and faecal contents than the control, while rats on the BSMI diet showed higher moisture level in the faecal samples only.

6.3.3.3 Lipid profiles

Rats on the BM and BI diets lowered total serum cholesterol level by 47.6% and 53.6%, respectively compared to those on the control diet (Figure 6.3), while those on the BS, BI and BSMI diets did not show a positive effect. Only the BM diet showed a significantly lower ($P < 0.05$) level of serum triglycerides (70.0%) than the control, while other diets did not show such effect. The synbiotic diets did not increase the beneficial HDL cholesterol level. On the contrary, rats on the BM diet showed a lower (28.1%) LDL cholesterol level compared to the control.

6.3.3.4 Microbial populations

Total aerobe counts decreased from cecal to faecal contents in all treatment groups and the control (Table 6.20), except rats on the BI and BSMI diets, which did not show any decrease in the aerobic population. Rats on all synbiotics showed a significantly lower ($P < 0.05$) population of aerobes in the colon than the control. However, the BI and BSMI diet caused a higher count of aerobes in the faecal samples than the control. The population of staphylococci remained constant in all intestinal contents for all treatment groups as well as the control. However, rats on the BI diet had a higher count of staphylococci across the intestine compared to the control. Similar to the control, the BS and BM diets showed reducing population of *E. coli* after the colon, while the BI and BSMI diets contributed to a constant count across the intestine. Despite this, the BSMI diet contributed to a lower count of *E. coli* in the cecum, colon and faeces compared to the control. The population of coliforms remained similar from the cecal to faecal contents with the BI diet, while the control and synbiotic diets showed reduced count of coliforms after the colon. The BS, BM and BI diets also showed lower count of coliforms in the cecal and colon contents than the control.

The population of total anaerobe remained similar in all intestinal contents for the control group and those on the BS, BI and BSMI diets, while the BM diet contributed to a reduced count after the colon (Table 6.21). Diets BS and BM also contributed to a lower count of total anaerobes in all the intestinal contents compared to the control. While rats on the BM, BI and BSMI diets showed a decreasing count of clostridia after the colon, those on the BS diet exhibited a decrease in the population of clostridia after the cecum. Only rats on the BSMI diet showed a lower count of clostridia in all intestinal contents compared to the control. The synbiotic diets did not have much effect on the population of bacteroides. Only rats on the BS and BM diets showed lower count of bacteroides in the cecal and colonic contents. The control group showed decreased population of *Bifidobacterium* after the colon while all other synbiotic diets had a constant count of *Bifidobacterium* in all intestinal contents studied. In addition, all synbiotic diets also showed a higher population of *Bifidobacterium* in the cecum, colon and faeces.

6.3.3.5 Concentration of organic acids

The concentrations of acetic acid decreased throughout the intestinal contents for the control as well as the synbiotic groups (Table 6.22). Diets BS, BM and BI contributed to a higher concentration of acetic acid in all intestinal contents than the control. The concentration of butyric acid was found to be higher in cecum, colon and faeces of rats on

the BS, BM and BSMI diets. The concentration of formic acid decreased after the cecum in the control group while it only decreased after the colon in rats on the BS, BM and BI diets. Rats on the BS and BM diets showed a higher concentration of propionic acid in the cecal, colon and faeces. It is interesting to note that all synbiotic diets contributed to a higher ratio of acetic:lactic acid than the control, where higher concentration of lactic acid than acetic acid was detected in all intestinal contents studied.

6.3.3.5 Translocation of *Bifidobacterium*

Samples from spleen, liver and kidney of each rat on the control, BS, BM, BI and BSMI diets were plated for the presence of total *Bifidobacterium*. No growth of *Bifidobacterium* was detected in any organs indicating the absence of translocation.

6.3.4 DISCUSSION

The experiments were conducted to investigate the combined effect of *B. infantis* ATCC 17930 and sorbitol, maltodextrin and/or inulin on serum lipid profiles, intestinal microflora and intestinal concentration of organic acids in rats. Translocation of *Bifidobacterium* to other intestinal organs was also examined. The feed intake between treatment groups and the control was not significantly different, except for those on diet BI. It appeared that the higher feed intake and lower weight gain contributed to the varied feed efficiency. Only rodent chow and synbiotics varied between control and the BI group. Considering that the fat and fibre content of rodent chow was low (< 5%), it should have minimal effect on the outcome of this study.

Rats on BS and BI diets had higher moisture content in samples collected after the cecum, indicating a stronger laxative effect, which was also reflected in the moist faecal samples compared to those on the control diet (personal observation). We wanted to determine the moisture content across the intestines to evaluate the effect of the prebiotics on lumen osmolality. Due to the nature of resistance towards digestion, osmolality across the intestines would be increased substantially by the presence of these prebiotics. In the present study, it appeared that the BS and BI diets were more resistant to digestion compared to the other synbiotic diets, and reached the colon to exhibit a laxative effect. Inulin is a fructan with linear fructose polymers and oligomers which are each linked by β(2-1) bonds, while the glucose molecule linked by an α(2-1) bond to the end of each fructose chain. Such bonds are unique as these linkages prevent inulin from being digested like any typical carbohydrate, reduced it caloric value and imposed dietary fibre effects (Niness, 1999). The

high number of fructosyl moities in inulin would also explain its resistance to digestion. Polyols such as sorbitol have been included as prebiotics due to their indigestible properties and has been widely used as an alternate to oligosaccharides. The supplementation of 0.8% sorbitol in a high-fat milk replacer diet has exerted prebiotic properties in veal calves (Bauchart *et al*., 1985).

Rats on the BM diet showed significantly ($P < 0.05$) lower total cholesterol, triglycerides and LDL cholesterol level than the control. A possible explanation could be the alteration of lipid metabolism by short-chain fatty acids (SCFA). Propionate was reported to inhibit fatty acid synthesis *in-vitro* (Delzenne and Kok, 2001) and in our study, rats that were on BM diet had a significantly ($P < 0.05$) higher concentration of propionic acid in all intestinal segments. Although a decrease in serum total cholesterol levels has been associated with a decrease in concentration of acetic acid which is a lipogenic substrate (Delzenne and Kok, 2001), our study did not indicate such an association. Results from our study also showed that all synbiotic diets studied had no effect on the level of HDL cholesterol, although fructooligosaccharides, the shorter polymer of inulin have been reported to increase the serum HDL level (Gibson and Roberfroid, 1995).

Results here also showed that while the BM and BI diet exhibited hypocholesterolemic effect, the BS diets did not, indicating that substrate preference may have played a role. Brown *et al.* (1999) found that soluble prebiotics was associated with a small decrease in total and LDL cholesterol, but exerted no effects on triacylglycerols and HDL cholesterol. Increasing soluble prebiotic intakes were found to contribute only a minor degree in dietary therapy to lower serum cholesterol. These may explain the insignificant hypocholesterolemic effect of the BS diet in rats, although our previous experiment showed that sorbitol significantly affected cholesterol removal *in-vitro* (Chapter 5.0, section 5.5.3.3). Wang and Gibson (1993) found that most species of bifidobacteria are capable of utilizing inulin in pure cultures, but needed an elaborated hydrolase in a mix cultures experiment. Such a competitive β-fructosidase enzyme was needed to catalyse a cleavage of the glycosidic linkages in inulin so that fructose is released for metabolism via the "bifidus" pathway (Imamura *et al.*, 1994). It was previously found that bifidobacteria has this advantage over other intestinal microflora due to its outer membrane that locates the related site of the enzyme activity (Gibson *et al*., 2000). This may explain the hypocholesterolemic effect of the BI diet. However, the inability of the BI diet to reduce LDL cholesterol and triglycerides levels remained unknown. The prebiotic properties of maltodextrin was contributed by its predominant α(1-4) glucosic linkages that escape digestion by the luminal enzymes, and its higher molecular mass components (DP 11) that made it less susceptible to

membrane digestion by disaccharidases on the epithelial surface of the small intestine (Olano-Martin *et al*., 2002). Bouhnik *et al.* (2004) also reported that maltodextrin were bifidogenic at a dose of 10g/day when consumed for just 8 days in humans. These may support the fermentability of the BM diet by *B. infantis* ATCC 17930 in the intestine and subsequently its serum hypocholesterolemic effects.

Rats on the BS, BM and BI diets showed suppressed growth of *E. coli*, total aerobes and bacteroides especially in the cecum and colon. Most inhibition of pathogenic bacteria was reported to be due to a decrease in intestinal pH (Swanson *et al*., 2002a). However, our study did not indicate such an association due to no changes in pH across the intestine. Our results indicated that the increased concentration of acetic acid and *Bifidobacterium* might have inhibited the growth of these microorganisms. It was previously reported that an increase in *Bifidobacterium* was often accompanied by a decrease in concentration of pathogenic bacteria (Gibson *et al*., 1995). *Bifidobacterium* may have suppressed growth of enteropathogenic *E. coli* via competitive inhibition, antagonistic activity, and/or inhibiting them from binding to the intestinal cells. The suppressions of these pathogenic microflora are beneficial considering that *E. coli* has been reported to cause diarrhoea and haemolytic-uremic syndrome (Friedrich *et al.*, 2003). Anaerobes have been associated with intestinal problems such as bloating, while bacteroides are reported to cause ulcerative colitis (Bamba *et al.,* 1995). It must also be noted that rats on the BI diet showed higher count of staphylococci across the intestine. This is supported by a previous study which reported better utilization of chicory oligofructose than glucose by staphylococci (Roberfroid *et al.*, 1998). Considering that the inulin used was a chicory polymer of oligofructose, its presence may have contributed to such an increase.

Bifidobacterium growing on glucose produces three moles of acetic acid and two moles of lactic acid, with a ratio of acetate: lactate of 1: 0.67. Deviations from the theoretical value have also been reported previously, mediated by the addition of prebiotics such as hi-maize and raftilose in the media (Bruno *et al.,* 2002). Such ratio was not exhibited in our current study possibly contributed by two main factors including the addition of prebiotics into the diets (Makras *et al.,* 2005) and the production of other organic acids by the indigenous gut microbial population including lactic-acid producers and acetate-converting bacteria (Bourriaud *et al.*, 2005). Although the concentration of lactic acid was higher than that of acetic acid, it is important to note that rats on all synbiotic diets exhibited higher concentration of acetic acid across the intestine, accompanied by a higher ratio of acetic: lactic acid than the control. The higher population of *Bifidobacterium* in cecal, colon and faecal contents of rats on the synbiotic diets than the control diet also suggested that the

prebiotics were beneficially used as substrates for growth. Our previous *in-vitro* study (Chapter 5.0, section 5.5.3.4) found that carbon sources from sorbitol, maltodextrin and inulin were responsible in modulating growth rate of *B. infantis* ATCC 17930, and thus may have exerted similar effects in the present *in-vivo* trial.

Results from this study also showed that a higher concentration of butyric acid was detected across the intestine from rats on the LS, LM and LSMI diets than the control. Our previous study (Chapter 5.0, section 5.5.3.4) showed that *B. infantis* ATCC 17930 did not produce butyrate. This led us to believe that the supplementation of sorbitol and maltodextrin may have accelerated the production of butyrate by other intestinal microflora. It has been reported that butyrate is the preferred energy source for colonic epithelial cells (Delzenne and Roberfroid, 1994) and has been found to stimulate *in-vitro* apoptosis in colonic cells (Hague *et al*., 1995). Considering this, the BS, BM and BSMI diets could beneficially reduce the risk of colon cancer which is often associated with decreased butyrate absorption in the bowel (Topping and Clifton, 2001).

Diets containing *Bifidobacterium* did not exhibit harmful translocation to organs such as spleen, liver and kidney. Results from this study suggested that the *Bifidobacterium* used was safe for consumption.

The combination of *B. infantis* ATCC 17930 and maltodextrin (BM) beneficially altered serum lipoprotein profiles, possibly contributed by increased concentration of propionic acid, while the other synbiotics had no major hypocholesterolemic effect. All synbiotic diets contributed to a higher *Bifidobacterium* count across the intestine than the control, accompanied by higher concentration of acetic acid. These may have contributed to the lower population of *E. coli*, total aerobes and bacteroides in rats fed *B. infantis* ATCC 17930 with individual prebiotics (BS, BM and BI) in the cecum and colon. The combination of *B. infantis* ATCC 17930 with all three prebiotics (BSMI) did not exhibit any specific benefits that could outweigh the benefits when single prebiotics were used. This study showed that the combination of *B. infantis* ATCC 17930 and maltodextrin was best for pathological cholesterol levels and healthier bowel microbial population without exhibiting harmful *Bifidobacterium* translocation.

Table 6.16. Composition of control, BS, BM, BI and BSMI diets.

Ingredients (g/kg)	Diets[1]				
	Control	BS	BM	BI	BSMI
SF00-245[2]	750.0	750.0	750.0	750.0	750.0
Copha[3]	50.0	50.0	50.0	50.0	50.0
B. infantis ATCC 17930	-	18.7	18.7	18.7	18.7
Sorbitol[4]	-	37.0	-	-	37.0
Maltodextrin[4]	-	-	23.1	-	23.1
Inulin[4]		-	-	38.4	38.4
Rodent chow[5]	200.0	144.3	158.2	142.9	82.8

[1]Diets include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.

[2]High-cholesterol diet (Specialty Feeds, Glen Forrest, Australia).

[3]Copha® (Unilever Australasia, Epping, Australia), hardened coconut oil 99%, soya bean lecithin 1%.

[4]Sorbitol (Sorbogem; SPI Polyol Inc., New Castle, USA); Maltodextrin (Maltrin M100; Grain Processing Corp., Muscatine, U.S.A.), dextrose equivalent (DE) of 9 to 12, average DP of 11; Inulin (Raftiline ST; Orafti Pty. Ltd., Tienen, Belgium), purity of 92%, average DP of 10.

[5]Rodent chow (Specialty Feeds, Glen Forrest, Australia), total protein 19.6%, total fat 4.6%, crude fibre 4.8%, trace mineral 2.9%, total vitamins 0.3%.

Table 6.17. Body weight gain, feed intake and feed efficiency of rats on control, BS, BM, BI and BSMI diets.

Component	Treatments[1,2]				
	Control	BS	BM	BI	BSMI
Body weight gain (g/rat/week)	34.0 ± 2.5^{a}	31.9 ± 4.2^{a}	32.2 ± 2.0^{a}	31.1 ± 3.6^{a}	35.3 ± 4.3^{a}
Feed intake (g/week)	140.5 ± 4.0^{a}	161.9 ± 1.6^{ab}	175.8 ± 9.8^{ab}	173.9 ± 8.8^{ab}	189.9 ± 6.0^{ab}
Ratio body weight: feed intake	0.24 ± 0.01^{a}	0.20 ± 0.02^{ab}	0.18 ± 0.00^{ab}	0.18 ± 0.00^{b}	0.19 ± 0.01^{ab}

[1]Treatments include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.

[2]Results are expressed as mean ± standard error of means. [ab]Means in the same row followed by different lowercase letters are significantly different ($P < 0.05$).

Table 6.18. pH values of contents obtained from the cecum, colon and faeces of rats on control, diets BS, BM, BI and BSMI.

pH	Treatments[1,2]				
	Control	BS	BM	BI	BSMI
Cecum	$6.4 \pm 0.1^{b,BC}$	$7.1 \pm 0.0^{b,A}$	$6.7 \pm 0.1^{b,AB}$	$6.8 \pm 0.0^{a,AB}$	$6.0 \pm 0.1^{b,C}$
Colon	$6.8 \pm 0.0^{b,BC}$	$8.2 \pm 0.1^{a,A}$	$6.9 \pm 0.1^{ab,B}$	$6.5 \pm 0.1^{a,BC}$	$6.3 \pm 0.1^{b,C}$
Faeces	$7.8 \pm 0.1^{a,B}$	$8.8 \pm 0.1^{a,A}$	$7.3 \pm 0.2^{a,C}$	$6.8 \pm 0.1^{a,C}$	$7.4 \pm 0.2^{a,BC}$

[1]Treatments include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.

[2]Results are expressed as mean ± standard error of means. [ab]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [ABC]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.19. Moisture content of cecal, colon and faecal contents of rats on diet control, BS, BM, BI and BSMI.

Moisture (%)	Treatments[1,2]				
	Control	BS	BM	BI	BSMI
Cecum	77.57 ± 1.63^{a,A}	85.12 ± 6.55^{a,A}	80.30 ± 3.03^{a,A}	83.72 ± 2.64^{a,A}	76.38 ± 7.41^{a,A}
Colon	58.05 ± 5.04^{b,B}	78.64 ± 8.17^{a,A}	69.12 ± 9.50^{b,A}	77.33 ± 5.34^{ab,A}	54.44 ± 4.02^{b,B}
Faeces	18.43 ± 1.52^{c,C}	68.27 ± 2.46^{b,A}	19.25 ± 10.16^{c,C}	72.22 ± 5.56^{b,A}	36.29 ± 5.38^{c,B}

[1]Treatments include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.

[2]Results are expressed as mean ± standard error of means. ^{abc}Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); ^{ABC}Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.20. Population of total aerobes, staphylococci, *Escherichia coli*, and total coliforms from cecal, colon and faecal contents of rats on diets control, BS, BM, BI and BSMI.

Log_{10}CFU/g dry mass	**Treatments**[1,2]				
	Control	**BS**	**BM**	**BI**	**BSMI**
Total aerobes					
Cecum	$9.393 \pm 0.047^{a,A}$	$8.938 \pm 0.149^{a,A}$	$7.890 \pm 0.069^{a,A}$	$8.915 \pm 0.103^{a,A}$	$8.993 \pm 0.081^{a,A}$
Colon	$9.413 \pm 0.032^{a,A}$	$8.607 \pm 0.129^{ab,B}$	$7.734 \pm 0.036^{ab,C}$	$8.681 \pm 0.038^{a,B}$	$8.756 \pm 0.034^{a,B}$
Faeces	$7.672 \pm 0.06^{b,BC}$	$8.127 \pm 0.035^{b,B}$	$7.276 \pm 0.055^{b,C}$	$8.743 \pm 0.027^{a,A}$	$9.008 \pm 0.097^{a,A}$
Staphylococci					
Cecum	$7.502 \pm 0.031^{a,C}$	$7.531 \pm 0.065^{a,C}$	$7.262 \pm 0.106^{a,C}$	$8.822 \pm 0.046^{a,A}$	$8.145 \pm 0.059^{a,B}$
Colon	$7.580 \pm 0.037^{a,BC}$	$7.441 \pm 0.013^{a,C}$	$7.315 \pm 0.146^{a,C}$	$8.694 \pm 0.089^{a,A}$	$7.971 \pm 0.079^{a,B}$
Faeces	$7.280 \pm 0.027^{a,B}$	$7.445 \pm 0.036^{a,B}$	$7.308 \pm 0.059^{a,B}$	$8.611 \pm 0.085^{a,A}$	$8.441 \pm 0.030^{a,A}$
Escherichia coli					
Cecum	$9.010 \pm 0.047^{a,A}$	$8.666 \pm 0.048^{a,AB}$	$8.458 \pm 0.075^{a,B}$	$8.728 \pm 0.090^{a,A}$	$8.212 \pm 0.118^{a,B}$
Colon	$9.027 \pm 0.062^{a,A}$	$8.542 \pm 0.101^{a,AB}$	$8.247 \pm 0.103^{a,B}$	$8.571 \pm 0.117^{a,AB}$	$8.174 \pm 0.117^{a,B}$
Faeces	$7.415 \pm 0.062^{b,BC}$	$7.846 \pm 0.089^{b,AB}$	$7.022 \pm 0.073^{b,CD}$	$8.264 \pm 0.086^{a,A}$	$6.767 \pm 0.029^{a,D}$
Coliforms					
Cecum	$9.057 \pm 0.113^{a,A}$	$8.381 \pm 0.034^{a,B}$	$8.351 \pm 0.053^{a,B}$	$8.207 \pm 0.070^{a,B}$	$8.581 \pm 0.108^{a,AB}$
Colon	$9.050 \pm 0.108^{a,A}$	$8.408 \pm 0.100^{a,B}$	$8.226 \pm 0.143^{a,B}$	$8.439 \pm 0.009^{a,B}$	$8.214 \pm 0.024^{a,B}$
Faeces	$7.431 \pm 0.033^{b,BC}$	$7.632 \pm 0.080^{b,B}$	$6.968 \pm 0.089^{b,C}$	$8.211 \pm 0.055^{a,A}$	$7.099 \pm 0.045^{b,C}$

[1]Treatments include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.

[2]Results are expressed as mean ± standard error of means. [ab]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [ABCD]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.21. Population of total anaerobes, clostridia, bacteroides and bifidobacteria from cecal, colon and faecal contents of rats on diets control, BS, BM, BI and BSMI.

Log_{10}CFU/g dry mass	Treatments[1,2]				
	Control	**BS**	**BM**	**BI**	**BSMI**
Total anaerobes					
Cecum	$9.102 \pm 0.074^{a,A}$	$7.795 \pm 0.097^{a,C}$	$7.809 \pm 0.030^{a,C}$	$8.533 \pm 0.013^{a,B}$	$8.723 \pm 0.064^{a,AB}$
Colon	$8.867 \pm 0.027^{a,A}$	$7.314 \pm 0.177^{a,B}$	$7.628 \pm 0.054^{a,B}$	$8.593 \pm 0.035^{a,A}$	$8.379 \pm 0.035^{a,A}$
Faeces	$8.725 \pm 0.050^{a,A}$	$7.557 \pm 0.137^{a,B}$	$7.220 \pm 0.057^{b,B}$	$8.767 \pm 0.061^{a,A}$	$8.465 \pm 0.103^{a,A}$
Clostridia					
Cecum	$5.101 \pm 0.115^{a,B}$	$5.999 \pm 0.071^{a,A}$	$5.072 \pm 0.281^{a,B}$	$5.243 \pm 0.043^{a,B}$	$3.870 \pm 0.075^{a,C}$
Colon	$4.540 \pm 0.022^{b,B}$	$4.901 \pm 0.142^{b,AB}$	$4.979 \pm 0.170^{a,AB}$	$5.234 \pm 0.161^{a,A}$	$3.728 \pm 0.166^{a,C}$
Faeces	$3.902 \pm 0.017^{c,C}$	$5.180 \pm 0.061^{b,A}$	$4.274 \pm 0.034^{b,BC}$	$4.596 \pm 0.228^{b,B}$	$3.113 \pm 0.073^{b,D}$
Bacteroides					
Cecum	$8.828 \pm 0.222^{a,A}$	$7.606 \pm 0.174^{a,B}$	$7.518 \pm 0.083^{a,B}$	$8.705 \pm 0.058^{a,A}$	$8.627 \pm 0.040^{a,A}$
Colon	$8.307 \pm 0.104^{a,A}$	$7.538 \pm 0.066^{a,B}$	$7.407 \pm 0.019^{a,B}$	$8.688 \pm 0.076^{a,A}$	$8.508 \pm 0.044^{a,A}$
Faeces	$7.333 \pm 0.047^{b,B}$	$7.227 \pm 0.087^{a,B}$	$7.548 \pm 0.068^{a,B}$	$8.667 \pm 0.040^{a,A}$	$8.844 \pm 0.035^{a,A}$
Bifidobacteria					
Cecum	$7.324 \pm 0.070^{a,B}$	$8.271 \pm 0.039^{a,A}$	$7.692 \pm 0.063^{a,AB}$	$8.529 \pm 0.059^{a,A}$	$8.289 \pm 0.071^{a,A}$
Colon	$7.074 \pm 0.067^{a,C}$	$8.364 \pm 0.229^{a,A}$	$7.582 \pm 0.028^{a,B}$	$8.442 \pm 0.049^{a,A}$	$8.174 \pm 0.021^{a,A}$
Faeces	$6.531 \pm 0.012^{b,D}$	$7.968 \pm 0.055^{a,B}$	$7.241 \pm 0.084^{a,C}$	$8.757 \pm 0.049^{a,A}$	$8.393 \pm 0.080^{a,AB}$

[1]Treatments include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.

[2]Results are expressed as mean ± standard error of means. [abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [ABCD]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

Table 6.22. Concentrations of organic acids from cecal, colon and faecal contents of rats on diets control, BS, BM, BI and BSMI.

mM/g dry mass	Treatments[1,2]				
	Control	**BS**	**BM**	**BI**	**BSMI**
Acetic acid					
Cecum	$14.40 \pm 0.94^{a,D}$	$31.89 \pm 1.13^{a,B}$	$21.23 \pm 0.30^{a,C}$	$39.43 \pm 2.12^{a,A}$	$25.32 \pm 2.17^{a,C}$
Colon	$5.66 \pm 0.06^{b,D}$	$27.54 \pm 2.72^{b,AB}$	$12.40 \pm 0.44^{b,C}$	$24.59 \pm 1.77^{b,B}$	$32.70 \pm 0.09^{b,A}$
Faeces	$2.25 \pm 0.49^{c,C}$	$5.11 \pm 0.08^{c,B}$	$7.19 \pm 0.29^{c,A}$	$9.03 \pm 0.04^{c,A}$	$4.57 \pm 0.37^{c,BC}$
Butyric acid					
Cecum	$19.51 \pm 1.93^{a,D}$	$53.73 \pm 1.70^{a,B}$	$30.86 \pm 0.44^{a,C}$	$19.96 \pm 5.04^{a,D}$	$65.81 \pm 0.67^{a,A}$
Colon	$16.16 \pm 3.41^{a,C}$	$41.27 \pm 1.48^{b,B}$	$35.49 \pm 2.75^{a,B}$	$6.27 \pm 0.18^{b,D}$	$50.99 \pm 3.62^{a,A}$
Faeces	$6.01 \pm 2.37^{b,B}$	$12.92 \pm 2.98^{c,A}$	$15.74 \pm 0.22^{b,A}$	$7.80 \pm 0.33^{b,B}$	$16.36 \pm 1.03^{b,A}$
Formic acid					
Cecum	$36.42 \pm 0.95^{a,A}$	$21.39 \pm 1.41^{a,B}$	$17.79 \pm 1.52^{a,B}$	$24.19 \pm 0.63^{a,B}$	$40.05 \pm 0.80^{a,A}$
Colon	$24.78 \pm 1.71^{b,AB}$	$12.03 \pm 1.62^{a,C}$	$16.22 \pm 0.81^{a,BC}$	$32.72 \pm 3.51^{a,A}$	$23.72 \pm 0.31^{b,AB}$
Faeces	$14.52 \pm 0.03^{c,AB}$	$4.61 \pm 0.72^{b,BC}$	$3.05 \pm 0.38^{b,C}$	$6.35 \pm 0.13^{b,BC}$	$17.74 \pm 0.51^{b,A}$
Propionic acid					
Cecum	$26.80 \pm 3.06^{a,D}$	$84.10 \pm 4.79^{a,A}$	$72.53 \pm 2.87^{a,B}$	$27.85 \pm 1.74^{a,D}$	$61.72 \pm 0.38^{a,C}$
Colon	$13.36 \pm 2.00^{b,C}$	$46.15 \pm 0.11^{b,A}$	$31.74 \pm 0.16^{b,B}$	$12.82 \pm 2.25^{b,C}$	$17.34 \pm 0.22^{b,C}$
Faeces	$3.83 \pm 1.10^{c,C}$	$39.16 \pm 0.18^{b,A}$	$15.16 \pm 1.08^{c,B}$	$16.70 \pm 2.31^{b,B}$	$12.17 \pm 1.99^{b,BC}$
Lactic acid					
Cecum	$130.00 \pm 1.41^{a,BC}$	$113.16 \pm 3.16^{a,C}$	$128.25 \pm 9.47^{a,BC}$	$138.19 \pm 4.14^{a,AB}$	$155.74 \pm 0.33^{a,A}$
Colon	$74.13 \pm 1.31^{b,AB}$	$46.55 \pm 2.12^{b,C}$	$95.40 \pm 12.16^{b,A}$	$56.27 \pm 4.05^{b,BC}$	$26.54 \pm 0.89^{b,D}$
Faeces	$32.78 \pm 0.49^{c,A}$	$3.70 \pm 0.12^{c,C}$	$15.08 \pm 1.90^{c,B}$	$20.43 \pm 1.61^{c,B}$	$28.56 \pm 0.51^{b,A}$
Ratio acetic: lactic acid					
Cecum	1: 9.03	1: 3.55	1: 6.04	1: 3.50	1: 6.15
Colon	1: 13.10	1: 1.69	1: 7.70	1: 2.29	1: 0.81
Faeces	1: 14.59	1: 0.72	1: 2.10	1: 2.26	1: 6.25

[1]Treatments include: Control, contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI, contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI, contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin.
[2]Results are expressed as mean ± standard error of means. [abc]Means in the same column followed by different lowercase letters are significantly different ($P < 0.05$); [ABCD]Means in the same row followed by different uppercase letters are significantly different ($P < 0.05$).

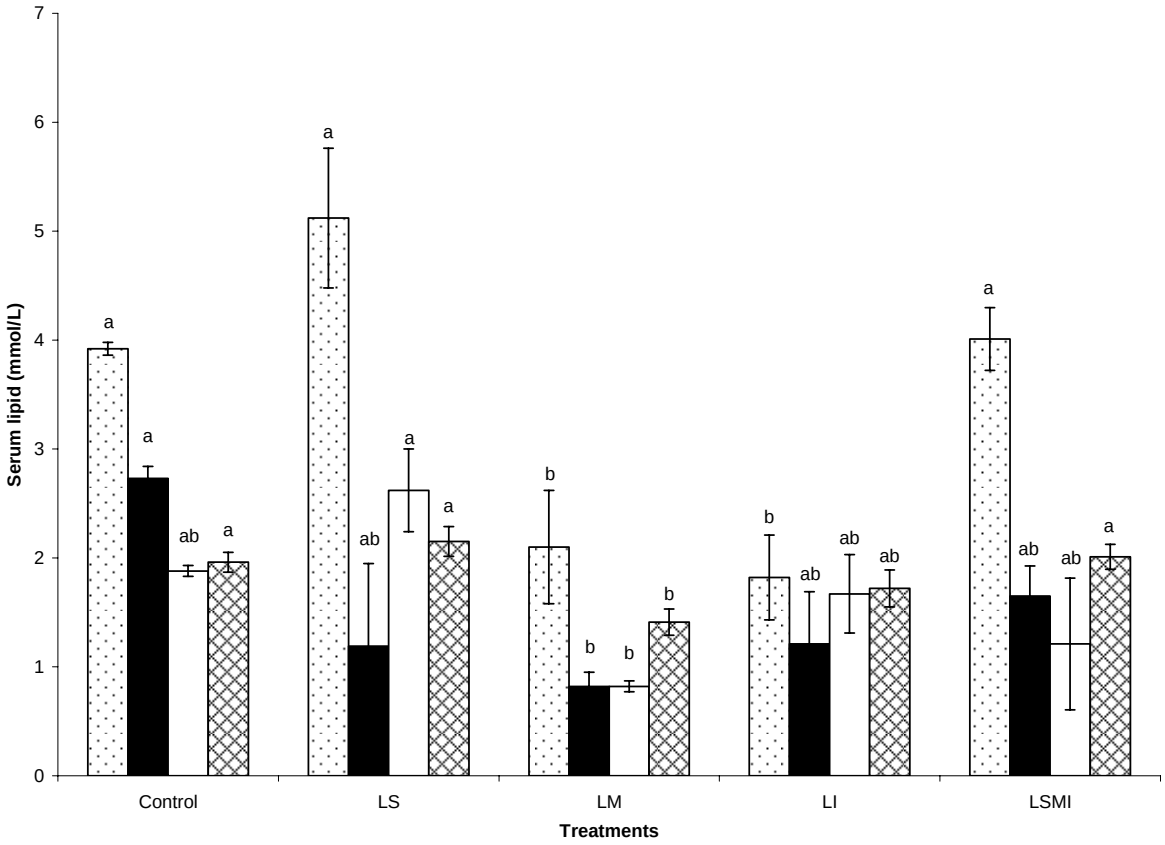


FIG. 6.3. Total cholesterol (⊡), triglycerides (■), HDL-cholesterol (□) and LDL-cholesterol (⊠) level of rats on with diets control, BS, BM, BI and BSMI diets. Control contained no *B. infantis* ATCC 17930, sorbitol, maltodextrin or inulin; BS contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.70% (w/w) sorbitol; BM contained 1.87% (w/w) *B. infantis* ATCC 17930 and 2.31% (w/w) maltodextrin; BI contained 1.87% (w/w) *B. infantis* ATCC 17930 and 3.84% (w/w) inulin; BSMI contained 1.87% (w/w) *B. infantis* ATCC 17930, 3.70% (w/w) sorbitol, 2.31% (w/w) maltodextrin and 3.84% (w/w) inulin. Results are expressed as mean ± standard error of means. [ab]Means within the same series followed by different lowercase letters are significantly different ($P < 0.05$).

7.0 Effects of a synbiotic containing *Lactobacillus acidophilus* ATCC 4962, FOS, mannitol and inulin on serum lipid profiles and morphology of red blood cells in hypercholesterolemic pigs on high- and low-fat diets

7.1 INTRODUCTION

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Reid *et al.*, 2003). Prebiotics are a more recent concept and are defined as chemical substances that act as substrates specifically for the host's intrinsic probiotic bacteria, and thus encourage their growth (Hamilton-Miller, 2004). A combination of probiotic and prebiotic is termed synbiotic, which has been used to improve various aspects of human health (Flickinger *et al*., 2000; Gibson and Roberfroid, 1995).

High-fat diets have been associated with hypercholesterolemia as indicated by higher than normal serum total cholesterol (Kenney, 2002). Studies have shown that one mmol higher than normal cholesterol level could increase the risk of coronary heart disease by 35 percent and the risk of coronary death by 45 percent, and a small reduction in the serum cholesterol of one per cent could reduce the risk of coronary heart disease by two to three percent (Chyou and Eaker, 2000). Numerous dietary approaches have been used to improve hypercholesterolemia including the usage of synbiotics. Although numerous studies have shown promising results in reducing triacylglycerol, total- and LDL cholesterol levels after consumption of probiotic and/or prebiotics (Jin *et al.*, 1998), their effects on serum lipids have been controversial with numerous studies indicating insignificant results (Lin *et al.*, 1989). Also, most of these studies involved continuous feeding of high cholesterol diets containing high concentrations of probiotics and/or prebiotics. In recent years, consumers are becoming nutritionally conscious, and adopt the diets containing monounsaturated- and polyunsaturated fatty acids, and minimize intake of total fat. Very little information is available on the cholesterol lowering effect of synbiotic in hypercholesterolemic subjects after cessation of high-fat diets.

A basic understanding of the metabolism of the plasma lipoproteins, and the major lipids that they transport is of primary importance. Because the metabolism of the plasma lipoproteins is highly interrelated, it is important to consider each of the lipoproteins and their properties instead of focusing only on the main lipid classes to better understand the complete lipid profile (Kwiterovich Jr., 2000).

This section has been submitted for publication: *Liong, MT, Dunshea, FR and Shah, NP. 2006. Effect of a synbiotic containing Lactobacillus acidophilus ATCC 4962, FOS, mannitol and inulin on serum lipid profiles and morphology of red blood cells in hypercholesterolemic pigs on high- and low-fat diets.* ***Am J Clin Nutr*** *(In review).*

When rabbits, guinea pigs and dogs were fed high cholesterol diets, morphological defects of the red blood cell (RBC) including the formation of spur cells and increased membrane rigidity were observed (Cooper *et al*., 1980). Any changes in the RBC membrane would involve modifications on the lipid order due to the fact that the RBC membrane lipid is an amphiphilic bilayer containing protein and cholesterol. Fluorescence probes are used to evaluate modifications in the membrane packing order via incorporation into different sites in the bilayer, namely the apolar regions, interface between the polar head and apolar tail and in the polar surface (Barshtein *et al*., 1997).

We have previously screened and developed a synbiotic product consisting of *L. acidophilus* ATCC 4962, mannitol, fructooligosaccharides (FOS) and inulin that specifically targeted removal of cholesterol in laboratory media containing cholesterol (Chapter 5.0, section 5.3). However, the effect of such synbiotic on serum lipid profiles and RBC in *in-vivo* models has not been studied. Thus, the aim of this study was to evaluate the effect of such a synbiotic on serum lipoprotein and their compositions in pigs given a high- and a low-fat diet. In addition, the effect of the synbiotic on the morphology of RBC was also investigated.

7.2 MATERIALS AND METHODS

7.2.1 Source of probiotic culture and prebiotics

L. acidophilus ATCC 4962 is a human derived strain that was obtained from the Australia Starter Culture Research Centre (ASRC; Werribee, Australia). Three commercially available prebiotics were used including mannitol (Mannogem; SPI Polyols Inc., New Castle, USA), FOS (Raftilose P95; Orafti Pty. Ltd., Tienen, Belgium) and inulin (Raftilene ST; Orafti). All working cultures, production of freeze-dried cultures and the properties of the prebiotics used in this study have been described in Chapter 5.0, section 5.1.2.1.

7.2.2 Animals and diets

Twenty four crossbred (Large White x Landrace) pigs (initial weights 33 ± 8 kg) were used. The study was approved by the Animal Ethics Committee of the Department of Primary Industry (Werribee, Australia). Pigs were housed in individual pens in a randomized block design according to their initial live weight and four treatments. The animals were kept on a basal diet containing 15% fat for two weeks to induce hypercholesterolemia. After this period, pigs were given either a high-fat (15% fat) or a low-fat (5%) diet for 8 weeks. The pigs on the synbiotic diet were supplemented with *L. acidophilus* ATCC 4962 (1.00

g/pig/day), FOS (1.25g/pig/day), mannitol (1.56 g/pig/day) and inulin (2.20 g/pig/day), while pigs on the control were not supplemented with synbiotic. The composition of the synbiotic was based on our previous *in vitro* optimization study where inoculum size of *L. acidophilus* ATCC 4962, and concentrations of mannitol, FOS and inulin were the significant factors for optimum removal of cholesterol (Chapter 5.0, section 5.3). The compositions of the high- and low-fat diets are shown in **Table 7.1**. Pigs were kept in a room with controlled temperature (20-22°C) and humidity (50-55%) and maintained in a cycle of light for 12 hours (6:00 to 18:00) and dark for 12 hours (18:00 to 6:00). The body weight of each pig was recorded weekly and the amount of the basal diet adjusted individually according to their energy intake per weight; 16.9 MJ/kg and 14.6 MJ/kg for high-fat diet and low-fat diet, respectively. Pigs were fed on a daily basis and allowed to consume water *ad libitum* during the experimental period.

7.2.3 Preparative procedures

Pigs were fasted overnight before blood was collected weekly. Fasting and prefeeding blood samples were obtained by venipuncture. Heparin was used as anticoagulant. Immediately after bleeding, pigs were fed their respective diets and postfeeding blood samples were obtained after three hours of feeding.

The whole blood was centrifuged for 20 min at 2714 x g (Sorvall RT7; Newtown, USA) to separate the plasma from the red blood cell (RBC) pellet. The plasma was recentrifuged for 30 min at 18879 x g (Beckman Coulter, Fullerton, CA, USA) to remove chylomicrons.

A discontinuous density gradient ultracentrifugal procedure was used to fractionate plasma lipoprotein as previously described (Chapman *et al.*, 1981). Chylomicrons-free plasma was adjusted with NaCl-KBr solution (1.346 g/mL) to desired densities of 1.006, 1.063 and 1.21 g/mL (Duhamel *et al.*, 1983). Gradients were centrifuged at 40,000 rpm for 20 hrs at 15°C (Beckman model TI ultracentrifuge with type 70.1 rotor) and subfractionated by successive downwards aspiration using a Pasteur pipette. Very-low density lipoprotein (VLDL) was isolated at density < 1.006 g/mL and the infranet obtained was refloated at density 1.063 g/mL to obtain low density lipoprotein (LDL; density 1.006-1.063 g/mL). Similarly, high density lipoprotein (HDL) was obtained at density 1.063-1.21 g/mL.

For the preparation of RBC ghosts, RBC pellets were washed twice with saline to remove any remaining plasma and buffy coat, followed by lysing in 30 volumes of 5 mM Na_2HPO_4 buffer, pH 8.0 (Dumaswala *et al.*, 1996) and isolating by centrifugation. RBC ghosts were washed with the same buffer until hemoglobin-free white ghosts were obtained.

7.2.4 Analytical procedures

Chylomicrons-free plasma was analysed for total-, high density lipoprotein (HDL)-, and low density lipoprotein (LDL) cholesterol and triacylglycerol using commercial kits (Thermo Electron Corp., Melbourne, Australia). Plasma subfractions of VLDL, LDL and HDL were analysed for triacylglycerol, protein, phospholipids, cholesteryl esters and free cholesterol levels. Triacylglycerol and total cholesterol were analysed using commercial kits (Thermo Electron Corp., Melbourne, Australia). Cholesteryl esters were determined using the Amplex Red Reagent Kit (Molecular Probes, Eugene, OR, USA) and free cholesterol was determined as the difference between total cholesterol and cholesteryl esters (Dhaliwal & Steinbrecher, 2000). The protein content was determined with bovine serum albumin (BSA) as the standard (Lowry *et al.*, 1951). A factor of 0.8 was used to convert BSA protein into lipoprotein protein (Sardet *et al.*, 1972). The total phospholipid content was measured as phosphate as previously described (Daly & Ertingshausen, 1972) and a factor of 25 was used for conversion into serum phospholipids (Cooper *et al.*, 1980).

The RBC count was carried out using a Newbauer hemocytometer. The morphology of RBC was assessed using Wright's stain smears (Cooper *et al.*, 1980). The RBC total cholesterol and phospholipids were determined after lipids extraction with acetone-ethanol (1:1). The total cholesterol and phospholipids were measured as described above.

The RBC membrane lipid order was determined by measuring the fluorescence anisotropy of lipid probes inserted into the RBC ghosts. Three fluorescence probes were used namely 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and 8-anilino-1-napthalenesulfonic acid (ANS). DPH and TMA-DPH were dissolved in tetrahydrofuran to a final concentration of 2 μM while ANS in ethanol to a final concentration of 6 μM (Barshtein *et al.*, 1997). The probe stock solutions were diluted (1:1000) in 0.155 M NaCl (Cooper *et al.*, 1980) with vigorous mixing before incubated with RBC ghosts. Working probe solutions were incubated with RBC ghosts solution (OD: 0.3) at a ratio of 3:1 at temperature of 37°C. The incubation time for DPH and TMA-DPH was 60 mins, while that for ANS was 90 mins. Fluorescence anisotropy was

measured using a luminescence spectrophotometer (LS-50; Perkin Elmer, Wellesley, MA, USA). Excitation wavelength for DPH and TMA-DPH was 365 nm while for ANS was 390nm. Emmision was determined at 445nm for DPH and TMA-DPH, and 490nm for ANS (Barshtein *et al.*, 1997). An unlabelled RBC ghost was used as a blank. Fluorescence anisotropy was calculated according to the equation:

$$A_r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$$

where I_{vv} and I_{vh} are the fluroscence intensities obtained from a vertical polarizer, and a vertical and horizontal analyser, while G is the instrumental grating factor. $G = I_{hv} / I_{vh}$ where I_{hv} is the intensity measured from a horizontal polarizer and a vertical analyser (Barshtein *et al.*, 1997).

7.2.5 Statistical analyses

A repeated measures analysis was used to compare the average means of the four treatment groups in a 2 x 2 factorial design (SPSS Inc., Version 10.0). The factors used were the supplementation of synbiotic (with and without), dietary fat (5% and 15%) and experimental period. ANOVA was used to perform multiple comparisons between means. All data are presented as mean ± standard error of means; n = 6.

7.3 RESULTS

7.3.1 Weight and feed intake

Pigs were in general healthy during the 8 weeks of experimental period. Growth rates, feed consumption and feed efficiency are shown in **Table 7.2**. Dietary treatments did not exhibit a significant effect on growth rates (P = 0.225), weekly feed intake (P = 0.415) and feed efficiency (P = 0.401).

7.3.2 Lipoprotein profiles

The effect of synbiotic supplementation and dietary fat on plasma lipid profiles is shown in **Table 7.3**. The plasma total cholesterol decreased in pigs supplemented with synbiotics on both dietary fats, while those without supplementation increased over 8 weeks (P = 0.001). Pigs on the high-fat diet also showed higher serum total cholesterol concentration than those on the low-fat diet (P = 0.016) although those supplemented with synbiotic and given the low-fat diet showed a larger decrease (18.16%) than those on the high-fat diet (1.51%) over 8 weeks. Similarly, pigs on the control diet also showed an increase in fasted triacylglycerol concentration of 17.64% (low-fat diet) and 49.69% (high-

fat diet) over 8 weeks, while those on the symbiotic diet had a decrease of 15.48% (low-fat diet) and 41.40% (high-fat diet) (P = 0.002). Changes in triacylglycerol were more prevalent between treatment groups and dietary fat in non-fasted samples. Pigs given the high-fat diet had significantly higher non-fasted triacylglycerol concentration than those on the low-fat diet (P = 0.001), while those given the synbiotic had a lower concentration than those without supplementation (P < 0.001). Dietary fat appeared to have no effect on the LDL-cholesterol concentration in pigs. However, the supplementation of synbiotic significantly lowered LDL-cholesterol concentration compared to the control (P = 0.045) over 8 weeks; pigs without the supplementation of synbiotic showed an increase of 46.63 to 59.19% while pigs given the synbiotic showed an increase of 13.11 to 28.11%. Dietary fat had no effect on the concentration of HDL-cholesterol, while the supplementation of synbiotic contributed to a smaller increase over 8 weeks compared to the control.

7.3.3 Lipoprotein subfractions

The subfractions of major lipoproteins are shown in **Figure 7.1**. Triacylglycerol content of VLDL decreased over 8 weeks (Figures 7.1A and 7.1D) for pigs on both dietary treatments and synbiotic supplementation. The low-fat diet had significantly lower concentration of triacylglycerol in VLDL than the high-fat diet (P = 0.008) over 8 weeks, while the supplementation of synbiotic similarly lowered the concentration of triacylglycerol in VLDL over the experimental period compared to pigs without supplementation (P = 0.008). This was accompanied by an increasing concentration of cholesteryl esters (CE) in VLDL over the experimental period for all treatment groups.

By weight, a large portion of LDL is CE and free cholesterol with little triacylglycerol (20). This was shown in pigs on all treatments studied (Figures 7.1B and 7.1E). Although the concentration of triacylglycerol was small in LDL and was decreasing over 8 weeks for all treatments, pigs supplemented with synbiotic had a significantly smaller decrease (P = 0.025) over 8 weeks (30.29-31.65%) than those without supplementation (63.07-77.58%), regardless of the dietary fat. However, dietary fat appeared to have no effect on the concentration of triacylglycerol in LDL. The supplementation of synbiotic also contributed to a larger decrease (P < 0.001) in the concentration of CE in LDL for both dietary fats (39.36-44.92%) over 8 weeks than those without supplementation (25.01-30.42%). Pigs on the high-fat diet also had a significantly larger decrease of CE in LDL than those given the low-fat diet (P = 0.05). There was a linear interaction (P = 0.002) between experimental period and supplementation of synbiotic such that the concentration of CE in

LDL decreased with time in pigs supplemented with synbiotic while the control remained relatively constant.

The HDL particles contain high amount of protein (approximately 50%) with small amounts of triacylglycerol (5%), cholesterol (20%) and phospholipids (25%) (21). These have been manifested in pigs on all treatment groups (Figures 7.1C and 7.1F). Although small, the concentration of CE in HDL increased (P = 0.036) when pigs were supplemented with synbiotic (25.86-35.09%) regardless of dietary fat over the experimental period, while those without supplementation showed a decrease of 20.34-29.87%.

7.3.4 Morphology of RBC

Morphological property of RBC was assessed using Wright's stain and is illustrated in **Figure 7.2**. The red cell morphology appeared to be normal for all treatments at the initial observation point (Figures 7.2A-D). However, at the end of the experimental period, the pigs on the control diet showed distinct characteristic of spur cells (Figures 7.2E and 7.2G), while those on the synbiotic diet were less affected. The occurrence of spur cells was also more prevalent in those on the high-fat diet (Figure 7.2F) than those on the low-fat diet (Figure 7.2H).

7.3.5 RBC membrane lipid properties

Phospholipids content of RBC membrane increased (P < 0.001) over the experimental period for all treatments studied (**Table 7.4**). The administration of synbiotic significantly (P = 0.005) contributed to higher concentrations of phospholipids compared to that without supplementation. Pigs given the high-fat diet also showed higher concentration of RBC phospholipids than those on the low-fat diet (P = 0.001). The diet with higher fat content contributed to higher concentration of cholesterol in the RBC compared to that with lower fat content (P < 0.001). There was a significant interaction (P = 0.017) between the dietary fat and supplementation of symbiotic; there was a largest increase in the concentration of cholesterol (157.67%) when pigs were not supplemented with synbiotic and was given the high-fat diet, while the concentration of cholesterol in RBC had the smallest increase (44.58%) when pigs were supplemented with synbiotic and was given the low-fat diet. The ratio of cholesterol/phospholipids (C/P; mol/mol) was significantly lower (P = 0.001) when pigs consumed synbiotic compared to those that did not. The C/P was also lower (P = 0.007) when pigs were given the low-fat diet compared to those on the high-fat diet. There was a significant interaction between the experimental period, supplementation of

synbiotic and dietary fat (P = 0.004) where a smaller increase in C/P over 8 weeks was observed in pigs supplemented with synbiotic but was given the high-fat diet (5.00%) compared to those given the low-fat diet (13.58%).

7.3.6 Fluorescence anisotropy (FAn)

Considering that the administration of dietary fat affected RBC membrane lipid content and the supplementation of synbiotic could alter such effects, we further evaluated the locations of cholesterol enrichment using fluorescence probes (**Table 7.5**). FAn of DPH increased juts after 2 weeks for all treatments studied except for pigs supplemented with synbiotic and given the low-fat diet which increased after 4 weeks (P < 0.01). The supplementation of synbiotic also reduced the FAn (P < 0.001) in pigs for both dietary fat treatments (10.87-19.57%) while those without supplementation showed an increase (2.27-39.39%) over the experimental period. The FAn was also higher (P = 0.012) when pigs were on the high-fat diet than those on the low-fat diet.

The experimental period significantly affected the FAn of ANS (P < 0.001) such that the low-fat diet contributed to a continuous decrease over 8 weeks regardless of synbiotic supplementation while the high-fat diet contributed to a decrease after 2 to 4 weeks. Also, pigs supplemented with synbiotic required a longer period before reducing the FAn of ANS compared to those without supplementation, accompanied by a smaller decrease of 26.47-36.59% over 8 weeks while those without supplementation decreased by 23.33-41.86% (P < 0.001).

There were significant changes (P = 0.004) in the FAn of TMA-DPH over the experimental period; pigs without the supplementation of synbiotic showed reducing FAn of TMA-DPH after 4 weeks for both the high- and low-fat diets, while the FAn for pigs supplemented with synbiotic only reduced after 6 weeks. The administration of synbiotic also reduced the FAn in pigs on both dietary fat treatments compared to those that did not consume the synbiotic (P < 0.001), accompanied by a lower increase over 8 weeks (0.00-11.76%) compared to an increase of 15.79-17.65% when pigs were not given the synbiotic. The high-fat diet also significantly contributed to a higher FAn (P = 0.040) of TMA-DPH than the low-fat diet.

7.4 Discussion

The current study showed that the supplementation of synbiotic reduced serum triacylglycerol (fasted and non-fasted), total- and LDL-cholesterol in pigs. Hypercholesterolemia has been associated with higher than normal total cholesterol (> 240 mg/dl) level. Although pigs given the high-fat diet remained hypercholesterolemic over the experimental period, the administration of synbiotic contributed to a decreased serum cholesterol concentration. When the low-fat diet was used, pigs without the supplementation of synbiotic remained hypocholesterolemic over 8 weeks, while those given the synbiotic showed a reduction to normal level over 8 weeks. Higher than normal level of LDL cholesterol (> 160 mg/dl) is also an attribute to hypercholesterolemia. Although the supplementation of synbiotic improved the LDL cholesterol content, it failed to achieve a normal level in all pigs.

There is very little information on the source and nature of abnormalities in lipoproteins (Jin *et al.*, 1998; Lin *et al.*, 1989). Hence, we wanted to evaluate further on the compositions of these lipoprotein classes in order to better understand the alteration of lipid components as affected by the synbiotic product that was developed in our previous study (Chapter 5.0, section 5.3).

VLDL is primarily synthesized in the liver and contains about 50 to 60% of triacylglycerol, which is the major fat that is transported from the liver into the bloodstream (Kwiterovich Jr.**,** 2002). Results from the present study showed that pigs on all dietary treatments had normal levels of triacylglycerol in the subfraction of VLDL at the initial point of the feeding trial, but were reduced over 8 weeks. VLDL particles are the precursors for LDL particles where the triacylglycerol in VLDL are exchanged for CE in the core of LDL (Kwiterovich Jr.**,** 2000). Results from the present study showed that pigs supplemented with the synbiotic decreased CE and increased triacylglycerol in LDL, complemented by a lower concentration of triacylglycerol in VLDL, indicating higher conversion of VLDL into LDL.

In addition, synbiotic also beneficially decreased the concentration of CE in LDL. Higher concentration of CE in LDL induced by diet was found to be associated with an increased risk for atherosclerosis in African green monkeys (Carr *et al.* 1992). More importantly, such triacylglycerol-enriched LDL particles are more susceptible to further hydrolysis (Kwiterovich Jr.**,** 2002) and are removed from blood via binding to LDL receptors, where the CE particles are hydrolysed into free cholesterol (Horton *et al.*, 2002). Loss of CE from the core of LDL forms smaller and denser LDL particles. Although smaller LDL appeared more atherogenic than larger LDL particles (Haffner, 2002), smaller LDL

formed as a result of dietary intervention was removed by plasma more rapidly than larger particles (Fernandez *et al.* 1993). Results from the present study clearly showed that the administration of the synbiotic decreased the concentration of CE coupled with increased concentration of triacylglycerol in the LDL subfraction, which may have contributed to the improved serum LDL-cholesterol in pigs supplemented with synbiotic.

The major function of HDL is to transport cholesterol back to the liver, and this could involve a process referred to as reverse cholesterol transport (Rader, 2002). Briefly, free cholesterol is transported into HDL particles and subsequently esterified to form CE. HDL contains mainly lecithin and protein, with small amount of cholesterol and triacylglycerol. Thus, matured HDL with bigger core containing more CE are transported back to the liver and hydrolyzed. These CE is transferred by cholesteryl ester transfer protein (CETP) and internalized by the LDL receptor. The HDL is then released for further reverse cholesterol transport (Kwiterovich Jr.**,** 2002). Experimental data from the present study showed that the administration of synbiotic increased the concentration of CE in the HDL subfraction, which indicated that cholesterol was removed by HDL in the form of CE. This explains as to why there was reduced serum total cholesterol in pigs supplemented with synbiotic.

Although results from this present study showed increased concentration of CE in the HDL particles, pigs supplemented with synbiotic also had lower serum HDL-cholesterol than those without supplementation. Low serum HDL-cholesterol is not an indicator of faulty removal of cholesterol through deficient activity of the reverse cholesterol transport pathway (Kwiterovich Jr.**,** 2002). Instead, the CE in the core of HDL may also be exchanged by CETP for the triacylglycerol in VLDL, producing a triacylglycerol-enriched but CE-depleted HDL, which appeared to be catabolized more rapidly by the kidney. The HDL-cholesterol level is thus decreased (Kwiterovich Jr.**,** 2000). Result from the present study showed that using direct concentration of serum HDL-cholesterol as an indicator for healthy lipoprotein profiles would be misleading, as higher concentration of serum HDL-cholesterol does not indicate high efficiency in cholesterol transport.

The occurrence of spur cells appeared to be improved by the supplementation of synbiotic as supported by the morphological representation and lower ratio of C/P. Cholesterol in the membrane lipid bilayer prevents fatty acid chains from coming together and crystallizing; however, cholesterol also decreases the permeability of the bilayer to small water-soluble molecules and thus reducing fluidity (Simons & Ehehalt, 2002). The normal C/P ratio of RBC is approximately 1.05 (Cooper *et al.*, 1978). At the end of the experimental

period, C/P ratio for the pigs with the supplementation of synbiotic was normal while those without supplementation were higher than the normal ratio. Such abnormality in lipid composition would result in an increase in the surface area of the cells, thus increasing rigidity and decreasing fluidity. These led to decreased ability of cell to deform and thus the occurrence of spur cells (Duhamel *et al.*, 1983).

Although our present results and previous studies (Cooper *et al.*, 1980) showed that dietary fat intervention contributed to the enrichment of cholesterol in RBC membrane, we were unable to distinguish the location of such enrichment in the membrane. Thus, we have used three different fluorescence probes to allow examination in different locations of the membrane bilayer. These probes exhibit strong fluorescence increase upon binding to lipids and have sensitive anisotropy responses to lipid order. DPH is an apolar fluorophore mainly incorporated into different apolar regions of the membrane (Kaiser & London, 1998). Our experimental data showed that pigs supplemented with the synbiotic had lower saturation of cholesterol within the apolar region of the RBC while a higher FAn of DPH in pigs without synbiotic indicated enrichment of cholesterol in the region close to the acyl chains of the phospholipid tails (Roy *et al.*, 2005). Higher fat content in the diet also contributed to increased membrane rigidity as supported by the higher FAn obtained. While the supplementation of synbiotic contributed to a reduced saturation within the apolar region of the RBC membrane in pigs on the high-fat diets, the synbiotic appeared to delay such saturation when pigs were given the low-fat diet.

ANS was used to monitor the outer membrane permeabilizing ability due to its high affinity towards the interface between the apolar tail and the polar head of phospholipids, and may be an indicator of changes in the surface (Barshtein *et al.*, 1997). The present study showed that the supplementation of synbiotic had little effect when pigs were given the low-fat diet. However, when pigs were on the high-fat diet, the supplementation of synbiotic had a reduced and delayed increase of cholesterol saturation in the upper phospholipids region. Increasing FAn of ANS has been associated with the insertion of positively charged molecules into the bilayer and thus increasing the packing order of the surface and the inner hydrocarbon region (Sujatha & Mishra, 1997). Results from the present study implied that synbiotic lessened such effects and thus improved membrane fluidity via increased permeability and decreased packing order; two attributes that determine the degree of membrane rigidity, and subsequently occurrence of spur cells.

TMA-DPH is a cationic probe with the fluorophore TMA incorporated in the head group and DPH incorporated in the acyl chain (Ben-Yashar & Barenholz, 1991), and is an

indicator of changes in the surface. Increased FAn of TMA-DPH has been associated with reduced water penetrability into the bilayer, more ordered bilayer structure and higher cholesterol levels in the head-group (Straume & Litman, 1988). Results from the present study showed that the supplementation of synbiotic reduced the packing order and saturation of cholesterol in the polar heads of the membrane bilayer, regardless of dietary fat contents, although higher fat content contributed to increased packing order of the bilayer structure compared to the diet containing lower fat content.

Our present study provided the experimental evidence that the supplementation of synbiotic reduced cholesterol saturation within the apolar tails of phospholipids, within the interfacial regions of the apolar tail and polar heads and improved penetrability of the head-group, which contributed to improved occurrence of spur cells. Also, it is interesting to note that the FAn of TMA-DPH only began to increase after the FAn of DPH and ANS started to decrease in all pigs. Considering that the occurrence of spur cells is only reversible after the termination of a high-fat diet (Cooper *et al.*, 1980), a consecutive and continuous series of membrane alterations are involved. We speculate that the occurrence of spur cells was initiated with the saturation of cholesterol in the apolar and interfacial regions, followed by increased packing order in the head group. Thus, before the saturation of cholesterol was entirely reversed when pigs were given the low-fat diet, cholesterol was partially relocated to another membrane region. This may explain the presence of spur cells in pigs even over 8 weeks on the low-fat diet.

In conclusion, the supplementation of synbiotic reduced serum triacylglycerol, total- and LDL-cholesterol in hypercholesterolemic pigs. Evaluation of compositions of individual lipoprotein classes suggested that the synbiotic may reduce cholesterol in the form of CE via the interrelated pathways of lipid transporters (VLDL, LDL and HDL). The synbiotic also appeared to lessen the effect of cholesterol enrichment in RBC membrane via improved membrane fluidity and permeability.

TABLE 7.1

Composition of the low-fat (5% fat) and high-fat (15% fat) basal diets.

Ingredient	Low-fat diet (%)	High-fat diet (%)
Wheat (11% crude protein)	78.40	61.64
Soybean Meal (48% crude protein)	7.82	14.29
Meat & Bone Meal (50% crude protein)	7.50	7.50
Blood Meal (85% crude protein)	2.50	2.50
Tallow	1.60	8.40
Sunflower oil	0.80	4.20
Dicalcium phosphate	0.72	0.73
Salt	0.20	0.20
Vitamin-mineral premix	0.20	0.20
Lysine	0.15	0.15
Methionine	0.07	0.10
Threonine	0.00	0.05
Tylan	0.04	0.04

TABLE 7.2

Effect of the supplementation of synbiotic and dietary fat on the growth rate, feed intake and feed efficiency of hypercholesterolemic pigs for 8 wk.

Treatment[1]	Control		Synbiotic		SE[3]	Statistical significance of effect: P[2]		
Dietary fat (%)	5	15	5	15		T	F	T x F
Growth rate (g/pig/wk)	7.98	8.50	7.88	8.05	0.22	0.225	0.124	0.433
Feed intake (g/pig/wk)	17.17	15.08	16.94	14.81	0.30	0.415	NA[4]	0.959
Growth rate: feed intake	0.46	0.56	0.47	0.54	0.01	0.401	NA[4]	0.315

[1]The control diet contained no synbiotic while the synbiotic diet contained *L. acidophilus* ATCC 4962 (1.00 g/pig/d), FOS (1.25g/pig/d), mannitol (1.56 g/pig/d) and inulin (2.20 g/pig/d).

[2]T, effect of treatment; F, effect of dietary fat.

[3]Standard error of the difference for T x F.

[4]Not applicable; feed was adjusted weekly according to energy intake per weight of pigs; 14.6 MJ/kg for those on the basal diet containing 5% fat and 16.9 MJ/kg for those on the basal diet containing 15% fat.

TABLE 7.3

Effect of the supplementation of synbiotic and dietary fat on plasma lipid profiles of hypercholesterolemic pigs for 8 weeks.

	Treatment[1]	Dietary fat (%)	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8	SE[3]	Statistical significance of effect: P[2] W	T	F
Total cholesterol (mg/dL)	Control	5	249.13	173.71	248.46	303.49	328.80	8.85	<0.001	0.001	0.016
		15	244.79	223.11	240.45	328.16	347.33				
	Synbiotic	5	268.13	149.17	220.44	230.75	219.44				
		15	267.47	211.77	196.10	278.14	263.44				
Triacylglycerol (fasted) (mg/dL)	Control	5	156.41	146.41	197.43	182.33	184.00	9.50	0.090	0.002	0.075
		15	142.07	207.39	207.77	202.67	212.67				
	Synbiotic	5	191.76	93.38	133.07	133.67	162.08				
		15	228.45	154.70	123.73	148.04	133.88				
Triacylglycerol (non-fasted) (mg/dL)	Control	5	296.15	471.57	427.33	404.87	423.60	35.46	0.018	<0.001	0.001
		15	307.82	649.33	608.01	672.67	620.07				
	Synbiotic	5	312.16	225.33	239.12	277.75	229.45				
		15	278.47	368.67	319.93	299.50	370.30				
LDL-cholesterol (mg/dL)	Control	5	164.90	196.98	225.94	243.02	262.50	9.85	<0.001	0.045	0.859
		15	174.69	226.04	244.27	250.73	256.15				
	Synbiotic	5	187.71	195.10	218.44	226.56	240.47				
		15	190.63	193.23	211.20	208.44	215.63				
HDL-cholesterol (mg/dL)	Control	5	18.85	22.22	32.56	32.32	30.71	1.02	<0.001	0.003	0.668
		15	21.33	26.11	32.26	31.99	38.26				
	Synbiotic	5	25.89	28.19	29.78	40.16	41.00				
		15	26.59	24.41	26.67	36.68	41.48				

[1]The control diet contained no synbiotic while the synbiotic diet contained *L. acidophilus* ATCC 4962 (1.00 g/pig/d), FOS (1.25g/pig/d), mannitol (1.56 g/pig/d) and inulin (2.20 g/pig/d).
[2]W, effect of experimental period; T, effect of treatment; F, effect of dietary fat.
[3]Standard error of the difference for T x F.

TABLE 7.4

Effect of the supplementation of synbiotic and dietary fat on concentration of cholesterol and phospholipids in RBC membrane of hypercholesterolemic pigs for 8 weeks.

	Treatment[1]	Dietary fat (%)	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8	SE[3]	Statistical significance of effect: P[2] W	T	F
Phospholipids ($\mu g/10^8$ RBC)	Control	5	0.44	0.66	0.70	0.66	0.65	0.03	<0.001	0.005	0.001
		15	0.53	0.62	0.69	0.78	0.85				
	Synbiotic	5	0.55	0.64	0.70	0.73	0.75				
		15	0.55	0.65	0.70	1.00	1.04				
Cholesterol[4] ($\mu g/10^8$ RBC)	Control	5	3.84	6.74	7.38	7.89	8.96	0.42	<0.001	0.076	<0.001
		15	5.15	11.07	11.36	11.24	13.27				
	Synbiotic	5	5.54	7.03	6.29	8.02	8.01				
		15	5.48	7.31	7.44	11.52	10.87				
Cholesterol/phospholipids[5] (mol/mol)	Control	5	0.72	0.83	0.87	0.97	1.19	0.05	<0.001	0.001	0.007
		15	0.78	1.47	1.35	1.17	1.30				
	Synbiotic	5	0.81	0.88	0.73	0.88	0.92				
		15	0.80	0.92	0.86	0.93	0.84				

[1]The control diet contained no synbiotic while the synbiotic diet contained *L. acidophilus* ATCC 4962 (1.00 g/pig/d), FOS (1.25g/pig/d), mannitol (1.56 g/pig/d) and inulin (2.20 g/pig/d).

[2]W, effect of experimental period; T, effect of treatment; F, effect of dietary fat.

[3]Standard error of the difference for T x F.

[4]T x F interaction, P = 0.017.

[5]W x T x F interaction, P = 0.004.

TABLE 7.5

Effect of the supplementation of synbiotic and dietary fat on fluorescence anisotropy of RBC ghosts in hypercholesterolemic pigs for 8 weeks.

Fluorescence anisotropy	Treatment[1]	Dietary fat (%)	Wk					SE[3]	Statistical significance of effect: P[2]		
			0	2	4	6	8		W	T	F
DPH	Control	5	0.44	0.68	0.51	0.41	0.45	0.02	<0.001	<0.001	0.012
		15	0.33	0.84	0.54	0.4	0.46				
	Synbiotic	5	0.46	0.56	0.68	0.29	0.37				
		15	0.46	0.71	0.45	0.33	0.41				
ANS	Control	5	0.43	0.42	0.27	0.24	0.25	0.01	<0.001	<0.001	0.231
		15	0.3	0.46	0.25	0.27	0.23				
	Synbiotic	5	0.41	0.41	0.37	0.3	0.26				
		15	0.34	0.40	0.44	0.27	0.25				
TMA-DPH	Control	5	0.19	0.19	0.27	0.21	0.22	0.01	0.004	<0.001	0.040
		15	0.17	0.23	0.4	0.23	0.2				
	Synbiotic	5	0.17	0.14	0.13	0.2	0.15				
		15	0.16	0.18	0.18	0.22	0.16				

[1]The control diet contained no synbiotic while the synbiotic diet contained *L. acidophilus* ATCC 4962 (1.00 g/pig/d), FOS (1.25g/pig/d), mannitol (1.56 g/pig/d) and inulin (2.20 g/pig/d).

[2]W, effect of experimental period; T, effect of treatment; F, effect of dietary fat.

[3]Standard error of the difference for T x F.

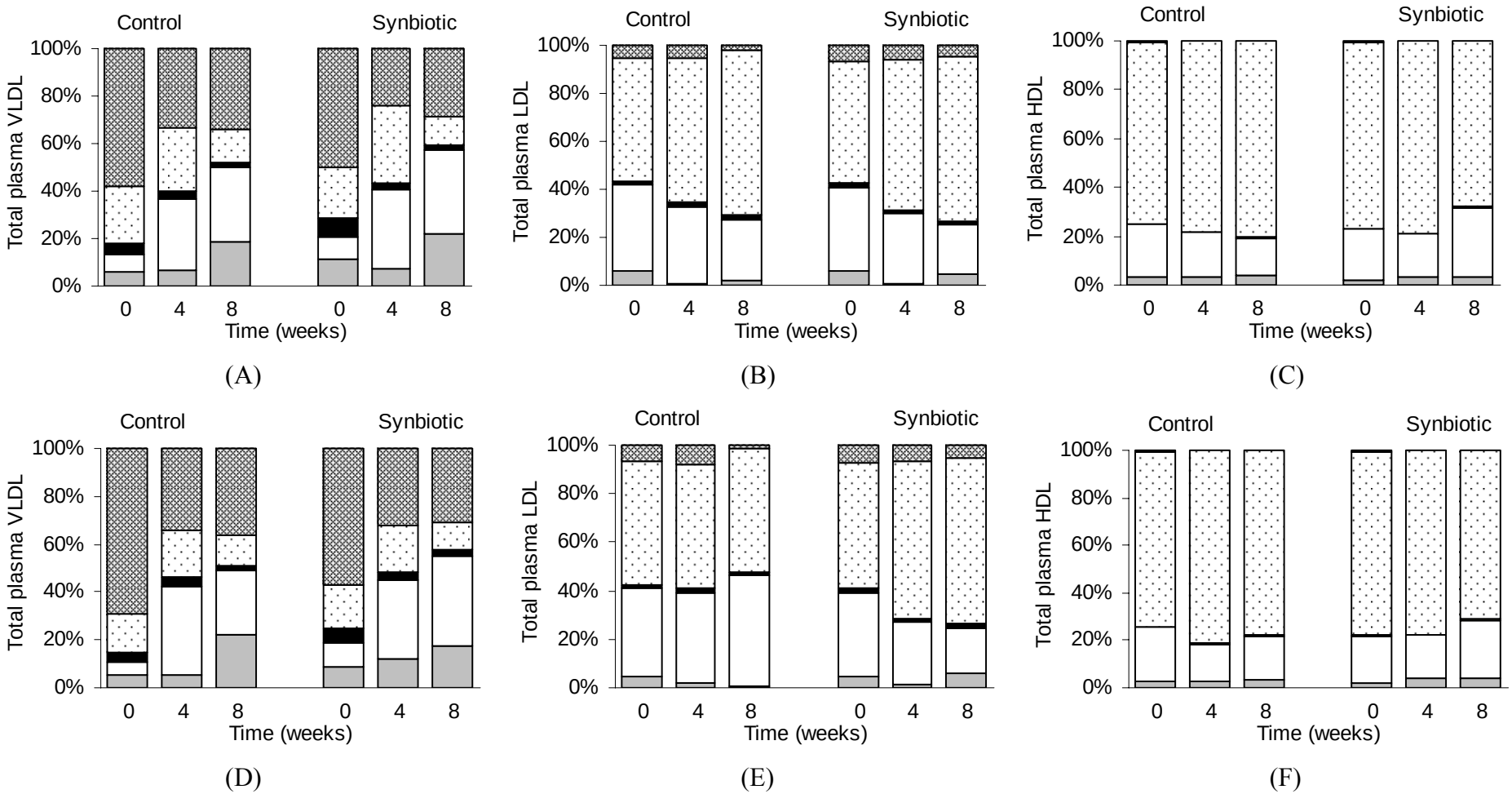


FIGURE 7.1 Subfractions of VLDL-, LDL- and HDL cholesterol of pigs fed the control and synbiotic diets with 5% fat (A,B,C) and 15% fat (D,E,F) for 8 weeks; triacylglycerol (⊠), protein (⊡), phospholipids (■), cholesteryl esters (□) and free cholesterol (▩). The control diet contained no synbiotic while the synbiotic diet contained *L. acidophilus* ATCC 4962 (1.00 g/pig/d), FOS (1.25g/pig/d), mannitol (1.56 g/pig/d) and inulin (2.20 g/pig/d). Results are expressed as percentage of total plasma lipoprotein.

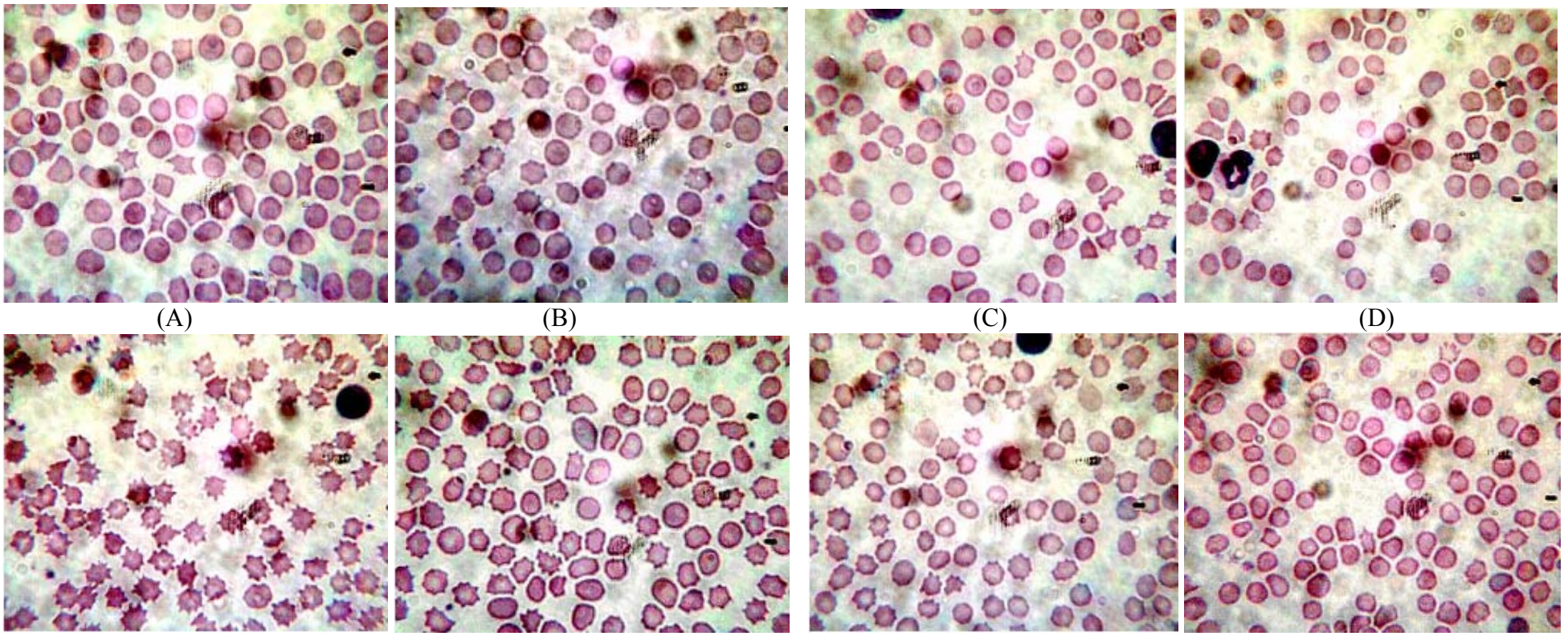


FIGURE 7.2 Morphology of RBC after staining using Wright's stain from pigs on the high-fat diet without (A) and with (B) synbiotic at the initial feeding period, pigs on the low-fat diet without (C) and with (D) synbiotic at the initial feeding period, pigs on the high-fat diet without (E) and with (F) synbiotic after 8 weeks, and pigs on the low-fat diet without (G) and with (H) synbiotic after 8 weeks. The synbiotic supplementation contained *L. acidophilus* ATCC 4962 (1.00 g/pig/d), FOS (1.25g/pig/d), mannitol (1.56 g/pig/d) and inulin (2.20 g/pig/d); n = 6.

8.0 Summary of Results

Eleven strains of lactobacilli and five strains of bifidobacteria were evaluated for their acid and bile tolerance, and their cholesterol removal ability *in-vitro*. Our results showed that all strains of *Lactobacillus* and *Bifidobacterium* studied were able to tolerate pH 2.0 for 2 hours at varying degrees. *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 were the most acid tolerant among lactobacilli while *B. longum* BB536 and *B. infantis* 17930 being the most acid tolerant among bifidobacteria. All strains showed tolerance to oxgall, cholic acid and taurocholic acid. All strains of *Lactobacillus* and *Bifidobacterium* could assimilate cholesterol at varying levels ranging from 4.17 to 32.25 μg/mL. The pattern of cholesterol removal showed similarities to that of bacterial growth, indicating that cholesterol removal was growth associated. Cholesterol was also removed by dead- and resting cells (0.79-3.82 mg/g dry weight). Considering that cells were not growing and cannot assimilate cholesterol, their ability to remove cholesterol would indicate that cholesterol may have been removed via binding to the cellular surface. We have also found that cells grown in the presence of cholesterol had higher saturated fatty acids and lower total unsaturated fatty acids than those grown in the absence of cholesterol, suggesting that cholesterol from the medium may be incorporated into the cellular membrane and altered the membrane lipid profiles.

The similar strains of *Lactobacillus* and *Bifidobacterium* that were used for acid and bile tolerant analyses were screened for their bile salt deconjugation ability, bile salt hydrolase activity (BSH) and coprecipitation of cholesterol with deconjugated bile. Using concentrations that resemble the molar ratio of sodium glycocholate and sodium taurocholate in human bile, our results showed that strains of *L. acidophilus* in general had higher deconjugation ability, which liberated substantial amount of cholic acid, ranging from 1.88 to 2.96 mM as compared to that by *L. casei*, which ranged from 1.14 to 2.69 mM. Of all the *Bifidobacterium*, *B. infantis* 1912 released highest amount of cholic acid at concentrations of bile that resembled the human ratio. All strains studied exhibited BSH activity, with preference towards glycine-conjugated bile than taurine-conjugated bile. Most strains of *Lactobacillus* and *Bifidobacterium* exhibited higher total BSH activity on bile mixtures compared to when bile were used individually. Our results also showed that cholesterol could co-precipitate with deconjugated bile, which increased with decreasing pH levels of the fermentation medium. Co-precipitation of cholesterol with cholic acid increased rapidly with decreasing pH levels below 5.0, with maximum co-precipitation at pH 1.0. Considering that the pH of intestine is unlikely to be lower than 6.0, results from our experiment indicate that co-precipitation of cholesterol with deconjugated bile would not be a major factor in controlling serum cholesterol levels.

L. acidophilus ATCC 4962, *L. casei* ASCC 292 and *B. infantis* ATCC 17930 were subsequently screened in the presence of six prebiotics, namely, sorbitol, mannitol, maltodextrin, high-amylose maize, fructooligosaccharide (FOS), and inulin, in order to determine the best combination of inoculum size and concentrations of prebiotics to remove the highest level of cholesterol *in-vitro*. Screening using a two-level partial factorial design showed that the combination of *L. casei* ATCC 292 in the presence FOS and maltodextrin was best for the removal of cholesterol. After determining the possible optimum region via steepest ascent, all factors were subjected to optimization via a central composite design. The second-order polynomial regression model estimated that the optimum condition of the factors for cholesterol removal by *L. casei* ASCC 292 was 1.71% (wt/vol) probiotic, 4.95% (wt/vol) FOS, and 6.62% (wt/vol) maltodextrin. The predicted responses were validated with actual experimentation. Results showed that the response surface method was reliable for developing the model, optimizing factors, and analysing interaction effects. Further evaluation of growth properties and fermentation of the prebiotics indicated that cholesterol removal was growth associated. The inoculum size of *L. casei* ASCC 292 had the most significant quadratic effect on all responses studied, except for substrate utilization and production of organic acids, where the interactions between the probiotic and both prebiotics played a bigger role. Highest amount of lactic acid was produced within the optimum region of cholesterol removal, followed by propionic, acetic, formic and butyric acid. Analyses of the production patterns of organic acids revealed that the production of lactic and acetic acids was sensitive to the end-product of maltodextrin fermentation. Increased concentration of FOS contributed to the increased production of propionic acid. The response surface of butyric and formic acid showed that the production of these acids were growth associated.

Using a similar methodology, we have found that *L. acidophilus* ATCC 4962 in the presence of FOS, mannitol and inulin was best for cholesterol removal *in-vitro*. Optimization via CCD showed that the optimum condition of the factors for cholesterol removal by *L. acidophilus* ATCC 4962 to be 2.64% (w/vol) inoculum size, 4.13% (w/vol) mannitol, 3.29% (w/vol) FOS and 5.81% (w/vol) inulin. The predicted responses were validated with actual experimentation. The response surface methodology was proven to be reliable in developing the model, optimizing factors and analysing interaction effects. The growth of the organism, mean doubling time and production of organic acids were also studied using quadratic models within the experimental region used for optimum cholesterol. Results here indicated that cholesterol removal and the production of organic acids were growth associated, and highly influenced by the concentrations of prebiotics. The response surfaces of acetic and formic acids showed that their productions were greatly influenced by the concentration of mannitol and was growth associated. The similarities between the response surfaces of

butyric and acetic acids indicated that the production of these acids were interrrelated. Increased production of lactic acid inhibited the growth of the organism, which was manifested in the cessation of growth in the response surfaced produced.

Initial screening of *B. infantis* ATCC 17930 in the presence of prebiotics showed that sorbitol, maltodextrin and inulin was the best prebiotics for the removal of cholesterol *in-vitro*. Subsequent optimization predicted that the optimum condition of the factors for cholesterol removal by *B. infantis* ATCC 17930 was 3.90% (wt/vol) inoculum size, 7.64% (wt/vol) sorbitol, 4.42% (wt/vol) maltodextrin and 7.60% (wt/vol) inulin. Validation experiments showed that the prediction by response surface methodology was reliable for developing the model, optimizing factors, and analysing interaction effects. Further evaluation of growth characteristics and production of organic acids were performed within the optimized regions for cholesterol removal to evaluate the relation between cholesterol removal, growth properties and end-product fermentation of the prebiotics. Responses surfaces produced showed that the removal of cholesterol was growth associated; increased removal of cholesterol was observed at increased growth of the organism and at higher substrate utilization rates. The polynomial models showed that the production of acetic, lactic and formic acids were dependent on the growth of the organisms and not just concentrations of prebiotics but types of prebiotics as well; acetic and lactic acids were repressed by high concentration of sorbitol, while maltodextrin induced the production of formic acid linearly.

The ability of the developed synbiotics to remove cholesterol was further evaluated using male Wistar rats (n = 6) on a high-cholesterol diet as a model. The individual effects of the prebiotics were also studied, with the control group receiving no prebiotic or probiotic.

Three synbiotic diets containing *L. casei* ASCC 292 were used including *L. casei* and FOS (LF), *L. casei* and maltodextrin (LM), and *L. casei*, FOS and maltodextrin (LFM). In addition, the changes in intestinal microflora, intestinal concentration of organic acids and the possibility of translocation of *Lactobacillus* were also investigated. The LFM diet showed the most promising hypocholesterolemic effect, with reduced serum total cholesterol and triglycerides levels. The LFM diet also contributed to a decreased population of pathogenic organisms such as staphylococci, bacteroides, *E. coli* and total coliforms in the bowel. No translocation of *Lactobacillus* to the spleen, liver and kidney was observed from the ingestion of all synbiotics studied.

Four synbiotic diets containing *L. acidophilus* ATCC 4962 were used including *L. acidophilus* and FOS (LF), *L. acidophilus* and mannitol (LM), *L. acidophilus* and inulin (LI) and *L. acidophilus* and all three prebiotics (LFMI). The LFMI diet showed greatest hypocholesterolemic effect, with reduced serum total cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol levels by 32.40%, 32.51% and 42.95%, respectively in rats. This diet also decreased the pH values in the cecum which was contributed to a reduced population of bacteroides. There was no translocation of *Lactobacillus* to other internal organs from all synbiotics studied.

The cholesterol lowering property of the synbiotic containing *B. infantis* ATCC 17930 was evaluated using five diets namely the control (without probiotic and prebiotics), diets that contained *Bifidobacterium infantis* and sorbitol (BS), *B. infantis* and maltodextrin (B), *B. infantis* and inulin (BI), and *B. infantis* and all three prebiotics (BSMI). The BM diet exhibited the mosy hypocholesterolemic effect, with reduced total cholesterol, triglycerides and LDL-cholesterol levels. This diet also increased the total *Bifidobacterium* in the cecum and colon, which contributed to an increased concentration of acetic acid in those segments. This was accompanied by decreased counts of total aerobes, *Escherichia coli* and bacteroides. There was no *Bifidobacterium* detected in the spleen, liver and kidney suggesting that all the synbiotics used were safe from translocation. Results from this study showed that the combination of *Bifidobacterium* and all prebiotics (BFMI) did not perform better than when the prebiotics were used independently.

The synbiotic containing *L. acidophilus* ATCC 4962, mannitol, FOS and inulin exerted better hypocholesterolemic effect that the other optimized synbiotics in rats, and was further evaluated using white male Landrace hypercholesterolemic pigs on a high- and low-fat diets.

Pigs supplemented with sthe synbiotic showed reduced serum total cholesterol ($P = 0.001$), fasted and non-fasted triacylglycerol ($P < 0.05$) and LDL-cholesterol ($P = 0.045$) when given both the high- and low-fat diets. After the experimental period of 8 weeks, pigs without the supplementation of synbiotic remained hypercholesterolemic with increased total-cholesterol level, while pigs given the synbiotic and on the high- and low-fat diets showed a decrease of 1.51% and 18.16%, respectively. Pigs on the low-fat diet supplemented with the synbiotic also achieved normal total-cholesterol levels.

Evaluation on compositions of individual lipoprotein classes suggested that the supplementation of synbiotic reduced cholesterol in the form of CE via the interrelated

pathways of lipid transporters (VLDL, LDL and HDL). Pigs given the synbiotic had a reduced concentration of CE ($P < 0.001$) and increased concentration of triacylglycerol ($P = 0.042$) in LDL suggested that LDL-cholesterol was reduced via the hydrolysis of smaller and denser LDL particles. Also, a higher concentration of CE in HDL of pigs supplemented with the synbiotic than those without the supplementation indicated high efficiency in cholesterol transport by HDL that subsequently led to reduction of total serum cholesterol.

Evaluation on the morphology of RBC after staining using Wright's stain showed that pigs without the supplementation of synbiotic had a more prevalent deformation than those given the synbiotic, as supported by the higher C/P ratio in RBC ($P = 0.001$). The high-fat diet also contributed to higher occurrence of spur cells than those on the low-fat diet, with higher cholesterol enrichment in the membrane ($P = 0.007$). The FAn of DPH, TMA-DPH and ANS that targeted the apolar, polar and interfacial regions, respectively of phospholipids in RBC of pigs was also significantly lower ($P < 0.001$) when pigs were supplemented with synbiotic compared to those without supplementation for both dietary fat diets, indicating improved membrane rigidity, fluidity and permeability.

Overall, the selected *L. acidophilus* ATCC 4962 proved to have the ability to tolerate acid and bile conditions *in-vitro* and was optimized with mannitol, FOS and inulin for maximum cholesterol removal *in-vitro*. These attributes were exhibited in *in-vivo* models using rats and pigs. In addition to its hypocholesterolemic effect, this synbiotic also showed the ability to improve intestinal microflora population, intestinal production of organic acids and deformities in RBC.

9.0 Future Research Directions

Our results showed that all strains of *Lactobacillus* and *Bifidobacterium* could remove cholesterol *in-vitro* via several possible mechanisms, with highest amount of cholesterol removed via assimilation. Although the results seemed promising, we remain oblivious of the microbial pathways that were involved and the fate of the assimilated cholesterol to whether it was metabolised, merely incorporated into the cells or excreted into the host. All strains also showed the capability to deconjugate bile via BSH activity, which was proven genetically to be present. However, the reason that triggered such deconjugation activity remained unclear. If the postulation that probiotics deconjugate bile to counteract toxicity from conjugated bile is true, it may exhibit harmful effects as bile in deconjugated form was reported to be toxic to the host. Also, the deconjugation of bile liberates amino acids (glycine and taurine), which may also affect the host. The information of the benefits *in-vivo* once ingested is also needed to assure the safety of probiotics on the host.

Our optimization experiments also showed that organic acids were produced within the experimental regions and were greatly affected by the type of prebiotics. Our study had formed the basis for their fermentation patterns. However, we remain unaware of the pathways involved, the effect of prebiotics on those specific pathways and the interrelation between the productions of the acids. Despite experimental evidence that the prebiotics such as mannitol, maltodextrin and FOS exhibited end-product inhibition on the production of acetic, lactic and formic acids, we are unaware of the reason that contributed to such a phenomenon. The use of radioactive prebiotics would be beneficial to study the pathways involved for more definite conclusions.

Despite proving that the developed synbiotics *in-vitro* also exhibited hypocholesterolemic effects in rats, we are still ignorant of the mechanisms involved. The synbiotics also altered intestinal microbial populations. Although a decrease in luminal pH levels and an increase in the concentration of lactic acid may contribute to this, we have also detected alteration of intestinal microflora when neither has been observed. This has led us to believe that the synbiotics exerted such antimicrobial effects via other means. Also, butyric acid was observed to decrease in the bowel upon ingestion of synbiotics, leading to the postulation that the synbiotics accelerated butyrate absorption by colonocytes. This matter is worth evaluating considering that butyrate reduces the risk of colonic cancer.

Analyses in the lipoprotein compositions in pigs were performed to generate more information on the hypocholesterolemic effects of synbiotics on serum lipid profiles. Our results showed that the synbiotic reduced serum LDL-cholesterol level by increased formation of smaller and denser LDL molecules, while serum total cholesterol was reduced

via increased formation of cholesteryl esters in the HDL particles. Despite this, the pathways involved and the effect of the synbiotic on these pathways remain unknown. Further evaluation is needed before a solid conclusion could be reached. The ingestion of synbiotic also improved the occurrence of spur cells in hypercholesterolemic pigs via decreased cholesterol enrichment in the membrane of RBC and delayed the saturation in the apolar and membrane regions of the membrane bilayer. No study has been previously conducted on the effect of synbiotic on RBC deformities. More information is needed to understand this phenomenon. Considering that our current study has laid the basis for further studies in this area, the use of tagged synbiotics or genetic manipulation would better assess the pathways involved.

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