Characterisation of Prebiotic Compounds from Plant Sources and Food Industry Wastes: Inulin from Jerusalem Artichoke and Lactulose from Milk Concentration Permeate

A thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy

Tatdao Paseephol
B.Sc., M.Sc.
(Food Science and Technology,
Chiang Mai University, Thailand)

School of Applied Sciences
Science, Engineering and Technology Portfolio
RMIT University
August 2008

DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

P. Tatdao

Tatdao Paseephol

14 November 2008

DEDICATION

This thesis is dedicated to my parents,

Chotsak and Pongchan Paseephol

for giving me life and love.

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere appreciation to Dr. Frank Sherkat as my senior supervisor for his guidance, encouragement, and constant support throughout my doctoral studies. Especially, I am thankful for his unfailing patience and critical outlook during the writing of this thesis and manuscripts for publication. My deep gratitude is also expressed to Dr. Darryl M. Small as co-supervisor for his assistance, advice and warm support during the course of the study.

My thanks also go to Mr. Tony Readshaw from Mandurah Australia Pty. Ltd. for his original suggestion of this project idea and providing commercial inulin samples for use in experimental trials.

I gratefully acknowledge RMIT University for providing financial support in the form of an International Postgraduate Scholarship (IPRS).

I am thankful to Food Science Laboratory Staff, Mr. Michael Kakoullis, Ms. Lillian Chuang and Mrs. Mary Pecar for their enthusiastic assistance and support in using the various laboratory and pilot plant equipment as well as for their friendship over the past three years. I wish to thank Mr. Paul Morrison of Applied Chemistry Staff for his support in HPLC analyses.

My thanks also go to my friends that I met during my many years as a student in Australia who offered warm friendship, wisdom and encouragement when it was most needed. Especially, I would like to thank my post-graduate friends, Ms. Marrissa Wijaya for the help in spray drying process, Mr. Ali Jafarpour for his help with running the rheometer, and Ms. Cecellia Widjaya for her assistance in texture analyser. I thank

Acknowledgements

Ms. Gina D. Kusuma who as an Honours student worked with me on the current research interests. Also, I would like to thank Mr. Hossein Daryaei and Mr. Kambiz Shamsi as the research team working together under the supervision of Dr. Frank sherkat for their friendship and useful discussions over the years.

I would also like to express my heartfelt thanks to my uncle, M.R. Chandram Sirischoke Chandratat for giving me the opportunity through financial assistance to pursue my studies here in Australia.

Finally, I would like to express my deepest appreciation to my dearest parents and my brother for their immense love and moral support in every step of my educational career.

Tatdao Paseephol

PUBLICATIONS AND PRESENTATIONS

Publications

Paseephol T, Small DM, Sherkat F. 2008. Lactulose production from milk concentration permeate using calcium carbonate-based catalysts. Food Chemistry 111(2):283-290.

Paseephol T, Small DM, Sherkat F. 2008. Rheology and texture of set yoghurt as affected by inulin addition. Journal of Texture studies (JTS-1051) – in press.

Paseephol T, Small DM, Sherkat F. 2007. Process optimisation for fractionating Jerusalem artichoke fructans with ethanol using response surface methodology. Food Chemistry 104(1):73-80.

Poster presentation

Paseephol T, Small DM, Sherkat F. 2006. Optimisation of inulin extraction from Jerusalem artichoke. Proceedings of the 39th Australian Institute of Food Science and Technology (AIFST) annual meeting – Festival of Food ; 2006 July 9-12; Adelaide, Australia.

Conference presentations

Paseephol T, Small DM, Sherkat F. 2008. Effects of fructans as fat replacers on set yoghurt texture and rheology. Proceedings of the 9th International Hydrocolloids Conference – Theoretical and Applied Aspects of Hydrocolloids Structure and Interactions with Natural Food Ingredients and Pharmaceuticals; 2008 July 15-19; Singapore.

Paseephol T, Small DM, Sherkat F. 2007. Value-adding to milk concentration permeate via isomerisation of lactose to lactulose. Proceedings of the 40th Australian Institute of Food Science and Technology (AIFST) annual meeting – Celebrating 40 years with 20/20 Vision; 2007 June 24-27; Melbourne, Australia.

Sherkat F, Small DM, Paseephol T. 2007. Lactulose production from milk concentration permeate using calcium carbonate-based catalysts. Proceedings of the International Dairy Federation Symposium – Lactose and its Derivatives; 2007 May 14-16, Moscow, Russia.

ABSTRACT

The development of processes for the preparation of prebiotic compounds, namely inulin from tubers of Jerusalem artichoke (JA-*Helianthus tuberosus* L.), and lactulose from milk concentration permeate (MCP) was examined. Inulin was extracted from the whole JA tubers using hydrothermal extraction process, followed by clarification and concentration. The concentrate was fractionated using two different procedures i.e. ethanol fractionation and cold precipitation (+4 and/or −24°C) into high- and low-molecular-weight components. The most satisfactory method was cold fractionation wherein the insoluble heavier inulin fractions were found to settle to the bottom and were separated and spray-dried to obtain inulin powder. Lactose in MCP was isomerised into lactulose using carbonate-based catalysts (oyster shell and egg shell powders) followed by clarification and concentration. The high-performance liquid chromatography with refractive index detector (HPLC-RID) chromatograms and changes in pH and colour values confirmed the conversion of lactose into lactulose and decomposition of lactulose into by-products. The results obtained showed the suitability of oyster shell powder for lactose isomerisation in lieu of egg shell powder. For preparing lactulose-enriched MCP with acceptable lactulose yield of 22%, the optimum reaction conditions were found to be catalyst loading of 12 mg per mL of MCP and isomerisation time of 120 min at 96°C.

The resulting products i.e. JAI concentrate and powder and lactulose-enriched MCP syrup (40°B) were tested for their prebiotic power in media broth and in fermented milk models. Prebiotic properties of these compounds were observed as supplementation levels increased from 0-2% to 3-4%. Based on the growth and acidification abilities of

the probiotic strains tested, the combination of *Lactobacillus casei* LC-01 with JAI, and *Lactobacillus acidophilus* LA-5 with lactulose-enriched MCP syrup were found to be the best for development of synbiotic yoghurt. The prebiotic effect of JAIP was then compared with the two commercial chicory inulin products (Raftiline® GR and Raftilose® P95). Probiotic yoghurts supplemented with 4% inulin powders were prepared from reconstituted skim milk using mixed cultures of *Lactobacillus casei* LC-01, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (1:0.5:0.5, w/w). The survival and acidifying activity of probiotic and lactic acid cultures were investigated during the shelf life of 28 days at 4°C. Incorporation of JAIP and chicory inulins resulted in a significant improvement in viability of LC-01 compared with non-supplemented yoghurt, maintaining $> 10^7$ CFU g^{-1} throughout storage time.

Additionally, the suitability of JAIP as fat replacer was determined in a set of fat-free yoghurt in comparison to three commercial chicory inulin products. Results of large deformation tests revealed that the firmness of JAIP-supplemented yoghurt was reduced to a similar level as the full-fat control yoghurt. However, small deformation results showed that the JAIP could not fully mimic milk fat to the same extent as Raftiline® HP with an average DP of 23. The rheological effects of JAIP addition were comparable to those of short-chain (Raftilose® P95 with an average DP of 4) and medium-chain inulins (Raftiline® GR with an average DP of 12).

TABLE OF CONTENTS

Table of contents

LIST OF TABLES

List of tables

LIST OF FIGURES

List of figures

LIST OF ABBREVIATIONS

*a**	redness
ΔA	hysteresis loop area
AACC	American Association of Cereal Chemists
AOAC	Association of Official Analytical Chemists
ANOVA	analysis of variance
A$_{up}$	upward curve area
B	*Bifidobacterium*
*b**	yellowness
BB	*Bifidobacterium bifidum*
CFU	colony forming unit
CHO	carbohydrate
DMRT	Duncan's multiple range test
DP	degree of polymerisation
E/S	ethanol-to-syrup ratio
ESP	egg shell powder
EU	European Union
FFCY	full-fat control yoghurt
G′,G″	elastic and viscous moduli
GIT	gastrointestinal tract
HPLC-RID	High-Performance Liquid Chromatography with Refractive Index Detector
INY	inulin-containing yoghurt
JA	Jerusalem artichoke
JAI	Jerusalem artichoke inulin
JAIP	Jerusalem artichoke inulin powder
JAIS	Jerusalem artichoke inulin syrup
JAY	JAIP-containing yoghurt
K	consistency index
L	*Lactobacillus*
*L**	lightness value
LA	*Lactobacillus acidophilus*

List of abbreviations

LAB	lactic acid bacteria
LB	*Lactobacillus delbrueckii* subsp. *bulgaricus*
LC	*Lactobacillus casei*
LCIY	long-chain inulin-containing yoghurt
LHSMP	low-heat skim milk powder
LVR	linear viscoelastic range
MCIY	medium-chain inulin-containing yoghurt
MCP	milk concentration permeate
MRS	de Man-Rogosa-Sharpe
MW	molecular weight
MWCO	molecular weight cut-off
_n_	flow behaviour index
NDOs	non-digestible oligosaccharides
NFCY	non-fat control yoghurt
OD	optical density
OF	oligofructose
OFY	oligofructose-containing yoghurt
OSP	oyster shell powder
PAHBAH	para-hydroxy benzoic acid hydrazide
PPO	polyphenol oxidase
_r_	correlation coefficient
_R²_	coefficient of determination for a regression curve or line
RSM	reconstituted skim milk
RT	room temperature
SCFAs	short-chain fatty acids
SD	standard deviation
ST	Streptococcus thermophilus
TA	titratable acidity
tan δ	loss tangent

UF	ultrafiltration
UHT	ultra-high temperature
UK	United Kingdom
USA	United States of America
UV-VIS	Ultraviolet-visible
WMP	whole milk powder

$\dot{\gamma}$	shear rate
η^*	complex viscosity
η_{app}	apparent viscosity
σ_o	yield stress
ω	angular frequency

EXPLANATORY NOTES

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. These are:

1. Where possible, the SI units have been used for the presentation of results. Temperatures have been given in degrees Celsius. Symbols of units used have followed the recent recommendations of the National Institute of Standards and Technology (NIST) (Thompson and Taylor 2008).

2. In the text, the spelling has conformed to The Oxford Advanced Learner's Dictionary of Current English (Hornby *et al*. 2005) and the British rather than the American spelling has been adopted.

3. If a lengthy name or expression is repeated, chemical symbols of elements have been used instead the name of compound (e.g. hydrochloric acid as HCl).

4. The citation and the list of references and information sources have followed the current recommendations of the Institute of Food Technologists (IFT) for the Journal of Food Science (IFT 2008).

Chapter 1

Introduction

Currently, consumers are becoming more interested in foods that contribute a positive effect on health beyond nutritional needs as they are more informed and more aware of the links between food and health than ever before. The demand by the consumers is driving food manufacturers towards new products with health promoting features. Among the functional foods, products containing prebiotic ingredients which are practically indigestible in the human gastrointestinal tract (GIT) are showing promising trends worldwide. These compounds exert beneficial effects on human health and well-being by positively promoting the growth of bifidobacteria and lactobacilli in the colon (Gibson 2004). A recent interest in prebiotic ingredients is to use them in combination with probiotic bacteria in synbiotic foods for enhanced functional properties and specific health benefits (Rastall and Maitin 2002; O'Neill 2008). Prebiotics also possess technological advantages through their reduced caloric value, non-cariogenic and fat-replacing effects. Most prebiotics originate naturally as native components in many plants e.g. onions, leek, artichoke, garlic and beans while a number of functional prebiotics are derived through diverse biochemical and/or enzymatic techniques (Van Loo *et al.* 1999; Nakakuki 2002).

Most of the commercial prebiotic products are fructan-based inulin and oligofructose (fructo-oligosaccharide) which are composed of β $(2{\rightarrow}1)$ linked fructosyl units extracted from many dicotyledonous plants (Tungland and Meyer 2002). They are currently manufactured in Belgium, France and the Netherlands from chicory roots and are marketed as Raftiline® (BENEO-ORAFTI Group, Aandorenstraat 1, 3300 Tienen,

Belgium), or Fibruline® (COSUCRA Groupe Warcoing, Rue de la Sucrerie 1, 7740 Warcoing, Belgium) or Frutafit® (Sensus Operations CV, Borchwerf 3, 4704 RG, Roosendaal, Netherlands) with increasing sales in Europe, the USA and Asia. Australia imports chicory inulin products from these European firms. Among other plants rich in prebiotics, Jerusalem artichoke (JA) accumulates similar levels of fructans as chicory roots (16-20% of fresh tuber) (Franck 2000), and is one of the most interesting plants for the industrial production of inulin, as it could be cultivated at a low cost with low input of fertilisers on any type of soil and cool climatic conditions (Parameswaran 1994). Several cultivars of JA have successfully been cultivated in Australia, especially in regions of Victoria and South Australia (Parameswaran 1996). Consequently, JA is an interesting candidate for the industrial production of inulin in Australia. The development of products from this plant would help Victorian agriculture and economy by reducing the volume of imported inulin.

Another category of prebiotic preparations is the disaccharide lactulose which consists of galactose and fructose moieties. In contrast to inulin-type fructans, lactulose does not occur naturally, but is spontaneously formed in heat-treated milk products and is also commercially produced by catalytic isomerisation of lactose under alkaline conditions (Schumann 2000). Lactulose has drug status in EU, USA as well as Australia, mainly designed for the treatment of constipation and hepatic encephalopathy (Murphy 2001). Moreover, as a prebiotic it has approval for food use as a gut health aid in Japan, the Netherlands and Italy (Schumann 2002). It is added to commercial infant formulae, baby food and several milk products. Owing to its greater sweetness and solubility than lactose, if produced economically, this sugar could also be used in baking and confectionary products instead of lactose (Parrish *et al*. 1979; Mizota *et al*. 1987).

Australian dairy industry is ranked third in the World in terms of adding value to farm gate products, valued at \$3.2 billion, and the fifth most important exporter valued at \$2.5 billion in 2006/07 (Dairy Australia 2007). Large volumes of waste streams such as whey and milk concentration permeate (MCP) are generated annually, representing major disposal problems. Milk concentration permeate is a by-product of milk ultrafiltration (UF) and contains mainly lactose along with some water-soluble minerals and vitamins. There are no microbial or rennet-induced casein fractions in MCP and practically no fat and protein because both macromolecules are rejected by the UF membrane and concentrate in the retentate (Hinrichs 2001). Some of the options available for MCP application are to use it for standardisation of milk which is currently a controversial issue for the Australian consumers of fresh pasteurised milk, or as a raw material for fermentation (alcohol or citric acid production) and/or processing into lactose powder (Harju 2001). Alternatively, the lactose in MCP could be converted to its derivatives such as lactulose, which is at least 10 times more valuable than lactose powder.

The aims of this study were therefore:

1. To develop processes for the preparation of prebiotic ingredients i.e. inulin from plant tissues and lactulose from MCP

2. To evaluate the prebiotic properties of the developed ingredients to support the growth and survival of probiotic bacteria in fermented milk

3. To study the fat-replacing properties of the extracted inulin in fat-free yoghurt.

The specific objectives of this study were:

1. To evaluate the suitability of JA grown in Australia for inulin production, and to establish the processing steps for extraction, clarification and fractionation to

   obtain JA-based inulin products in various forms i.e. syrup, precipitate and powder

2. To evaluate the suitability and catalytic activities of pulverised limestone, egg shell powder (ESP) and oyster shell powder (OSP) for isomerisation of lactulose in MCP and to optimise the process parameters e.g. catalyst loading, temperature and time for maximum lactulose transformation

3. To evaluate the growth of *Lactobacillus casei* LC-01, *L. acidophilus* LA-5, and *Bifidobacterium bifidum* BB-12 in fermented milk models in the presence of lactulose-enriched MCP syrup, and JA inulin syrup (JAIS) and powder (JAIP)

4. To incorporate JAIP at optimum supplementation level into yoghurt milk and determine its ability to support the survival of the above-mentioned probiotic bacteria during the shelf life of yoghurts at 4°C for 4 weeks

5. To evaluate the feasibility of using JAIP as fat replacer in fat-free set yoghurt.

The outcome of this study is presented eight chapters. Chapter 1 introduces the study and its aims and objectives. Chapter 2 presents an overview of prebiotic, probiotic and synbiotic concepts. Chapter 3 covers the details of material, chemical, reagents, equipment and analytical methods used in this study. Chapter 4 details the extraction of inulin-type fructans from JA tubers and reports the process optimisation to fractionate the high-molecular-weight components from JA concentrate by two different techniques. Chapter 5 covers the evaluation of the three carbonate-based catalysts for lactose isomerisation into lactulose from MCP and focuses on process optimisation for maximum lactulose yield. Chapter 6 presents the effectiveness of experimentally-prepared JAIS and JAIP, and lactulose-enriched MCP syrup in supporting the growth and acid production by *L. casei* LC-01, *L. acidophilus* LA-5 and *B. bifidum* BB-12 in media broth and in fermented milk models. Additionally, the chapter compares the

effects of JAIP and commercial chicory inulin products on survival and acid production by selected probiotic bacteria during the refrigerated storage at 4°C. Chapter 7 compares the fat-replacing effects of incorporated commercial chicory inulins with experimentally-manufactured JAIP in terms of rheological profiles of fat-free set yoghurt. The overall conclusions and the future directions of research are highlighted in Chapter 8. All references are listed in the final section.

Chapter 2

Background and literature review

The purpose of this chapter is to provide the background and review the current state of knowledge concerning prebiotics with emphasis on inulin-type fructans and lactulose as interesting prebiotics in this study. Areas covered include the definition of prebiotics, sources and occurrence, production, the significance on human health and the application in food formulation. This review also presents relevant information on probiotics i.e. the definitions and the characteristics of probiotic bacteria, the utilisation in food preparations, and the claimed benefits of the ingestion of these bacteria.

2.1   Prebiotics

2.1.1   Definition and types of prebiotics

Gibson and Roberfroid (1995) first introduced the term prebiotics by exchanging the prefix "pro" from the term "probiotic," meaning "for life" to "pre" which means "before" or "for". They defined a prebiotic as "A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health." The definition of prebiotics overlaps significantly with the dietary fibre definition; with the exception of its selectivity for certain genera or species of indigenous bacteria. Presently, only poorly digested carbohydrate (CHO) molecules, a range of di-, oligo- and polysaccharides, resistant starches and sugar polyols have been claimed to have prebiotic properties.

According to Salminen *et al*. (1998) and Gibson (2004), any food ingredient considered to be an effective prebiotic must demonstrate the following characteristics:

- Non-digestibility and non-absorption in the upper part of gastrointestinal tract (GIT);

- Fermentability by the microflora colonising the GIT;

- Selective stimulation of a one or a limited gut microflora known as probiotics;

- An ability to alter the colonic microflora towards a healthier composition by increasing the number of saccharolytic species and reducing putrefactive micro-organisms e.g. *Clostridia* and *Enterobacteriaceae*.

The prebiotic concept, similar to that of probiotic is aimed to improve the microflora content in GIT through dietary means. However, while exogenous bacteria are introduced into the colonic microflora under the probiotic concept, the prebiotic concept assumes that there is already beneficial microflora in human GIT and prebiotics stimulate the growth and metabolic activities of those bacteria (Saxelin *et al*. 2003). Using prebiotic ingredients in food formulation offers the benefits over the probiotic strategy by reducing the problems of keeping the organisms alive during transit through upper part of GIT as well as during storage (Crittenden 1999).

Most researched prebiotics fall into non-digestible oligosaccharides (NDOs), molecules containing 3 to 10 monosaccharide residues connected by glycosidic linkages (Niness 1999). Most of these occur naturally as native components in plants e.g. raffinose and stachyose in beans and peas, and oligofructose (OF) and inulin in chicory, garlic, artichoke, onion and leek (Van Loo *et al*. 1995). It is specifically noted that the term inulin used throughout this review, refers to chicory inulin unless specified otherwise. Lactose-based galacto-oligosaccharides are naturally found in small quantities in

breast milk. Some prebiotics can spontaneously occur in food products, like isomalto-oligosaccharides that are found in miso and soy sauce. However, a number of functional prebiotics are industrially synthesised from monomers, small oligosaccharides and/or natural polymers through chemical and enzymatic methods (Delzenne 2003). In some instances, lactitol and xylitol are produced by hydrogenation of lactose and xylose, respectively, while sorbitol is manufactured by reduction of glucose or fructose (Livesey 2003).

The prebiotic CHOs normally have similarity in chemical structure, however, their fermentation profiles and application advantages are often different. For example, despite the same empirical formula and molecular weight, the effect of lactitol on bacterial metabolism, faecal pH and transit time is much less pronounced than lactulose (Schumann 2002).

2.1.2 Fermentability of prebiotics

The human intestinal tract is colonised with > 400 different species of bacteria, divided into harmful, beneficial and neutral groups, at concentrations up to 10^{14} bacteria per gram (Angus *et al.* 2005). The presence of these micro-organisms and their metabolised products play an important role in the health and well-being of the host (Gibson 2004). At the time of birth, the intestine of the foetus is sterile. However, soon after the birth coliform, enterococci and/or *Clostridium* begin to colonise the large intestine. During the period of breastfeeding, bifidobacteria appear and immediately become predominant. In adults, harmful micro-organisms of the gram-positive, anaerobic genera *Bacteroides*, *Eubacterium* or anaerobic cocci predominate in the large intestinal instead of bifidobacteria. In the elderly, smaller numbers of bifidobacteria are found due to diminishing secretion of gastrointestinal juices and the increased number of harmful

bacteria (Holzapfel and Schillinger 2002). The enormous evidence from a wide range of research support the view that beneficial bacteria in the large intestine could be proliferated by prebiotics, which in turn allow strengthening of pathogen-suppressing colonisation by lowering pH in the gut and maintaining good health (Roberfroid 1998; Manning and Gibson 2004).

Figure 2.1 summarises the behaviour and effects of prebiotics in human digestive system. Orally ingested prebiotics escape digestion in the upper GIT including mouth, stomach and small intestine (Kolida *et al.* 2002; Schumann 2002) as human digestive enzymes (α-glucosidase, maltase, isomaltase, sucrase) are limited for β-glycosidic linkages with the exception of lactose (Roberfroid and Delzene 1998). They arrive virtually intact in the large intestine where the beneficial bacteria i.e. bifidobacteria and some lactobacilli ferment them successfully as a growth factor. The longer-chains NDOs allow the stimulation of microflora metabolism in more distal parts of the colon, whereas the short-chain prebiotics are fermented in the proximal part of the colon (Rastall 2003). The metabolism of prebiotics produces a variety of products, mainly bacterial cell mass, short-chain fatty acids (SCFAs) (acetic, propionic and butyric), lactic acid, and gases (CO_2, H_2, CH_4), which have varying effects on host health (Cummings *et al.* 2001). The type of SCFAs produced during the fermentation is dependent on the microflora which is stimulated by prebiotics. Van Loo *et al.* (1999) reported that inulin increases the level of SCFAs, with acetate being the primary component, followed by butyrate and propionate, while transgalacto-oligosaccharides show an increased production of acetate and propionate, and xylo-oligosaccharides increase acetate levels only. The metabolism of lactulose produces acetate and lactate (Crittendon 1999). Up to 95% of the three main SCFAs are not metabolised in colonocytes, but are absorbed from the ascending part of the colon, transported via the

circulation and further metabolised in the gut epithelium (butyrate), liver (propionate) or muscles (acetate) to provide energy (Gibson 2004). Likewise, the gases are not metabolised but excreted to faeces, breath and flatus.

Mouth ⟹ No hydrolysis, minimal bacterial breakdown

Esophagus

Stomach ⟹ No acid hydrolysis, no absorption

Small Intestine ⟹ No enzymatic hydrolysis, no absorption

Large Intestine ⟹ Supply energy → | Growing bacterial mass utilises N_2 compounds & Amino acid breakdown |

Rectum

Decrease faecal pH & → Increase osmotic pressure | Decrease colonic transit time |

Change bacterial metabolism → | Decrease bacterial urea degradation & Increase fermentation by lactobacilli & bifidobacteria |

⟱

No excretion

Figure 2.1   Behaviour and effects of prebiotics in human GIT
Source: Kolida *et al*. (2002); Schumann (2002)

Fermentability of prebiotics differs with their structure. Rycroft *et a*l. (2001) compared the fermentation properties of commercial prebiotics. The results from fluorescence in situ hybridisation (FISH) tests indicated that xylo-oligosaccharides caused the highest increase in bifidobacteria numbers at 5 h fermentation, followed by lactulose, while at 24 h fermentation the highest increases were equally recorded with isomalto-oligosaccharides and lactulose. The biggest increases in lactobacilli were found in

fructo-oligosaccharides after 5 h and soybean-oligosaccharides after 24 h of fermentation, while transgalacto-oligosaccharides resulted in the largest decreases in the number of *Clostridia*. Gas production was lowest with isomalto-oligosaccharides and highest with inulin. The results of this comparative study revealed that galactose-containing oligosaccharides supported higher bifidobacteria numbers and lower gas levels than fructose-containing oligosaccharides.

2.1.3   Inulin and oligofructoses

Inulin was first discovered by Rose, a German scientist, who in 1804 isolated "a peculiar substance of plant origin" from a hot water extract of the roots of Elecampagne (*Inula helenium*), a genus of perennial herbs of the group *Compositae*. The substance was named inulin by Thomson in 1818, but it was also called by other names e.g. helenin, alantin, meniantin, dahlin, sinanternin, and sinisterin (Franck and De Leenheer 2005).

2.1.3.1 Description and chemical structure

Inulin and oligofructoses (OF) are natural food components belonging to a class of carbohydrates known as fructans which consist of a series of oligo- and polysaccharides of fructose with β (2→1) linkages, where the terminal sugar in most chains is glucose. The β-configuration of the anomeric C2 in fructose monomers prevents fructans from digestion and this is responsible for its reduced caloric value and dietary fibre effects (Kaur and Gupta 2002). Inulin and OF are represented by the general formula GF_n and F_m, wherein G is glucosyl unit, F is fructosyl unit, n is an integer number of fructose units linked to the terminal glucose unit, and m is an integer number of fructose units linked to each other in the carbohydrate chain. The molecular structures of inulin and OF are shown in Figure 2.2 (Franck 2000).
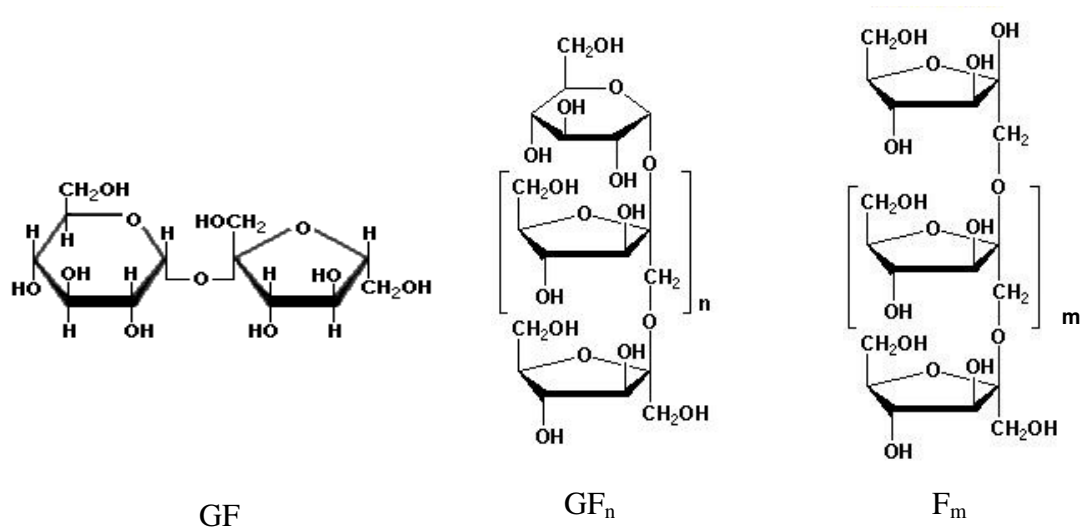
Figure 2.2   Chemical structures of sucrose (left), GF_n-type inulin (centre), F_m-type oligofructose (right)

Inulin is a mixture of GF_n molecules with $2 < n < 60$ while OF is a subgroup of inulin which is composed of GF_n and F_m with $2 \leq n$, and $m \leq 10$ (Franck 2000; Niness 1999). The term OF was introduced as a synonym for fructo-oligosaccharide in 1989 for labelling purposes (Coussement 1999). Oligofructose is manufactured by two different processes which lead to slightly different end-products. Those produced via partial enzymatic hydrolysis of inulin contain both fructose chains (F_m) and fructose chains with terminal glucose units (GF_n), whereas OF produced via transfructosylation of sucrose contains only GF_n. Both GF_n- and F_m-molecules have very similar physico-chemical properties except that F_m-type products are reducing, but GF_n-type compounds are not (Franck 2002; Roberfroid 1998).

The number of saccharides in the fructans molecule is commonly referred to as the degree of polymerisation (DP). The DP of plant inulin is rather low and depends on plant source, growing stages, climatic conditions and the duration and conditions of post-harvest storage. Native inulin which refers to inulin extracted from fresh

roots/tubers without fractionation procedure has an average DP of 10-12 while inulin from which smaller oligosaccharides have been removed has an average DP of 27-29 (De Leenheer and Hoebregs 1994).

2.1.3.2 Sources and occurrence

Fructans are present as storage polysaccharides in > 36,000 plant species, including common vegetables and fruits. Primary fructan-containing plants belong to mono- and dicotyledonous families, either *Liliaceae* (3,500 species) e.g. leek, onion, garlic and asparagus, or *Compositae* (25,000 species) e.g. dahlia, chicory and yacon (Kaur and Gupta 2002). Important sources of inulin and OF are summarised in Table 2.1. Currently, two species are suitable to produce inulin: Jerusalem artichoke (JA-*Helianthus tuberosus*) and chicory (*Cichorium intybus*), the latter being by far the most commonly used source (Franck 2000). Many cereals and other grasses contain high fructans content, but are not used for industrial production. Chicory is native to Europe and has been cultivated in several temperate areas since the 16th century. Its roots and greens are widely used for human consumption, especially as a coffee substitute after roasting. Chicory inulin is mainly stored in the fleshy root representing about 70-80% of the root's dry weight (Kaur and Gupta 2002).

Fructans are also produced by bacteria and fungi. The majority of levan-producing bacteria are found among the *Pseudomonaceae*, *Enterobacteriaceae*, *Streptococcaceae,* *Actinomycetes* and *Bacillaceae*. Bacterial inulin has a much higher DP (up to 100,000) than plant inulin (up to 200).

Table 2.1   Inulin and oligofructose content of plants commonly used in human nutrition (% of fresh weight)

Source	Edible part	Solid content	Inulin	Oligofructose
Artichoke	Leaves-heart	14-16	3-10	< 1
Banana	Fruit	24-26	0.3-0.7	0.3-0.7
Barley	Cereal	NA	0.5-1.5*	0.5-1.5*
Burdock	Root	21-25	3.5-4.0	NA
Camas	Bulb	31-50	12-22	NA
Chicory	Root	20-25	15-20	5-10
Dandelion	Leaves	50-55*	12-15	NA
Garlic	Bulb	40-45*	9-16	3-6
Jerusalem artichoke	Tuber	19-25	16-20	10-15
Leek	Bulb	15-20*	3-10	2-5
Murnong	Root	25-28	8-13	NA
Onion	Bulb	6-12	2-6	2-6
Rye	Cereal	88-90	0.5-1*	0.5-1*
Salsify	Root	20-22	4-11	4-11
Wheat	Cereal	NA	1-4	1-4
Yacon	Root	13-31	3-19	3-19

Source: Van Loo *et al*. (1995); Franck (2000)
NA, data not available
*Estimated value

2.1.3.3 Technological properties

Commercial inulin is available as a white amorphous hygroscopic and odourless powder. Its solubility is less in cold water than in hot water. Inulin has a good thermal stability although it decomposes to caramel either when heated at $\geq 178°C$ or boiled with alkali, as well as hydrolyses to fructose when heated with dilute acids. The taste of inulin ranges from slightly sweet to bland, depending on the levels of mono-, di- and oligosaccharides (Franck 2000).

Oligofructose is available as powder and clear syrup (75% dry substance). It is much more soluble than inulin (*ca*. 80% in water) at room temperature (RT) and has a good stability during usual food process operations (Franck 2000), although β-bonds between the fructosyl units are hydrolysed under acidic conditions. The taste of OF is slightly sweet without aftertaste and off-flavour. The moderate reducing power of OF gives rise to slight browning reactions during baking.

The nutritional properties of inulin and OF are similar, thus the decision to formulate with these ingredients is dependent on the desirable attributes of the finished product. For example, high DP inulin that has a creamy, fat-like mouthfeel with no added sweetness would be the suitable choice when formulating a low-fat table spread. Conversely, when formulating a low-calorie fruit preparation for yoghurts using high intensity sweeteners, OF could enhance the fruity flavour, balance the sweetness profile and mask any undesirable aftertaste (Angus *et al*. 2005).

2.1.3.4 Common intakes in diet

Inulin and OF have always been part of the daily human diet. The most commonly consumed inulin-containing vegetable is probably onion. In the 19th century, the daily intake of inulin- and OF-containing plant foods was 160-260 g by the Australian aborigines and 25-32 g by European populations. The current-day consumption is significantly lower with estimated 3-11 g in Europe and 1-4 g in the USA (Van Loo *et al*. 1995). Although there is no recommended daily intake of inulin and OF, Roberfroid (1998) suggests a minimum dose of 4 g per day for adults while doses greater than 20 g per day might induce some side effects, such as stomach cramps, flatulence, abdominal bloating and diarrhoea.

2.1.3.5 Legal and regulatory status

Nowadays inulin and OF are isolated in pure form for use in many food formulations. They are legally classified as "food" or "natural food ingredient" in all EU countries, including Switzerland and Norway. Both of these countries are excluded from food additive status in the standard lists from the EU or from Codex Alimentarius (Coussement 1999; Prosky 1999), instead they comply with the Codex Alimentarius definition of dietary fibre. The Authorities in Australia, New Zealand, Israel, Canada and Japan came to the same conclusion. In the USA, the Food and Drug Administration has accepted the "Generally Recognized as Safe (GRAS)" status of inulin and OF since 1992. In these countries, inulin and OF can be used without any specific limitation in foods and drinks. In many countries, claims concerning the dietary fibre and bifidogenic effects of inulin and OF are legally made. The name "inulin" is an accepted name for the ingredient list. Either "fructo-oligosaccharides" or "oligofructose" can be labelled for OF, but it is not acceptable for inulin to be labelled as OF.

2.1.3.6 Production of inulin and oligofructoses

In the early 1920s, inulin was first produced on a pilot scale in Deutsche Kulorfabrik by Schöne and later was extracted on an industrial scale in 1927 by Belval. In 1931, an improvement of the extraction process was patented by Raffinerie Tirlemontoise, Belgium (Franck 2000). The extraction of inulin and OF from the plant tissue is similar to that of extraction of sucrose from sugar beets. The production process involves three general steps: extraction of raw inulin by hot water diffusion, followed by purification of the inulin extract and then drying of the purified juice to a pure inulin powder (Angus *et al.* 2005). The finished powder typically contains 6-10% glucose, fructose and sucrose and has an average DP of 10-12 (Niness 1999). Optionally, inulin extract is

fractionated to remove mono-, di- and oligosaccharides to achieve various grades of inulin.

Several techniques for inulin extraction from various plants have been reported e.g. extracting ground dry JA tubers or dried slices of JA tubers (Marchand 1951; Yamazaki *et al.* 1989), wet milling or crushing chicory roots (Leite Toneli *et al.* 2007), globe artichoke bracts (Lopez-Molina *et al.* 2005), JA tubers (Laurenzo *et al.* 1999) or dahlia tubers (Mitchell and Mitchell 1995) or preferably, soaking freshly sliced or shredded chicory roots with hot water (Berghofer *et al.* 1993). The extraction is performed at varying temperatures between 60 and 85°C sometimes up to 100°C for 20 min to 1 h. Recently, ultrasound has been applied to facilitate the extraction of inulin from JA tubers with the maximising yield of 84% under optimum conditions (Lingyun *et al.* 2007). Inulin extract is subsequently subjected to a depuration method (i.e. liming and carbonation or another flocculation technique, and filtration), followed by refining (i.e. treatment over ion-exchange resin column, treatment with active carbon and filtration). Alternatively, ultrafiltration (UF) membranes with appropriate molecular weight cut-off (MWCO) can be employed to purify inulin extract where non-inulin components e.g. ash and nitrogenous substances pass into the permeate while inulin is kept in the retentate (Berghofer *et al.* 1993; Laurenzo *et al.* 1999; Lopez-Molina *et al.* 2005). Inulin is widely commercialised in powder form due to ease of handling, transportation, storage and application, and various methods are applied to convert liquid extract of inulin to dry form i.e. spray drying, drum drying, freeze-drying and microwave vacuum drying. To reduce energy costs of drying, the liquid extract is frequently concentrated by evaporation before drying.

The presence of mono- and disaccharides makes inulin difficult to dry, handle and store due to their hygroscopic nature. On the other hand, in liquid inulin products, the high DP inulin fractions tend to settle to the bottom during the storage. Therefore, many attempts are made to narrow DP range of products and achieve uniform quality products. Moreover, the properties of inulin are highly DP dependant, for example, the higher DP fractions are used as fat substitutes while the lower DP components are used as sugar replacers. In the following section, a series of methods employed to enrich high DP inulin fractions are reviewed.

- **Chromatography**

In US Patent 5,660,872, Van Loo *et al.* (1997) described a process for fractionating inulin by column chromatography techniques. After being purified through a cation-exchange column (K^+ form) at 65-75°C, mono- and disaccharides are removed effectively, and the inulin with an average DP of 10 and less than 1% low-molecular-weight (MW) sugars is obtained.

- **Solvent precipitation**

In US patent 2,555,356 Marchand (1951) proposed the addition of acetone to the aqueous solution to decrease the solubility of high DP inulin. Later in US Patent 6,303,778 Smits *et al.* (2001) precipitated long-chain inulin from inulin syrup in the presence of alcohol, and separated it by filtration or centrifugation. By this method, inulin with almost double or higher DP than that of the source inulin was obtained with an average yield of 30-35%.

- **Ultrafiltration**

Thomann *et al.* (1995) proposed the separation of short-chain inulins from long-chain inulin by UF or nanofiltration. Various UF membranes were tested for their suitability

to separate inulin from mono- and disaccharides. In US Patent 5,968,365, Laurenzo *et al.* (1999) employed a series of UF membranes with different MWCO ranges to achieve a series of inulin fractions with different MW distribution.

- **Crystallisation**

Inulin with a higher DP was prepared by crystallisation techniques as described by Silver (2003) in US Patent 6,569,488. The process principally includes holding the inulin extract in a settling tank at 0 to +7°C for 24-48 h. Inulin fractions with a higher DP are less soluble at low temperature and thus precipitate to the bottom at the faster rate than those with a lower DP. The precipitated inulin is then siphoned out, drained out or separated by centrifugation from the mixture. Lopez-Molina *et al.* (2005) reported that the precipitation yield of inulin was higher at lower temperature and longer precipitation time, and found the optimum conditions for maximising the yield to be −24°C and 12 h. Leite Toneli *et al.* (2007) verified phase separation when concentrated inulin syrup was subjected to low temperature treatment.

Similarly, Berghofer *et al.* (1993) and Mitchell and Mitchell (1995) reported the crystallisation of concentrated inulin extract (40-70%) after subsequent cooling, grafting with inulin crystals and storing at +1 to −15°C for 12-30 h.

Moerman *et al.* (2004) compared three different methods (UF, crystallisation, and solvent precipitation) to enrich the high-MW fractions of chicory and dahlia inulins. It was noted that the utilisation of membranes with MWCO between 2 and 5 kDa increased the average DP of chicory inulin from 8 to 22 and that of dahlia inulin from 29 to 43. The crystallisation reaction of 10% dahlia inulin solution at RT for 6 days gave the same DP as using UF, but with a much higher yield. With solvent-precipitation, acetone was shown to be the best solvent system to increase the DP,

followed by ethanol and methanol. In the case of ethanol, the DP could be raised to 25 for chicory inulin and up to 40 for dahlia inulin.

- **Enzymatic treatment**

In the Japanese Patent 04211388, Manfred (1992) disclosed the enzymatic treatment to achieve inulin products with low glucose, fructose and sucrose contents. The enzyme α-glucosidase was added to inulin solution at pH 5.4 to break down the low DP fractions (mono- and disaccharides) which were then removed by column chromatography. The resulting product contained > 50% of tri-, tetra- and pentasaccharides. Similarly, in US Patent 5,478,732, Kunz *et al*. (1995) prepared long-chain inulins with an average DP of > 20 using inulinase (NOVO, SP 230). The optimum reaction conditions were inulin syrup solids content of 40-50%, pH of 4.8-5.0 and 40-60°C, which resulted in a yield of up to 45% long-chain inulin.

When low DP inulin fraction i.e. OF is the desired product, a hydrolysis step using either acids or enzymes is included after extraction stage. Using an inulase enzyme, inulin is broken down into short-chain lengths, varying from 2 to 10 with an average DP of 4. The resulting products also contain *ca*. 5% glucose, fructose and sucrose on dry basis. Alternatively, OF is synthesised from sucrose by the transfructosylation using enzyme β-fructofuranosidase which attaches additional 2-4 fructose monomers to sucrose molecule (Angus *et al*. 2005). By-products of the process e.g. glucose, fructose and unreacted sucrose can be removed by chromatography (Crittenden and Playne 1996).

2.1.4 Lactulose

2.1.4.1 Structure and general properties

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a disaccharide derived from lactose (4-O-β-D-galactopyranosyl-D-glucose) with similar MWs (342.3) and the same empirical formula ($C_{12}H_{22}O_{11}$) as shown in Figure 2.3 (Schumann 2002). In contrast to lactose, the β (1→4) glycosidic bond of lactulose is not degraded by human and animal digestive enzymes but is metabolised by saccharolytic colonic bacteria (Kontula *et al*. 1998; Salminen *et al*. 1998). In the large intestine, lactulose stimulates the growth of selected micro-organisms i.e. *Bifidobacterium*, *Lactobacillus* and *Streptococcus* and crowds out potentially harmful bacteria i.e. *Bacteroides*, *Clostridium*, coliforms and *Eubacterium* (Crittenden 1999).
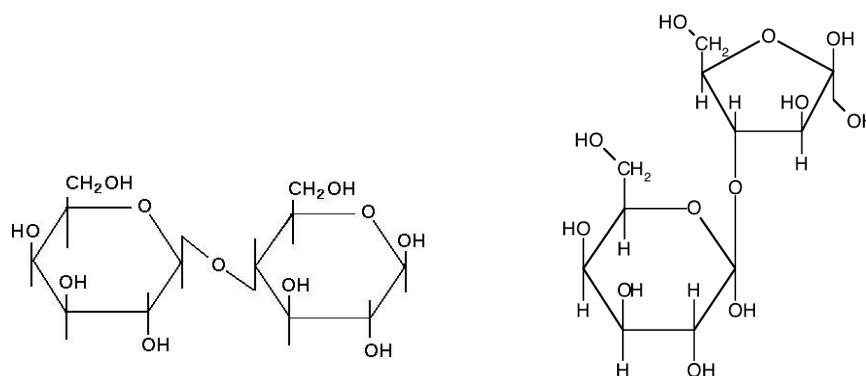
Figure 2.3   Chemical structures of lactose (left) and lactulose (right)
Source: Schumann (2002)

Commercially available lactulose is produced at *ca*. 50,000 tonnes per annum mainly in Germany, Denmark, Austria, Italy, the Netherlands, UK and Japan, and is marketed in two major forms: dry lactulose as a powder or granulate, and lactulose syrup. Lactulose syrup is a yellowish, clear and odourless liquid with a sweet taste due to the presence of other sugars. Dry lactulose is a white, crystalline and odourless powder with sweetness 60-80% that of sucrose and similar humectancy to sucrose (Schumann 2002). The powder

is soluble in water, poorly soluble in methanol and insoluble in ether. An overview of the chemical composition of dry and liquid lactulose is summarised in Table 2.2.

Table 2.2   Principle composition of dry and liquid lactulose

Compositions	Powder (g per 100 g)	Syrup (g per 100 mL)
Lactulose	> 95.0	63.4-70.0
Galactose	≤ 2.5	≤ 15.0
Lactose	≤ 2.0	≤ 9.0
Epilactose	≤ 1.5	≤ 7.0
Tagatose	≤ 3.0	≤ 3.0
Fructose	≤ 1.0	≤ 1.0

Source: Schumann (2000)

2.1.4.2 Sources and occurrence

Lactulose is not present in nature but is found in small amounts as a secondary product of catalyst-free isomerisation of lactose in high heat-treated milk. It has been proposed by the International Dairy Federation (IDF) and by the European Commission (EC) as an indicator of the severity of heat treatment and for the classification among pasteurised, UHT and in-container sterilised milks. Both international bodies suggest 600 mg L^{-1} of lactulose as a marker for distinguishing between the UHT and sterilised milk while in Germany, a limit of 400 mg L^{-1} is adopted to protect the quality of milk from excessive heat-load (Marconi *et al*. 2004).

Lactulose synthesis is based on the molecular rearrangement of lactose under alkaline conditions, known as a Lobry de Bruyn-Alberda van Ekenstein, in which the glucose moiety is converted into a fructose residue (Holsinger 1999). Figure 2.4 shows a scheme of lactose alkaline isomerisation. Besides the epimerisation of lactose into lactulose, the reaction routes comprise the subsequent degradation of keto-disaccharides to galactose

and isosaccharinic acids by alkaline-peeling and β-elimination, and the isomerisation of lactose into small amounts of epilactose.
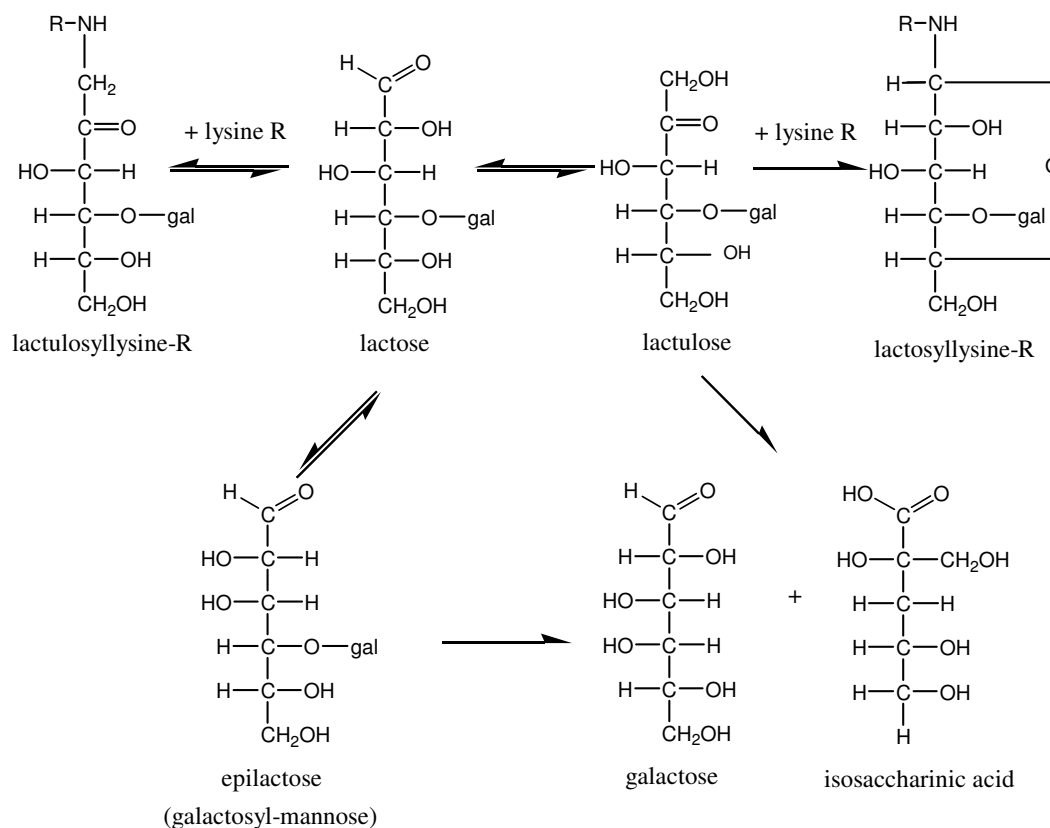
Figure 2.4   Model of the alkaline isomerisation of lactose
Source: Andrews (1986); Berg and van Boekel (1994)

2.1.4.3 Legal and regulatory status

Lactulose is known as a medicinal product for humans use since 1950s and has drug status in over 100 countries, being an over-the-counter drug in EU and a prescription drug in the USA, but not as a food additive which clearly differs from other prebiotics. The two major uses are in the treatment of hepatic encephalopathy (up to 90 g per day) and chronic constipation (10-40 g per day). It is also suggested for the treatment of salmonella carrier state in some countries. Lactulose is accepted for use as food or drink additive and as a pure prebiotic in Italy and the Netherlands (Schumann 2000), and as

special food for health maintenance and for protection against enteric infections in Japan since 1992 (Holsinger 1997).

2.1.4.4 Production of lactulose

Various catalysts are used as proton acceptors to facilitate the conversion from aldose form of lactose to ketose form of lactulose. Aider and de Halleux (2007) listed the important criteria for choosing the catalysts:

- Offering a maximum yield of lactulose with minimum level of by-products;

- Non-toxic and safe for environment;

- Low cost and available in great quantity;

- Giving repetitive results of isomerisation; and

- No difficulty for removal after isomerisation.

However, the catalysts used currently present both positive and negative aspects and the ideal one has not yet been found. Depending on the catalytic systems, the conversion of lactose to lactulose may vary from 20 to 80%. This review summarises various catalysts known for the preparation of lactulose solution.

- **Lactose isomerisation by alkali hydroxides**

Lactulose was prepared for the first time by Montgomery and Hudson in 1930, by heating lactose in aqueous solution in the presence of calcium hydroxide at 35°C for several days. Later, stronger alkaline reagents i.e. potassium hydroxide, barium hydroxide and sodium hydroxide (NaOH) were studied for their catalytic abilities. A process is described by Nagasawa *et al*. (1974) in US patent 3,816,174 where lactose solution was combined with 0.27-0.54% NaOH (lactose base) and heated > 70°C. Dendene *et al*. (1994) determined the kinetics of lactulose formation and lactose

degradation in NaOH medium at different OH$^-$ concentrations and temperatures. Their experimental data showed that the isomerisation of lactose (375 g L^{-1}) at pH 9.5 and 70°C resulted in a maximum lactulose production (20%) within 60-65 min, in accordance with those calculated from kinetic models. Zokaee *et al*. (2002a) investigated the formation of lactulose in sweet cheese whey permeate using NaOH as a catalyst. The optimal pH and temperature for the isomerisation were found to be 11.5 and 70°C, respectively.

Preparation of lactulose by alkali hydroxides produces low yield and large number of by-products which are coloured. Moreover, lactulose must be isolated from unreacted lactose, degradation products and metal salts. In most cases, lactulose syrup is purified and demineralised by ion-exchange resins, bromolysis and electrolysis.

- **Lactose isomerisation by amines**

A further group of processes use amines for lactulose preparation. Hough *et al*. (1953) used ammonia for the isomerisation, but the use of primary and secondary amines led to the formation of glycosylamines and Amadori compounds as side reaction by-products. Later, in US Patent 3,514,327, Parish (1970) employed triethylamine, a tertiary amine for the conversion of lactose into lactulose and achieved 32% lactulose yield.

- **Lactose isomerisation by phosphates and sulphites**

The fact that sulphites and phosphates have the characteristic to prevent oxidation of disaccharides allows the use of high temperature and high lactose concentrations in the process (Aider and de Halleux 2007). According to US Patent 4,264,763 (Gasparotti 1981), 0.5-2 M disodium phosphate (2.1-8.6% lactose base) was added to lactose monohydrate solution (55-65% concentration), followed by heating at 104°C for 20-240 min. The maximum yield of lactulose was 20% within 120 min.

- **Lactose isomerisation by aluminates and borates**

Another group of catalysts used are amphoteric electrolytes, including borate and aluminate which shift the reaction in favour of lactulose and prevent its degradation to by-products. In US Patent 4,957,564 (Carobbi and Innocenti 1990) a 25-50% lactose solution was added with sodium aluminate solution at a molar ratio of 0.3:1 up to 1:1 calculated on lactose basis (pH 11-12), and heated at 50-70°C for 30-60 min. At the end of the process, the mixture was adjusted to a pH of 4.5-8.0 with sulphuric acid. However, the elimination of sodium sulphate produced under these conditions was difficult. An improvement to this work was proposed in the US Patent 6,214,124, where Carobbi *et al*. (2001) proposed using CO_2 gas under pressure to neutralise the reaction mixture, instead of sulphuric acid, followed by using filter press to separate aluminum hydroxide from the lactulose solution.

The preparation of lactulose using borax as catalyst was mentioned in US Patent 5,071,530 by Krumbholz and Dorscheid (1991). An aqueous lactose solution at pH 8.3 was heated in the presence of borax to above 80°C. This catalyst facilitated the reaction with a higher lactulose yield (70-80%) and minimum by-products, however, the process was not deemed satisfactory for industrial production because of the difficulty presented in the elimination of boric acid.

Other works investigate the use of boric acid mixed with other catalysts. One example is a study by Hicks and Parrish (1980) who achieved a high yield of lactulose (up to 87%) and low levels of monosaccharides from degradative side reaction with the combination of tertiary amines and boric acid. The addition of tertiary amines was aimed to achieve the desired pH with minimal use of boric acid. Hicks *et al*. (1984) demonstrated that adjusting the pH of sweet cheese whey permeate to 11 with either triethylamine or

NaOH before adding an equivalent mole of boric acid resulted in high yields of lactulose (> 80%). Five purification procedures were also mentioned to obtain lactulose syrup.

Zokaee *et al*. (2002b) compared three catalytic systems (i.e. NaOH, boric acid and NaOH, and sodium aluminate) for lactose isomerisation. The maximum conversion achieved was *ca*. 20% and total by-product was 5-7%. On the other hand, treatment of aqueous solutions of lactose-boric acid (mole ratio 1:1) with NaOH at pH 11 increased lactulose yield to 77-80%. The use of sodium aluminate at a mole ratio of 2 also resulted in a high yield of 68-70% with fewer by-products than that of borate system.

- **Lactose isomerisation by sepiolites**

In addition to homogenous catalysts, a group of processes employ heterogeneous catalysts for lactose isomerisation. Troyano *et al*. (1996) first investigated the catalytic activity of natural sepiolite (a hydrated magnesium silicate) at varying catalyst loadings, temperatures and reaction times. Besides lactulose small amounts of epilactose, galactose, tagatose and 3-hydroxypentulose were also produced. To attain a 20% conversion of lactulose, sepiolite loading of 6% (w/v) at 100°C was needed. In 1999, de la Fuente *et al*. reported that the replacement of Mg^{2+} of sepiolite with alkaline ions (Li^+, Na^+, K^+ and Cs^+) significantly improved the catalytic ability of the sepiolite, where similar conversion was obtained at lower temperature (90°C) and lower sepiolite loading of 1.5% (w/v). Villamiel *et al*. (2002) broadened the use of alkaline-substituted sepiolites (Na^+ or K^+) for lactulose isomerisation in milk permeates. It was found that the activity of Na^+ sepiolite was higher than that of K^+ form and the control of washing cycles of sepiolites during their preparation was critical for process optimisation.

Chapter 2

- **Lactose isomerisation by ion-exchange resins**

Another method for lactulose production is using strongly alkaline ion-exchange resins for the isomerisation. Carubelli (1966) reported that lactose was converted into lactulose when subjected to ion-exchange chromatography on a column of Dorex I (borate) at RT. By this method, the addition of catalyst was unnecessary and the final products could be used in functional foods without the additional stages for demineralisation and decolourisation (Aider and de Halleux 2007). However, these results are not consistent with those of Mahran *et al*. (1995) who employed anion-exchanger in either borate form or OH^- as isomerising agents and found no lactulose formation in the reaction mixture.

Most lactulose is marketed as impure syrup containing *ca*. 80% solids, with a lactulose content of 66% and varying amounts of related saccharides as shown in Table 2.2. The presence of these by-products is acceptable for food applications purpose, but undesirable for pharmaceutical purpose and specialised foods. Therefore, attempts have been made to remove such by-products after the isomerisation. In Japanese Patent 02-124895, Fumihiko (1990) proposed the use of two types of ion-exchange resins for purification of lactulose syrup. The syrup was first subjected to a packed bed of Na^+-type strongly acidic cation-exchange resin, followed by the Ca^{2+}-type resin to eliminate mono- and disaccharides, respectively. Water was used as an eluent and the temperature during treatment was kept at 50 to 80°C. Recently, several extraction procedures e.g. supercritical fluid extraction, solid-phase extraction and pressurised liquid extraction have been developed for the purification of lactulose from a mixture with lactose to replace traditional processes (Ruiz-Matute *et al*. 2007).

Excess colour of lactulose syrup is also undesirable. In an attempt to prevent the development of brown colour during isomerisation, several workers have suggested

using a mixture of catalysing and a reducing agents e.g. sodium sulphite, sodium bisulphite or sodium phosphate. According to US Patent 4,536,221 by Carobbi *et al.* (1985), lactose solutions of 60-70% were added with equal parts of magnesium oxide (catalyst) and sodium hydrosulphite (reducing agent) at the rate of 0.05-0.2% by weight of lactose. Thereafter, the mixture was heated to 90-100°C for 10 min, cooled, followed by filtration or centrifuging. The clear solution obtained was then passed through ion-exchangers to remove the ions of Mg and Na and organic acids. In Russian Patent 2133778, Dykalo *et al.* (1999) described the use of sodium sulphite mixed with NaOH in lactose solution of 20-65%. The catalyst concentration was 0.1-0.5 mole per kg lactose to raise the pH to 10.5-12.0 and the temperature used was 80-105°C. At the end of the process, the solution was treated by electrodialysis for purification from reagents.

Several studies have aimed to remove coloured by-products after the isomerisation. In the US Patent 5,026,430, de Haar and Pluim (1991) described a method for preparing lactulose products with an acceptable colour for pharmaceutical purposes by the treatment with hydrogen peroxide (H_2O_2). As oxidant agent, H_2O_2 can convert the degradation products of lactulose into acids which later are removed by ion-exchangers. It is suggested to add H_2O_2 to lactulose syrup at a temperature of *ca.* 50°C. In the study by Montilla *et al.* (2005) who treated milk permeate with egg shell powder, decolourisation of lactulose syrup was effectively achieved with treatment with activated carbon and membrane filtration. Activated carbon, added at a rate of 5-15 mg per mL removed 65-92% of the colour within 5 min.

2.1.5 Health-benefits of prebiotics

A number of health-related benefits have been ascribed to prebiotic consumption (O'Neill 2008), including relieving constipation (Roberfroid 1993), reducing the risk of atherosclerosis by modulating lipid metabolism (Delzenne and Williams 2002), decreasing the risk of osteoporosis by improving mineral absorption (Cashman 2002; Scholz-Ahrens *et al.* 2001), reducing the risk of colon cancer (Rumney and Rowland 1995), preventing intestinal infections (Naughton *et al.* 2001; Manning and Gibson 2004) and stimulating the immune system of the body (Saavedra *et al.* 1999; Macfarlane and Cummings 1999). Figure 2.5 summarises the currently proposed mechanisms of human heath benefits by prebiotics.
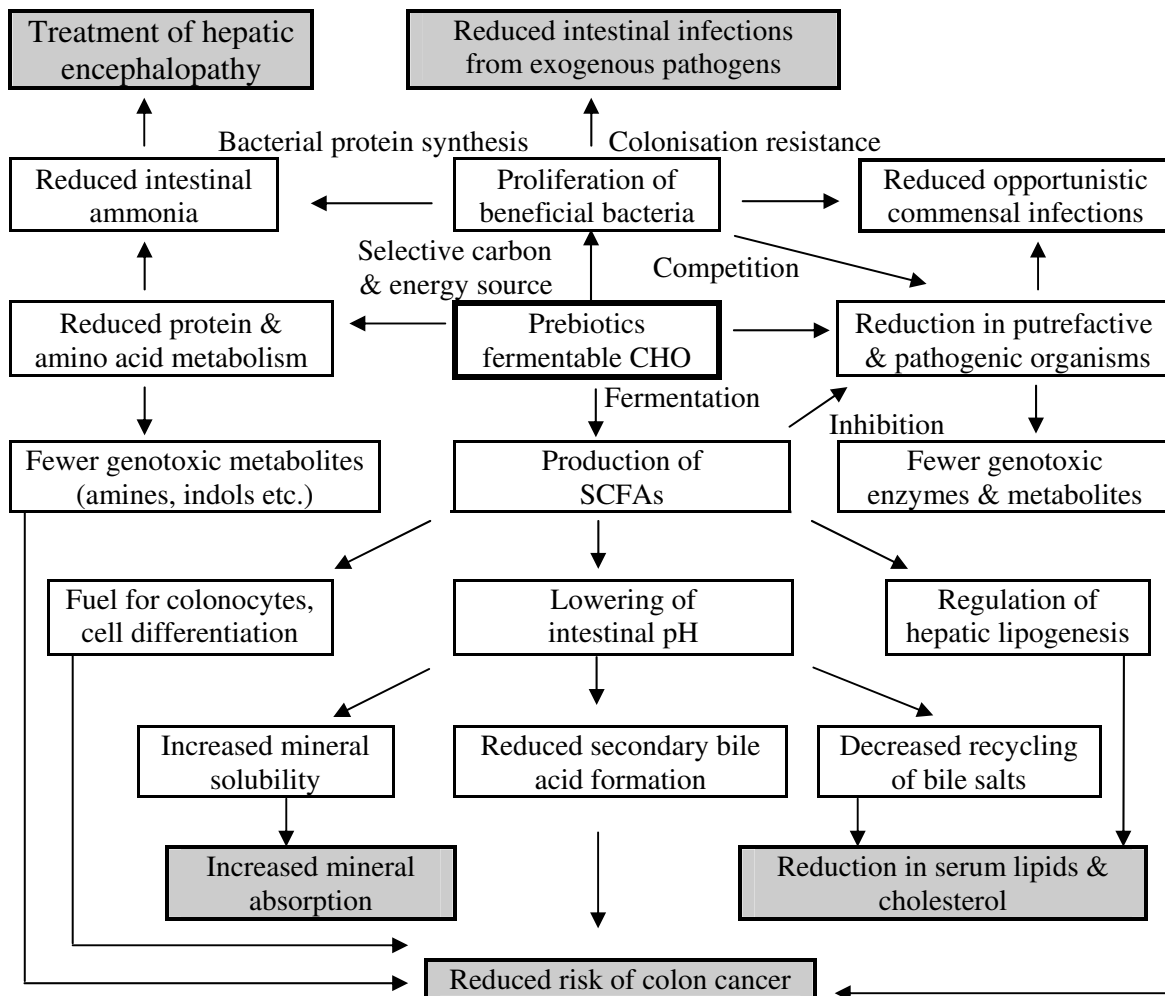
Figure 2.5   Proposed mechanisms of prebiotic effects on human heath benefits
Source: Crittenden (1999)

2.1.6   Prebiotics in food application

Prebiotic foods are "food products that contain prebiotic ingredients in an adequate matrix and in sufficient concentration, so that after their ingestion, the postulated benefit is obtained" (Saxelin *et al*. 2003). The potential for prebiotic is gaining interest through its reduced energy value, hypocariogenic and bifidogenic properties, and dietary fibre effects, depending on its chemical structure, MW and levels of mono- and disaccharides (Roberfroid 1993). The main dairy products formulated with prebiotics include yoghurts, yoghurt drinks, spreads, fresh cheeses, and milk. Other emerging food products are kefir, sport products, functional waters, nutrition bars, weight loss products, soymilk, green foods, mineral supplements, nutraceuticals and also pet foods (Kaur and Gupta 2002; Niness 1999).

2.1.6.1 Bifidus promoting agents

Inulin-type fructans are amongst the most studied and well-established prebiotic ingredients. Their selective stimulation of the growth of bifidobacteria and the production of SCFAs as end products of fermentation has been confirmed in many *in vitro* and *in vivo* studies (Gibson and Wang 1994; Roberfroid 2001). They are increasingly used in functional foods, especially dairy products and breads at typical amounts of 1-6% or *ca*. 3-8 g per serving to allow the bifidogenic claims (Coussement 1999; Franck 2000).

Compared to inulin-type fructans, other types of NDOs are either branched or composed of several types of glycosidic bonds, which makes them less readily accessible for bacterial hydrolysis. Oligofructose is somewhat more prone to upper GIT hydrolysis than inulin (Roberfroid 1998). However, F_m- and GF_n-type molecules had a similar prebiotic effect in humans (Menne *et al*. 2000). The other commercially available

prebiotics are lactulose, galacto-, xylo- and soybean-oligosaccharides (Murphy 2001; Cummings *et al*. 2001). Lactulose is the original prebiotic, know since 1957 and is currently marketed as bifidus factor for infant formula. Its ingestion contributes to the growth of gut microflora in bottle-fed babies in the same way as breast-fed babies (Strohmaier 1998; Salminen *et al*. 1998).

2.1.6.2 Fibre enhancer

Another interesting functionality of prebiotics in food formulations is their roles as dietary fibre. The dietary fibre is defined as "remnants of plant cells resistant to hydrolysis by the human digestive enzymes" (Trowel and Burkitt 1986). Unquestionably, several prebiotic substances i.e. inulin, OF and resistant starch fall under this definition (Flamm *et al*. 2001). Moreover, from a physiologic point of view the effects of these prebiotics on intestinal function, blood lipid parameters and caloric value meet the properties of dietary fibres (Roberfroid 1993; Gibson *et al*. 1995; Hidaka *et al*. 1986). These effects are related to reduced risk of coronary heart disease, colon cancer and other colonic disorders and fit into the concept of dietary fibre.

Compared to insoluble fibre e.g. bran, soluble prebiotic ingredients are more palatable and have superior functional properties (Dreher 1999). Resistant starch is related to increased fibre content in baked goods and pasta products without the grainy or discoloured appearance (Murphy 2001). The use of inulin or OF in baked goods allows not only fibre enrichment, but also better moisture retention properties and improved texture (Franck 2000; Tungland and Meyer 2002). Their solubility also allows fibre incorporation in drinks, dairy products, soup and table spread. Such additions are in the range of 3-6 g per serving and increase up to 10 g in extreme cases (Coussement 1999).

2.1.6.3 Sugar replacer

Several types of NDOs and polyols can replace sugar sweeteners due to their physiological characteristics i.e. having minimal contribution to energy intake and performing bulking properties. They are termed sugar substitutes, sugar replacers, or alternative sweeteners.

Oligofructose possesses functional properties similar to glucose syrup and is frequently used to replace sugar in various foodstuffs, mainly in dairy and bakery products e.g. chocolate filling, biscuits, chewing gums, confectionary, dairy desserts, ice-cream and fruit preparations in the range of 2-6 g per serving (Franck 2000). Oligofructose contributes humectancy to baked goods, depresses the freezing point of frozen desserts and acts as a binder in nutrition bars, in much the same way as sugar. The solubility of OF is higher than sucrose but its sweetness is *ca*. 30% of sucrose (Angus *et al*. 2005). In combination with intense sweeteners e.g. acesulfame K and aspartame, OF provides a desired sweetness profile and a better-sustained flavour with reduced aftertaste (Weidmann and Jager 1997; Kaur and Gupta 2002).

A number of studies with lactulose have been carried out on yoghurt, cookies, cake, chocolate, etc. in aspects of flavour enhancing properties, a favourable browning behaviour and the behaviour during processing which allow replacement of sucrose (Schumann 2002). However, because of its laxative characteristics, lactulose is utilised in limited quantities in foods.

The fact that the sugar alcohols e.g. sorbitol, mannitol, xylitol and lactitol, and NDOs e.g. OF and lactulose contribute fewer calories (1-2 kcal g^{-1}) than sugar (4 kcal g^{-1}) (Salminen *et al*. 1998; Oku and Nakamura 2002; Murphy 2001) allows the development of sugar-reduced low-energy products. This is particularly true in sugar-free confections

e.g. hard candies, chewing gums and marshmallows, sugar-free added baked goods and ice-creams. More importantly, these low-calorie ingredients offer advantages over traditional digestible CHOs like sucrose, glucose and fructose in terms of having low-glycaemic index (potentially helpful for diabetics and those suffering from cardiovascular disease), low-insulinaemic (potentially useful for obesity) and non-cariogenic (sugar-free tooth-friendly) (Hidaka *et al.* 1986; Schumann 2002).

2.1.6.4 Fat replacer

Specific kinds of prebiotic oligosaccharides have been developed as fat replacers and texture modifiers. The specific functions are:

- To reduce total fat or partial fat content

- To modify smoothness and creaminess

- To improve mouthfeel and/or increase perception of body and richness

- To improve an overall eating quality and an acceptable appearance.

Inulin is an example of such prebiotics. Inulin is well-recognised for its ability to replace fat in the manufacturing of low-calorie foods. When inulin is mixed with aqueous liquid or water, it forms gels composed of a tri-dimensional gel network of insoluble sub-micron crystalline inulin particles with large amounts of immobilised water. This inulin gel provides the same texture and mouthfeel as fat (Franck 2000, Silva 1996). The chain length of inulin plays a key role in gel quality. A high DP inulin facilitates gel formation at lower concentrations and can be formulated to replace fat up to 100%. Fat replacement by inulin is successfully applied in most water-based foods e.g. dairy products, frozen desserts, dressings, table spreads, sauces, soups and even in meat products, but not in dry foods e.g. snacks, bakery and confectionery products (Murphy 2001). Typically, 1 g of fat can be replaced by a 0.35 g of inulin and

consequently amounts of 2-6 g per serving are practically used in most foods (Coussement 1999). Formulating foods with inulin also helps to maximise freeze-thaw stability and minimise phase separation of emulsions, dispersions, mousses, foams and creams due to its ability to immobilise water and to work synergistically with most gelling agents e.g. gelatine, alginate, carrageenan, gellan gum and maltodextrin (Bishay 1998). Inulin also gives a richer texture to liquid products and spreads and provides crispness and expansion to extruded snacks and cereals.

In addition to inulin, resistant starch is also used as a fat mimetic and a texture enhancer in low-moisture foods e.g. crackers and cookie. In extruded cereals, the use of resistant starch improves crispness and expansion (Murphy 2001).

Chapter 2

2.2 Probiotics

2.2.1 Definition of probiotics

The term "probiotics" derived from Latin and Greek, meaning "for life" has been defined in many ways in the past. In 1965, Lilly and Stillwell introduced probiotics as "substances secreted by one micro-organism which stimulates the growth of another". In 1971, Sperti applied the term to tissue extracts that stimulate microbial growth (Schrezenmeir and de verse 2001). Later in 1974, Parker proposed the term for organisms and substances which influenced the intestinal microflora and had beneficial effects on animals. The term "substances" is imprecise which would include even antibiotics. Therefore, in 1989 Fuller defined probiotics as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". In 1992, Havenaar *et al*. agreed to broaden Fuller's definition of a probiotic as "a viable mono- or mixed culture of micro-organisms which applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora" (Suskovic *et al*. 2001; Lourens-Hattingh and Viljoen 2001). In 1996, Salminen defined probiotic as "a live microbial culture or cultured dairy product which beneficially influenced the health and nutrition of the host". Schaafsma (1996) also re-defined probiotics as "living micro-organisms which upon ingestion in certain numbers, exert health benefit beyond inherent basic nutrition". This definition fits well with that of functional foods. Recently, Schrezenmeir and de verse (2001) proposed the definition of probiotic as "a preparation of a product containing viable, defined micro-organisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in the host". This definition is close to the definition given by Havenaar *et al*. (1992).

2.2.2 Characteristics of probiotic bacteria

The lactic acid bacteria (LAB) have long been used for food fermentation and preservation. Renewed interest in the role of LAB for human health originated from the observations of Metchnikoff in 1908, that the longevity and well-being of Bulgarians were attributed to their high yoghurt consumption (Lourens-Hattingh and Viljoen 2001). Many decades later the importance of fermented products in human nutrition and the significance of an indigenous microflora in the GIT as a natural resistance factor against pathogenic micro-organisms together with the emergence of antibiotic-resistant bacteria have contributed to the concept of probiotic. Probiotic bacteria are known for their protective role in the host against colonisation of non-indigenous micro-organisms and are increasingly included in human diets to re-establish the intestinal microflora balance and help maintain good health (Kalantzopoulos 1997).

There is no agreement in the criteria for classifying a strain of bacterium as a probiotic, however, according to O'May and Macfarlance (2005); Ziemer and Gibson (1998) the probiotic micro-organisms must possess the following characteristics:

- The bacterium must survive harsh conditions of the upper GIT (e.g. gastric acid in the stomach and bile in the small intestine) and then flourish in the intestine;

- The bacterium must be able to compete with the normal microflora, including the same or closely related species, and potentially resistant to bacteriocins, acids, and other antimicrobials produced by residing microflora;

- The organism, its fermentation products or cell components must not be toxic, pathogenic, mutagenic or carcinogenic;

- The bacterium should be antagonistic towards carcinogenic and pathogenic micro-organisms and must be genetically stable; and

- The bacterium must be easily reproducible and remain viable during processing and storage.

Probiotics are usually bacterial members of the normal human intestinal flora. The predominantly selected probiotics are species of *Lactobacillus* (naturally found in the human small intestine) and *Bifidobacterium* (naturally found in the human large intestine) while probiotic yeasts i.e. *Saccharomyces boulardii* have limited uses in the food industry (Tamime *et al*. 2005) although it has been used since the 1950s for the prevention of various diarrhoea. Because of not typical of human microflora, propionic acid bacteria are also not of much interest. Mostly, these species are used to produce flavour and eyes in Swiss-type cheese (Champagne *et al*. 2005). Table 2.3 shows lists of micro-organisms used in dairy and pharmaceutical probiotic preparation. In views of technological aspects, lactobacilli may be a preferred choice to incorporate into dairy food products as they are facultative anaerobes and tolerate exposure to oxygen during processing, transport and storage, however, in aspects of biological activity, bifidobacteria produce more potent anti-microbial activities than lactobacilli (Rastall and Gibson 2004).

2.2.2.1 Genus *Bifidobacterium*

Bifidobacteria was first called *Bacillus bifidus* and were also assigned to several genera such as *Tissieria*, *Nocardia*, *Lactobacillus*, *Actinomyces*, *Bacterium* or *Corynebacterium* (Doleyres and Lacroix 2005). They are among the first micro-organisms to colonise the intestine of a newborn infant and account for up to 95% of all culturable bacteria in the colons of breast-fed infants. Bifidobacteria constitute only 5-10% of the total intestinal flora of children and adults. The numbers of bifidobacteria

decline with age and changes in eating habits, with the elderly demonstrating the lowest populations of bifidobacteria (Champagne *et al*. 2005).

Table 2.3   Micro-organisms used as probiotics

Genus *Lactobacillus*	Genus *Bifidobacterium*	Other LAB	Non -lactics[a]
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*[b]	*Saccharomyces boulardii*
L. amylovorus	*B. bifidum*		
L. casei	*B. breve*	*Enterococcus faecium*	*Propionibacterium freudenreichii*[b]
L. cremoris	*B. infantis*		
L. crispatus	*B. lactis*[c]		
L. fermemtum	*B. longum*	*Sporolactobacillus inulinus*[b]	*Bacillus cereus*[b]
L. gallinarum[b]			
L. gasseri			*Escherichia coli*[b]
L. helveticus			
L. johnsonii			
L. lactis			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			
L. salivarius			

Source: Macfarlance and Cummings (1999); Holzapfel and Schillinger (2002); Tamine *et al*. (2005).
[a] Mainly as pharmaceutical preparations.
[b] Mainly applied for animals.
[c] Reclassified as *B. animalis*.

Bifidobacteria are classified as Gram positive, non-sporing, non-motile and catalase negative, obligate anaerobes and are polymorphic with shapes including short, curved rods, club shaped rods and bifurcated V- or Y-shaped rods (De Vries and Stouthamer 1969). Although considered as obligate anaerobes, some bifidobacteria can tolerate oxygen while some species can tolerate oxygen in the presence of CO_2, depending on the species and culture medium (Doleyres and Lacroix 2005). The optimum pH for their

growth is 6.5-7.0, with virtually no growth at pH values 4.5-5.0 and below or at pH 8.0-8.5 and above. The optimum growth temperature is 36-38°C for bifidobacteria of human origin and 41-43°C for those of animal origin with virtually no growth below 20°C and above 46°C, while *B. bifidum* dies at 60°C (Ballongue 1998). So far, 29 different species of *Bifidobacterium* have been identified, 9 of which are from human sources e.g. oral caries, faeces and vagina and the remaining 20 species are from fermented milk, animal intestinal tracts, sewage, anaerobic digesters and honeybees (Champagne *et al.* 2005). Bifidobacteria are found in the large intestine and to a lesser extent in the lower part of the small intestine. The main species of humans original are *B. adolescentis* and *B. bifidum* found in infants, and *B. infantis*, *B. breve* and *B. longum* found in adults (Ballongue 1998).

In the manufacture of fermented milk, *B. bifidum* is the species most commonly incorporated, followed by *B. longum* and *B. breve*. Bifidobacteria are heterofermenters producing both acetic and lactic acids at a mole ratio of 3:2 (Shah 1997). Small amounts of succinic acid and CO_2 are also produced by some strains and during the degradation of gluconate (Doleyres and Lacroix 2005). Besides glucose, they can ferment galactose, lactose and fructose. Utilisation of CHOs and synthesis of water-soluble vitamins e.g. B_6, B_{12}, folic acid of probiotic bacteria appears to be strain- dependent (Ballongue 1998). It is evident that *B. adolescentis* can utilise a wide range of CHOs (up to 20), followed by *B. breve*, *B. infantis* and *B. longum* whereas *B. bifidum* can ferment only five CHOs (Tamime *et al.* 1995). Bifidobacteria grow slowly in milk due to lack of proteolytic activity and suffering from the presence of oxygen, hence adding nitrogen sources e.g. casein hydrolysate (Klaver *et al.* 1993) and cysteine (Shah 1997) or sometimes co-culturing with the yoghurt cultures (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) and/or other proteolytic lactobacilli species such as *L. acidophilus* are

necessary to enhance the fermentation process (Tamime *et al*. 2005). A large number of bifidobacteria species are also able to use complex CHOs for growing e.g. D-galactosamine, D-glucosamine, amylose and amylopectin (Shah 2006), as well as carbonate or bicarbonate, but not fatty acids and organic acids (Shah 1997).

2.2.2.2 Genus *Lactobacillus*

Lactobacilli are Gram positive, non-spore forming, non-flagellated rods or coccobacilli, and are either micro-aerophilic or anaerobic and strictly fermentative (Hammes and Vogel 1995). They are lacking catalase enzyme, but pseudo-catalase is found in rare cases. Lactobacilli are found in dairy products e.g. cheese and yoghurt, fermented meat, sour dough, fermented vegetables, silage, wine (Champagne *et al*. 2005), and in a various habitats such as the vagina, intestinal and respiratory tracts of humans and animals, on plants, in sewage, and in spoiled food (Suskovic *et al*. 2001). Lactobacilli have complex nutritional requirements e.g. for CHOs, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins. They are traditionally defined by formation of lactic acid as a sole or main end-product from CHO metabolism. Lactobacilli can either be homofermentors which predominantly convert almost exclusively glucose to lactic acid (> 85%) by the Embden-Meyerhof-Parnas (EMP) pathway or heterofermentors producing lactic acid and additional products i.e. CO_2, ethanol and/or acetic acid. While currently at least 56 species of lactobacilli have been described (Shah 2006), the heterofermentative lactobacilli of human use include *L. reuteri* and *L. fermentum,* whereas homofermentative lactobacilli are divided into three groups: (i) the *L. acidophilus* groups, mainly with strains of *L. acidophilus*, *L. gasseri*, *L. crispatus*, *L. amylovorus* and *L. johnsonii*; (ii) the *L. salivarius*; and (iii) the *L. casei* groups, mainly with strains of *L. paracasei*, *L. zeae* and *L. rhamnosus* (Champagne *et al.* 2005; Holzapfel and Schillinger 2002).

Strain of *L. acidophilus* is one of the most prominent strains with considerable industrial and medical interest. The possible health benefits relevant to *L. acidophilus* are the reduction of blood cholesterol and the protection against vaginal Candida infections (Kalantzopoulos 1997). It is described as obligatory homofermentative lactobacilli and is *ca*. 0.6 to 0.9 μm in width and 1.5 to 6.0 μm in length with rounded ends (Hammes and Vogel 1995). Its cells may appear singularly or in pairs or in short-chains. Most strains of *L. acidophilus* have moderate lactase activity and can ferment cellobiose, glucose, fructose, galactose, maltose, mannose, salicin, trehalose and aesculine but not gluconate and pentoses due to lacking of phosphoketolase. Strain of *L. acidophilus* can tolerate acid ranging from 0.3 to 1.9% titratable acidity and temperatures as high as 45°C, but the optimum conditions are pH of 5.5-6.0 and temperature of 35-40°C (Shah 2006). It is resistant to bile and gastric acids and survives the gastrointestinal transit. However, many *L. acidophilus* strains do not grow well in milk and survive poorly in fermented products.

2.2.3   Health-effects of probiotics

The scientific evidence obtained through various studies on *Lactobacillus* and *Bifidobacterium* spp. has strengthened the positive effects of these micro-organisms on human health. Such examples are presented in Table 2.4.

It is noted that no strain provides all the proposed health benefits and strains of the same species often exhibit distinct effects (Table 2.4), therefore, the health properties of each strain need to be investigated independently (Doleyres and Lacroix 2005). Strain of *L. rhamnosus* GG (Valio) is the most extensively studied probiotic in human clinical trials (Fonden *et al*. 2003), particularly involving in the management of rotavirus diarrhoea, and antibiotic-associated diarrhoea (*Clostridium difficile*). Strains of

L. acidophilus NCFB 1748, *B. lactis* Bb 12, *L. plantarum* DSM9843 (299V), *L. reuteri* (BioGaia Biologics), *L. johnsonii* La-1 and *L. casei* Shirota (Yakault) are also well established for the clinical effects (Shah 2006; Fonden *et al*. 2003).

Table 2.4   Currently available probiotics and their reported effects

Strains	Reported effects in clinical studies
L. acidophilus NCFM	Treatment of lactose intolerance; production of bacteriocin; lowering faecal enzyme activities
L. acidophilus NCFB 1748	Prevention of radiotherapy-related diarrhoea; lowering faecal enzyme activities; decreasing faecal mutagenicity; improvement of constipation
L. casei Shirota	Prevention of intestinal disturbance; balancing intestinal flora; inhibition of superficial bladder cancer; lowering faecal enzyme activities
L. reuteri	Colonising the intestinal tract in animal studies; shortening of duration of rotavirus diarrhoea
L. rhamnosus GG	Antagonistic against anticarcinogenic bacteria; prevention and treatment of rotavirus diarrhoea; prevention and treatment of relapsing *C. difficile* diarrhoea; prevention of acute diarrhoea; alleviation of Crohn's disease
L. johnsonii La-1	Balances intestinal flora; immune enhancement; adjuvant in *Helicobacter pylori* treatment
L. gasseri (ADH)	Faecal enzyme reduction
B. lactis Bb12	Balancing microflora; shortening of duration of rotavirus
B. bifidum	Treatment of rotavirus diarrhoea; balancing intestinal flora
Saccharamyces boulardii	Prevention of antibiotic associated diarrhoea; treatment of *C. difficile* diarrhoea
Enterococcus faecium Gaio®	Reduction in cholesterol

Source: Lee and Salminen (1995); Shah (2006); Salminen *et al*. (1998)

Chapter 2

The mechanism of probiotic action is still not fully known, but three main mechanisms of action have been developed as summarised in Figure 2.6.
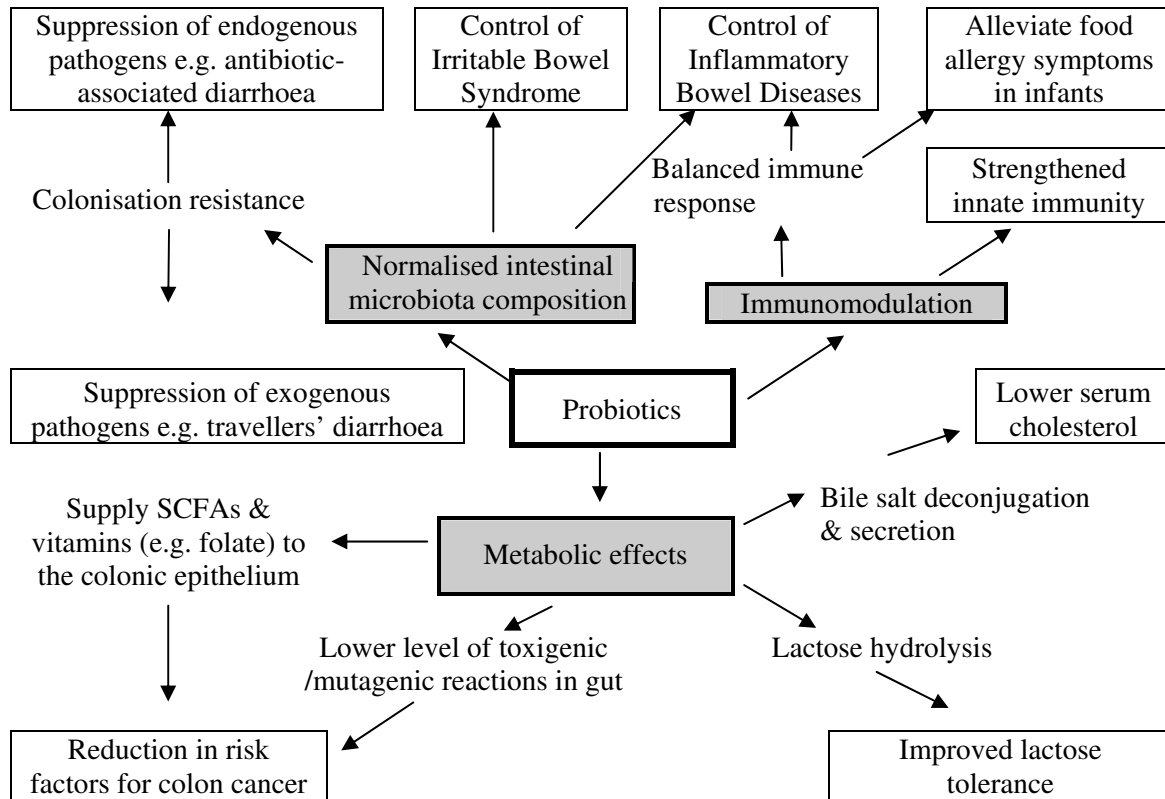
Figure 2.6   Proposed mechanisms of probiotic effects on human heath benefits
Source: Saarela *et al*. (2002)

2.2.4   Applications of probiotics in functional foods

Probiotics are widely used in dairy products, particularly yoghurts where the fermentation is often carried out with strains of *Lactobacillus* spp., primarily *L. acidophilus*, *L. johnsonii*, *L. casei/paracasei*, and *Bifidobacterium* spp. Probiotic yoghurts may be produced with probiotic LAB only or with the assistance of a supporter culture (Fonden *et al*. 2003). In Germany, the mixture of *L. acidophilus* and *B. bifidum* were introduced during the late 1960s for producing mildly acidified yoghurts, later known as "AB yoghurt" due to their expected adaptation to the intestine and the sensory benefits. Later the trend has been to incorporate *L. casei* in addition to *L. acidophilus* and bifidobacteria as these strains are believed to act synergistically on each other.

Typical examples of other probiotic products available in the market are probiotic drinks including drinking yoghurts, fruit juices, fermented soy products, sour cream, buttermilk, ice-cream and frozen desserts, spread, cheeses, and milk powders (Lourens-Hattingh and Viljoen 2001). Currently, probiotic milk drinks are manufactured in various ways. The bacteria may be added without fermentation, so-called sweet milk or the milk is cultured with probiotic bacteria such as Yakault (Tamime *et al*. 2005). Fermented milks containing *B*. *longum* or *B*. *breve* have obtained "foods for specific health uses" (FOSHU) approval in Japan (Champagne *et al*. 2005). In recent times, probiotics have also been marketed as dietary supplements consisting of freeze-dried bacteria, mainly *L*. *acidophilus* in tablet, capsule or powder form (Hamilton-Miller 2005). Some of the commercial companies producing such dietary supplements include Blackmores Ltd. (Balgowlah, NSW, Australia), Probiotics International Ltd. (Stoke-sub-Hamdon, Somerset, UK) and Natren Inc. (Westlake Village, CA, USA).

Industrial interest in developing probiotic foods is driven by the market potential for foods that target health and well-beings. To date over 100 bifidus- and acidophilus-containing products are available worldwide (Tamime *et al*. 2005). In Japan, probiotic-containing foods have been launched since the 1920s and more than 53 different types of milk products are estimated to be on the market (Shah 2006). Using probiotic is largely restricted to the manufacturing of yoghurt in Europe and acidophilus milk in the USA. It was estimated that in 2000 Europeans spent $899 million on probiotic yoghurts and milks and on average the market share of probiotic yoghurts was *ca*. 10% of the total yoghurts (Stanton *et al*. 2001). In 2007 the Australian market for yoghurt and dairy desserts accounted for 17% of dairy category sales value (Dairy Australia 2007) with probiotic yoghurt a leader (Anon 2003), growing up by 12% (Anon 2007).

2.3   Synbiotic foods

Synbiotic is defined as "A mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" (Gibson and Roberfroid 1995). The synbiotic concept is a promising trend in the functional food sector as the combination of probiotics and prebiotics may confer superior benefits to using individual ingredients. It is expected that adding prebiotic would benefit the survival of bifidobacteria during the shelf life of the dairy products (Lourens-Hattingh and Viljoen 2001).

The effect of synbiotics on faecal microflora of experimental animals is demonstrated by increasing the total anaerobes, aerobes, lactobacilli, and bifidobacteria counts and decreasing in *Clostridia*, *Enterobacteriaceae* and *Escherichia coli* counts (Suskovic *et al*. 2001). The synbiotic also showed the potential over either a probiotic or a prebiotic alone for reducing the total number of aberrant crypt foci in the colon of rats (Roberfroid 1998). In humans, Bouhnik *et al*. (1996) reported an overall increase in faecal bifidobacterial numbers in healthy volunteers after the consumption of synbiotic mix of inulin and *Bifidobacterium* spp. Other human feeding studies have focused on the synergistic effects of prebiotics and probiotics on human health. Kiebling *et al*. (2002) observed the significant decrease in LDL/HDL cholesterol ratios after long-term consumption of synbiotic yoghurt (*L. acidophilus* and *B. longum* plus inulin), similar to the previous observation of Schaafsma *et al*. (1998).

Synbiotic products available in the EU markets are currently probiotic yoghurts and dairy drink. These products often combine inulin-type fructans with bifidobacteria, and

lactitol or lactulose in conjunction with lactobacilli. In Australia, while synbiotic products appear on supermarket shelves, they are not being positioned as such due to low levels of consumer awareness and understanding of synbiotics (Anon 2003). The future of synbiotic foods depends on not only their effectiveness on human health and safety but also the organoleptic characteristics of the products and communication to consumers.

2.4 Summary of the literature review

It is clear that the pre-, pro- and synbiotic approaches are promising tools for the development of functional foods. The implantation of live bacteria into the human or the use of dietary CHOs to selectively increase certain resident bacterial genera has led to an increase in barrier function and reduction in risk of intestinal infections. Relevant mechanisms of their effects and human data are accumulating. With prebiotic concept, inulin-type fructans and lactulose are the leading sources amongst food ingredients that meet the needs of the food industry today. Unquestionably, inulin and OF have many interesting nutritional and functional properties. The use of lactulose is clearly for medical reasons, but it also finds the way in food formulation.

On the basis of the literature reviewed above, it appears that the large number of research has focused on the production and bifidogenic effects of chicory root inulin and less in JAI. Equally clear is that although a wide range of catalysts has been employed for lactose isomerisation into lactulose, the potential of carbonate-based catalysts is little known. On this basis, the current project involves the development of process for inulin extraction from JA and lactose isomerisation from dairy industry wastes and the task for assessment of the viability of commercial probiotic cultures in the presence of these prebiotic compounds.

Chapter 3

Materials and methods

The purpose of this chapter is to describe the starting materials, chemicals, reagents, equipment and methods of physico-chemical and microbiological analyses used in this study.

3.1 Materials

3.1.1 Jerusalem artichoke (JA)

For the extraction of Jerusalem artichoke inulin (JAI), JA tubers were obtained from local markets in Melbourne, Victoria, Australia during the harvest seasons (June to August) from 2005 to 2007. The tubers were cleaned in cold water to remove soil then soaked in 100 ppm sodium hypochlorite solution at 15°C for 30 min to reduce microbial and fungal load, then, rinsed in cold water and drained overnight at room temperature (RT) before storing at 4°C.

3.1.2 Milk concentration permeate (MCP) and lactose solution

The MCP used for lactose isomerisation was collected from the UF plant of a local dairy processor in Victoria, Australia and kept in an ice box during transportation, followed by freezing upon arrival to the University's pilot plant. To obtain MCP, pasteurised skim milk was passed through a 30,000 kDa MWCO polyethersulfone spiral wound membrane (Synder™ MK-4333, Synder Filtration, Vacaville, CA, USA) at 45°C and 120 kPa. The collected MCP contained 5% (w/v) solid-non-fat (SNF) including *ca.* 4% lactose, *ca.* 0.5% protein and 0.5% ash. It is not uncommon to have some proteins contaminating the MCP especially at the start of the process, which was the case with MCP used in this project. When needed, the frozen MCP was thawed and filtered

through Whatman No.1 filter paper, and the resulting turbid green-yellowish liquid was used for isomerisation trials with or without de-proteination pre-treatment.

Control Lactose Solution: A 4% (w/v) solution of α-lactose (95% pure, Table 3.1) was prepared in distilled water with pH adjusted to 6.7 using 0.1 M NaOH, filtered and used as reference (control) solution.

3.1.3 Carbonate-based catalysts: egg shell powder (ESP) and oyster shell powder (OSP)

Two types of catalysts for lactose isomerisation were prepared in the laboratory as follows: raw chicken egg shells were prepared by removing the shell membrane and washing off the residual albumen under tap water following the procedure of Montilla *et al*. (2005). The cleaned egg shells were then dried overnight in an oven at 102°C, ground after cooling the following day in a micro hammer mill at 600 rpm and sieved through a 120 mesh screen (Table 3.2). The ESP thus obtained was a cream-coloured powder with an average particle size of 117 μm. For preparing OSP, oyster shells obtained from seafood restaurants in Melbourne, Victoria, Australia were cleaned, washed and dried overnight at 102°C. The dried shells were broken into smaller pieces using a mortar, followed by grinding and sieving in the same way as the ESP, resulting in an off-white coloured fine powder. The powders were stored in glass vials and placed in a desiccator at RT until further use.

3.1.4 Reconstituted milks and starter cultures

Whole milk powder (WMP, 26% milk fat, 26% protein) and low-heat skim milk powder (LHSMP, 0.9% milk fat, 34% protein) supplied by Bonlac Foods Limited (Melbourne, VIC,

Australia) were used for reconstituted milk preparation and for yoghurt making at different concentration levels (12, 13, 14, 15 and 16%).

Freeze-dried starter cultures of *L. acidophilus* LA-5, *L. casei* LC-01, *B. bifidum* BB-12 and YC-380 [Yoflex®, a 50:50 mixture of *S. thermophilus* (ST) and *L. delbrueckii* subsp. *bulgaricus* (LB)] were obtained from Chr. Hansen Pty. Ltd. (49 Barry St, Bayswater, VIC, Australia) and were kept at −22°C until required for yoghurt preparation as direct vat set (DVS).

3.1.5 Chemicals and reagents

The commercial preparations of non-digestible oligosaccharides used as reference prebiotic compounds in the current study included lactulose (95% purity, Sigma, St Louis, MO, USA), oligofructose (Raftilose® P95 with an average DP of 4), medium-chain inulin (Raftiline® GR with an average DP of 12), and long-chain inulin (Raftiline® HP with an average DP of 23). The three later compounds were extracted from chicory roots and supplied by Mandurah Australia Pty. Ltd. (Dandenong, VIC, Australia).

Other chemicals including growth media and supplements used in the preparation of JAI and lactulose-enriched MCP, and analytical procedures were of the analytical-reagent grade or unless otherwise stated. Mono- and disaccharides having ≥ 95% purity were used in this study as chemical standards for analytical purposes and as carbon sources for micro-organisms. Tap water was used for washing and cleaning the JA tubers and the equipment. Distilled water was used for reconstitution of the chemicals and culture ingredients, and was filtered through a Milli-Q-ultrapure water purification system (Millipore Australia Pty. Ltd., North Ryde, NSW, Australia) before using for HPLC

analysis. The list of all chemicals used and the respective suppliers are presented in

Table 3.1.

Table 3.1   List of chemicals used in this study and their suppliers

Chemicals	Suppliers
Sucrose, Zinc sulphate, Potassium ferrocyanide, Calcium chloride dihydrate, Sodium metabisulphite, Sodium acetate, Activated carbon powder, Phenolphthalein, Dipotassium phosphate	Ajax Chemicals International Pty. Ltd., Level 24, 270 Pitt St, Sydney, NSW, Australia
Lactose, Calcium carbonate, Hydrochloric acid, Sodium hypochlorite, Tri-ammonium citrate, Magnesium sulphate, Sulphuric acid, Glycerol, Hydrogen peroxide, Diethyl ether, Potassium dihydrogenphosphate, Ethanol absolute, Tri-sodium citrate dihydrate	BDH Chemicals, Australia Pty. Ltd., 207 Colchester Rd, Kilsyth, VIC, Australia
Anhydrous D-(+) glucose	Fluka Chemie Gmbh, Gruenwalder Weg 30, Deisenhofen, Germany
Acetonitrile, Glacial acetic acid	Selby-Biolab, 2 Clayton Rd, Clayton, VIC, Australia
Fructan Assay kit (Sucrase/β-amylase/ pullulanase/maltase, Fructanase, Fructan control powder, D-fructose standard solution)	Megazyme, Deltagen Australia Pty. Ltd., 31 Wadhurst Drive, Boronia, VIC, Australia
Manganese sulphate, Bromocresol green, Sodium hydroxide	Merk KGaA, Frankfurter St 250, Darmstadt, Germany
Peptone water, M17 agar, MRS agar, MRS broth, Lab Lemco Powder, Buffered peptone water, Casein hydrolysate (acid), Bacteriological agar, Yeast extract, Bacteriological peptone	Oxoid Australia Pty. Ltd., 20 Dalgliesh St, Thebarton, Adelaide, SA, Australia
Lactulose, α-lactose, D-fructose, D-ribose, Maleic acid, Phenol, Sodium borohydride, para-hydroxy benzoic acid hydrazide, Cysteine hydrochloride, Calcium hydroxide, Phosphoric acid, Calcium hydroxide, Tween 80	Sigma Chemical Co., 6050 Spruce St, St Louis, MO, USA
Lactic acid	Purac bioquimica, Gran Vial 19-25, Montmelo, Barcelona, Spain

Chapter 3

3.2   Apparatus and auxiliary equipment

The items of process equipment and analytical instruments used in this study, along with the details of manufacturers and model numbers are presented in Tables 3.2 and 3.3, respectively. The HPLC system components and ancillary items used are described in Table 3.4.

Table 3.2   List and suppliers of the processing equipment used

Equipment	Manufacturer/supplier	Model No
Stephan Kettle	A. Stephan U. Sohne GmbH & Co., Stephanplatz 2, Hameln, Germany	UMM/SK 24E
Climbing film evaporator	James A Jobling & Co. Ltd., Wear Glass Works, Sunderland, England	CEF 1
Laboratory spray drier	N/S Niro atomiser, Gladsaxevej, Soborg 305, Denmark	-
Rotary evaporator	BÜCHI Labortechnik AG, Meierseggstrasse 40, Postfach, Flawil 1, Switzerland	R-114
Hot-plate stirrer	Industrial equipment & control Pty. Ltd., 61-65 McClure St, Thornbury, Melbourne, Australia	CH 2093-001
Micro hammer mill	Glen Mills Inc., 220 Delawanna Ave, Clifton, New Jersey, USA	-
Sieve (120 μm)	N. Greening & Sons Ltd., Hayes, Middx, England	BS 410/1943
Portable microprocessor thermometer	Hanna instruments Pty. Ltd., 18 Fiveways Boulevarde, Keysborough, VIC, Australia	HI 93503
Erma Abbe Refractometer	Erma Inc., Yushima 2-31-6, Bunkyo-ku, Tokyo, Japan	ER-98
Water bath (thermostatically controlled)	Watson Victor Ltd., 95-99 Epping Rd, North Ryde, NSW, Australia	BW6T

Table 3.3   List and suppliers of analytical instruments used in this study

Equipment	Manufacturer/supplier	Model No
Minolta Chroma Meter	Minolta Camera Co. Ltd., 3-13, Azuchi-machi 2-chome, Chuo-ku, Osaka, Japan	CR 121
UV-VIS spectrophotometer	Shimadzu Scientific Instruments, Unit 102, 45 Gilby Rd, Mount Waverly, VIC, Australia	UV-1601
Rheometer	Haake Rheometer, Dieselstrasse 4, Karlsruhe, Germany	RS 50
Texture Analyser	Stable Micro System Ltd., Vienna Court, Lammas Rd, Godalming, UK	TA-XT2
Bench-top pH-meter	Orion Research Inc., 829 Main St, Boston, MA, USA	520A
Hot air oven (102°C)	H. B. Selby & Co. Pty. Ltd., 35 York St, Sydney, Australia	Cat No. 303
Centrifuge	Beckman Instruments, Inc., 2500 Harbour Boulevard Box, Fullerton, CA, USA	GS-15R
Analytical balance, Ohaus Adventurer™ Pro	Ohaus Australia Pty. Ltd., 448 Boundary Rd, Derrimut, VIC, Australia	AR 2140
Steam bath	Conterm Scientific Ltd., 27 Cornish St, Petone, Wellington, New Zealand	330
Muffle furnace	Techtrader Pty. Ltd., Unit 1, 3 Lanceley Place, Artarmon, NSW, Australia	Cat No. 102C
Incubator	Watson Victor Ltd., 95-99 Epping Rd, North Ryde, NSW, Australia	IA 24 S
Stomacher	Seward Ltd., Southdownview Way 4a, Worthing, West Sussex, UK	400
Autoclave	A E Atherton & Sons Pty. Ltd., 364 Darebin Rd, Thornbury, VIC, Australia	-
Sterilise filter units, Millex®- HA	Millipore Corporation, 80 Ashby Rd, Bedford, MA, USA	0.45 μm,
Anaerobic jars with Gas-pack System	Oxoid Australia Pty. Ltd., 20 Dalgliesh St, Thebarton, Adelaide, Australia	2.5 L
Colony counters	Suntex Instruments Co. Ltd., Level 13, 31 Kang Ning St, Hsi-Chih, Taipei, Taiwan	560

Table 3.4   Description of HPLC system components and ancillary items

Equipment	Manufacturer	Model No
Carbohydrate Analysis column, 3.9×300 mm	Waters Associates Inc., 34 Maple St, Milford, MA, USA	PN 84038
Refractive Index Detector (RID)	Waters Associates Inc., 34 Maple St, Milford, MA, USA	R401
Integrator	Hewlett-Packard, Strasse 8, Waldbronn, Germany	3396A
Pump	Waters Associates Inc., 34 Maple St, Milford, MA, USA	M-6000A
Injector, 20 μL	Rheodyne, Inc., 600 Park Court, Rohnert Park, Cotati, Ca, USA	Z161
Filter units, PTFE Philic PP	Bonnet Equipment Pty. Ltd., P.O. Box 2042 Taren Point, NSW, Australia	0.45 μm

3.3   Physico-chemical analysis of Jerusalem artichoke

In all experiments, except otherwise stated triplicate sub-samples of each sample were tested at least in duplicate as described for the individual analysis procedure. In reporting the data, the results of individual samples are expressed as the mean ± standard deviation (SD).

3.3.1   Determination of fructans content

The enzymatic, spectrophotometric method described by McCleary and Blakeney (1999) was used for quantitative determination of fructans in JA tubers, JAI extract and concentrate. All samples were analysed using the Megazyme Fructan Assay kit (Table 3.1). In summary, the method involved enzymatic hydrolysis of sucrose, starch and maltodextrins that may be present in the sample into D-fructose and D-glucose by the combined actions of sucrase, β-amylase, pullulanase and maltase. The resulting reducing sugars were then converted to sugar alcohols in the presence of alkaline

borohydride. The solution was neutralised and excess borohydride was removed using diluted acetic acid. In the next stage, fructanase was applied to hydrolyse inulin and/or OF into glucose and fructose which were then measured with para-hydroxybenzoic acid hydrazide (PAHBAH) reducing sugar method as follows:

- **Preparation of reagents**

Sodium hydroxide (50 mM, 1 M, 2 M): To prepare 50 mM, 2 g of NaOH was dissolved in 900 mL distilled water and the volume was adjusted to 1 L, while for 1 and 2 M, 40 and 80 g of NaOH were used, respectively.

Acetic acid (0.2 M): A 11.6 mL of glacial acetic acid was made up to 1 L with distilled water.

Alkaline borohydrate: A 50 mg of sodium borohydride was dissolved in 5 mL of 50 mM NaOH. This solution was stable for 4-5 h at RT.

- **Preparation of buffer solutions**

Buffer I (Sodium maleate buffer, 0.1 M, pH 6.5): A 11.6 g of maleic acid was dissolved in 900 mL distilled water and adjusted pH to 6.5 with 2 M NaOH. The solution was made up to 1 L and stored at 4°C.

Buffer II (Sodium acetate buffer, 0.1 M, pH 4.5): A 5.8 mL of glacial acetic acid was diluted in 900 mL distilled water and the pH was adjusted to 4.5 with 1 M NaOH. The solution was made up to 1 L and stored at 4°C.

- **Preparation of enzymes**

Sucrase (100 U) plus β-amylase (500 U), pullulanase (100 U) and maltase (1,000 U): A freeze dried powder of the entire enzyme contents was dissolved in 22 mL of Buffer I and stored at −20°C.

Chapter 3

Fructanase (10,000 U): A freeze dried powder of fructanase was dissolved in 22 mL of Buffer II and stored at −20°C.

- **Preparation of reducing sugar assay reagent**

Stock solution A: A 10 g of PAHBAH was dissolved in 60 mL distilled water and mixed well with a magnetic stirrer. The slurry was added with 10 mL conc. HCl and made up to 200 mL with distilled water. This solution was stable for 2 years at RT.

Stock solution B: A 24.9 g of tri-sodium citrate dihydrate was dissolved in 500 mL distilled water, added with 40 g NaOH with thorough mixing and then diluted to 2 L with distilled water. This solution was stable for 2 years at RT.

PAHBAH working solution: Before use, 20 mL of stock solution A were made up to 200 mL with the stock solution B, mixed thoroughly and stored on ice and used within 4 h.

- **Preparation of the sample solution**

One gram of the sample was accurately weighted into a dry beaker and added with 80 mL hot distilled water (80°C). The beaker was heated on a hot-plate stirrer at *ca*. 80°C for 15 min. The solution was cooled to RT and diluted to 100 mL with distilled water before filtration through a Whatman No. 1 filter paper. The resultant filtrate was used as the sample solution.

- **Preparation of controls and standard solutions**

With each set of determinations, reagent blanks, D-fructose standards and fructan controls were included and analysed at the same time as the samples.

Reagent blank: Duplicate reagent blanks were prepared by mixing 0.3 mL of Buffer II with 5 mL of PAHBAH working solution.

D-fructose standard: To 0.2 mL of D-fructose standard solution (1.5 mg mL^{-1}) (Table 3.1) 0.9 mL of Buffer II was added. Quadruplicate aliquots (0.2 mL) of this solution

(containing 54.5 µg of D-fructose) were transferred into test tubes, followed by adding 0.1 mL of Buffer II and 5 mL PAHBAH working solution into each tube.

Fructan control: Freeze-dried powder of fructan (Table 3.1) was used to prepare the fructan control solution following the same procedure as the sample.

- **Assay procedure**

A 0.2 mL aliquot of the sample solution and fructan control solution were individually dispensed into glass test tubes, added with 0.2 mL sucrase/amylase solution and subsequently incubated at 40°C for 30 min. A 0.2 mL of alkaline borohydride solution was added to the tube with vigorous stirring and incubation was continued for 30 min. The reaction was then stopped by the addition of 0.5 mL of 0.2 M acetic acid. The resulting aliquots (0.2 mL) were transferred into three separate glass test tubes. A 0.1 mL of fructanase solution was added to two of these tubes (designated as samples) and 0.2 mL of Buffer II was added to the third tube (designed as sample blank). All tubes were incubated at 40°C for 20 min and then mixed with 5 mL of PAHBAH working reagent.

All tubes, containing sample solution and fructan control sample, along with reagent blanks and D-fructose standards were heated in a boiling water bath for exactly 6 min and immediately cooled in cold water (20°C) for *ca*. 5 min (see flow diagram 3.1).

| JA and/or Fructan control (1 g) | Buffer II (0.3 mL) | D-Fructose std sol^n (0.2 mL) |

← Hot water (80°C, 80 mL)

Heat (80°C/15 min)

Cool to RT

Make up to 100 mL& filter

Transfer test solution (0.2 mL) into test tube

← Sucrase/amylase sol^n (0.2 mL)

Incubate 40°C/30 min

← Sodium borohydride (0.2 mL)

Incubate 40°C/30 min

← 0.2 M Acetic acid (0.5 mL)

Transfer into duplicate test tubes

← Buffer II (0.9 mL)

Transfer quadruplicate aliquots (0.2 mL) into test tubes

← Buffer II (0.1 mL)

Transfer duplicate aliquots (0.2 mL) into test tubes

Transfer aliquot (0.2 mL) into test tube

← Fructanase sol^n (0.1 mL)

← Buffer II (0.1 mL)

Incubate 40°C/20 min

Incubate 40°C/20 min

← PAHBAH reagent (5 mL)

Incubate 100°C/6 min

Cool to 20°C/5 min

Measure absorbance at 410 nm

Figure 3.1   Flow diagram for fructans analysis

The absorbance of all solutions was rapidly measured at 410 nm against the reagent blank. The average absorbance for each sample was used in the calculation of fructans content using the following equations:

$$\% \text{ Fructans content } = \frac{\Delta_A \times V \times F \times 2.48}{W}$$

Where Δ_A =   Sample absorbance – sample blank absorbance

       V   =   Volume of extract used (100 mL)

       F   =   Factor to convert absorbance values to μg of D-fructose

           =   (54.5 μg of D-fructose)/(absorbance for 54.5 μg of D-fructose)

       W   =   Weight of sample extracted (mg)

       2.48   =   Conversion factor suggested by the manufacture

3.3.2   Determination of total carbohydrates (CHOs)

Total CHO present in JA tubers, JAI extract and concentrate was determined colourimetrically using the phenol-sulphuric acid method (Southgate 1991). This method is based on the measurement of absorbance at 490 nm of a coloured aromatic complex formed between phenol and CHO. The assay was set up by preparing a series of standard solutions, sample tubes and blanks as follows:

Standard tubes: Standard glucose solutions were prepared in the range 10-100 μg mL^{-1}. For this purpose, 1 mg glucose was dissolved in 10 mL distilled water and used as stock solution. Aliquots of the stock solution were accurately pipetted into 10 test tubes in 0.1 mL increments ranging from 0.1 to 1.0 mL. The liquid volume in each tube was then adjusted to 1 mL with distilled water followed by the addition of 1 mL of 5% phenol and 5 mL of 96% sulphuric acid.

Chapter 3

Sample tubes: A sufficient amount of each CHO sample was weighed into a 100 mL volumetric flask and diluted with distilled water to a final assay solution concentration of *ca*. 10-70 μg mL^{-1}. Then 1 mL of the diluted sample solution, 1 mL of phenol solution and 5 mL of sulphuric acid were transferred to duplicate test tubes giving a final volume of 7 mL.

Blanks: A blank solution was prepared by substituting sample solution for 1 mL of distilled water.

Sulphuric acid converts all non-reducing sugars to reducing sugars, therefore it was important to mix the solution thoroughly before incubating all test tubes in water bath at 30°C for 20 min. The absorbance of yellow-orange colour thus developed was measured at 490 nm using a spectrophotometer (Table 3.3), and the amount of CHO present was determined from the calibration curve.

The standard curve was prepared using Microsoft® Excel 2000 software where the absorbance values of standard solutions were plotted against their concentrations. A linear regression equation of the type [y = mx + c] along with its R^2 value was generated and recorded. The analyses were repeated when the R^2 value was < 0.98. The mean absorbance value for each sample tested was then used in the calculation of CHO concentrations of the sample solutions using the linear equations. The results were calculated using following equation:

$$\% \text{ CHO content } = \frac{C \times D}{S_w \times (10^6)} \times 100$$

Where C = Concentration of CHO calculated from the standard curve (μg mL^{-1})

D = Dilution factor

S_w = Amount of sample originally weighed (g or mL)

10^6 = Conversion factor so that result is expressed in units of g of CHO

3.3.3   Determination of reducing sugars

The amount of reducing sugars present in JA tubers, JAI extract and concentrate was determined spectrophotometrically at 440 nm using PAHBAH reagent with modified method of Southgate (1991). Samples were diluted with distilled water to give a working solution containing *ca*. 10-70 µg mL^{-1} sugar, and 1 mL aliquots of this solution were used for the assay. In a series of test tubes, 1 mL of blank and/or standard solutions was added. The blanks were prepared by substituting sample solution with distilled water while the glucose stock solution (0.1 mg mL^{-1}) was diluted into 10 different standard solutions in the range 10-100 µg mL^{-1} (see 3.3.2). All tubes were mixed with 5 mL of PAHBAH working reagent (see 3.3.1) and subsequently boiled for exactly 6 min. After cooling the tubes in cold water at 20°C for *ca*. 5 min, the absorbance of the coloured hydrazide product developed in alkaline solution was measured against the reagent blank and the amount of reducing sugar was determined by interpolating from standard curve.

3.3.4   Determination of soluble solids content

An Abbe refractometer (Table 3.2) was used for routine checking of the soluble solids content of JA tubers juice, the JAI extract during hot water extraction and clarification, as well as the JAI concentrate during syrup preparation. To prepare JA tubers juice, representative samples of the tubers were cut into small pieces and duplicate 10 g portions were separately pulped in a mortar. The resulting pulps were then pressed separately through muslin cloth and a few drops of each transparent juice were applied on the main prism of refractometer, and the soluble solids content was read on the lower scale and expressed as °Brix.

Chapter 3

3.3.5 Determination of total solids

Total solids of test samples, including JA tubers, JAI extract, concentrate and powder were determined gravimetrically following AOAC (2000a) method 990.20. All analyses were carried out at least in duplicate on triplicate samples. Approximately 3-5 g of each sample were weighed into empty aluminium moisture dishes with lids which were previously dried at $102 \pm 2°C$ for 1 h, cooled in a desiccator containing silica gel desiccant for 30 min and weighed. The uncovered dishes containing liquid samples were pre-dried on a steam bath for 30 min before further drying in a hot air oven at 102°C followed by cooling and weighing. The oven drying was repeated until a constant weight was attained. The net weight of residue was used to calculate total solids of the samples using the following equation:

$$\% \text{ Total solids } = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

Where W_1 = Mass of empty moisture dish (g)

W_2 = Mass of moisture dish plus sample before drying (g)

W_3 = Constant mass of moisture dish plus sample after drying (g)

3.3.6 Determination of ash content

Ash content of all samples was determined gravimetrically using AOAC method 945.46 (AOAC 2000b). Five grams of triplicate samples were placed into pre-ignited, pre-weighed crucibles. Crucibles were evaporated to dryness on a steam bath, charred over a low flame until smoke emission was ceased, and further heated in a muffle furnace at 550°C for 4 h. After crucibles were removed from the furnace and cooled to RT, the residue was moisten with minimum distilled water and reheated in the furnace until a white ash free from carbon residues was obtained. The crucibles were transferred to a desiccator for 1 h before weighing. The ash content was calculated from the formula:

$$\% \text{ Ash content } = \frac{M_3 - M_1}{M_2 - M_1} \times 100$$

Where M_1 =   Mass of empty crucible (g)

   M_2 =   Mass of crucible plus test sample (g)

   M_3 =   Constant mass of crucible plus ash (g)

3.3.7   Colour measurement of JAIP

Colour measurement of JAIP was performed in the tristimulus L^*, a^*, b^* measurement mode using a portable Minolta Chroma Meter (Table3.3). Calibration was made using the standard white tile supplied by the manufacturer. The samples were placed into Petri dishes and five readings were taken on each sample by randomly moving the measuring head to different locations on the surface of the sample and three different colour parameters, L^*, a^* and b^* were recorded. The L^* value measures the degree of whiteness to darkness and the higher the L^* value, the lighter the sample colour. The a^* value indicates the intensity of redness to greenness of the sample with positive values corresponding to red colour and negatives indication green colour. The b^* value indicates the intensity of yellowness (+) to blueness (−). For a^* and b^* readings, values closer to zero mean less intense colour while values away from zero indicate more intense chroma characteristics (Hutchings 1999).

3.4   Physico-chemical analysis of MCP

3.4.1   Determination of lactose and lactulose content

High performance liquid chromatography (HPLC) method was used to determine the degree of isomerisation of lactose into lactulose, following the procedure of Zokaee *et al*. (2002b).

Chapter 3

- **Preparation of solutions**

Carrez I: $K_4Fe(CN)_6.3H_2O$ (7.2 g) and *Carrez II*: $ZnSO_4.7H_2O$ (14.4 g) were weighted accurately and made up to 100 mL with Milli-Q water in separate volumetric flasks.

Mobile phase: The mobile phase chosen was a mixture of acetonitrile-Milli-Q water (80:20 v/v) which was prepared freshly on each analysis day. Before use in the HPLC analysis, the solution was degassed and filtered through a 0.45 μm filter.

- **Preparation of standard solution**

To prepare standard solutions, 500 mg of each of lactose and lactulose were accurately weighed into individual 100 mL volumetric flasks, dissolved with minimum Milli-Q water and made up to the mark with a mixture of acetonitrile and Milli-Q water (50:50 v/v).

- **Preparation of sample solution**

A 5 mL aliquot of sample (MCP and/or lactose solution) was pipetted into a 25 mL volumetric flask and added with 500 μL each of Carrez I and Carrez II reagents to remove protein and fat. The solution was then made up to 25 mL with 50:50 v/v acetonitrile and Milli-Q water mix and rested for at least 30 min, followed by filtration through Whatman No.1 filter paper. The resultant filtrate was designated as sample solution. All measurements were carried out at least in duplicate within 24 h following the isomerisation process.

- **Assay procedure and calculation**

All standard solutions and prepared samples were filtered through 0.45 μm PTFE Philic PP filters before injection into the HPLC system (Table 3.4) via an injection valve fitted with a 20-μL injection loop. The separation was performed on a 3.9×300 mm

Carbohydrate Analysis column at ambient temperature with a flow rate of 0.8 mL min^{-1} and refractive index detection (RID). The total run time required for each injection was less than 20 min. Each sample solution was injected in duplicate. Qualitative HPLC analysis and identification were performed by direct comparison with external standard sugars. The HPLC operating conditions applied did not provide a complete baseline separation of lactose and lactulose, therefore, the amount of each sugar was estimated from peak heights, instead of peak areas following the formula:

$$\% \text{ Sugar content } = \frac{H_m \times C_s \times V}{H_s \times S_w}$$

Where H_m = Peak height of lactose or lactulose in sample test solution

H_s = Peak height of lactose or lactulose in standard solution

S_w = Amount of the sample originally applied (mL)

V = Total quantity of sample solution used (25 mL)

C_s = Concentration of lactose or lactulose in standard solution (g 100 mL^{-1})

3.4.2 Determination of soluble solids content

The soluble solids content of MCP during isomerisation and syrup preparation was determine using an Abbe refractometer (Table 3.2).

3.4.3 Determination of pH

The pH of MCP and lactose solution was determined using a bench-top pH-meter (Table 3.3) at 20°C which was previously calibrated with pH 7.0 and 4.0 standard buffers. All analyses were carried out in duplicate.

3.4.4 Colour measurement of MCP and lactose solution

For turbid liquid samples i.e. MCP the sample was poured into a clear cylindrical glass vial (15 mm × 45 mm) and placed in the light path of the portable Minolta Chroma Meter (Table 3.3) for colour reading and mean values of b^* of five readings are

reported. On the other hand, the colour of clear liquid samples i.e. de-proteinated MCP and lactose solution was estimated as the absorbance value measured at 420 nm following the method of Moreno *et al*. (2003).

3.5   Physico-chemical analysis of cultured milk and set yoghurt

3.5.1   Determination of titratable acidity

The titratable acidity (TA) of cultured milks was determined in triplicate according the AOAC (2000c) titration method 947.05. A 10 g sample was placed in a 250-mL volumetric flask, mixed gently with 10 mL CO_2-free water, mixed with 3-4 drops of 0.5% phenolphthalein indicator and titrated with 0.1 M NaOH until the pink end point (pH 8.6) persisted for *ca*. 30 s. The amount of NaOH was used to calculate the % lactic acid using the following formula:

$$\% \text{ TA} = \frac{\text{mL of 0.1 M NaOH} \times 0.0009 \times 100}{\text{Initial weight of sample}}$$

3.5.2   Determination of pH

The pH of cultured milks was determined following the procedure in section 3.4.3.

3.5.3   Determination of fat content

The method used in fat analysis involved acid hydrolysis and solvent extraction as described by Pearson (1976). About 5 g of yoghurts was weighed into an empty, dry, pre-weighed Mojonnier tube and mixed with 5 mL of distilled water. Ten millilitres of conc. HCl were added, the tube was stoppered and the content was mixed well, followed by immersing the tube in boiling water to achieve complete digestion of proteins. At this stage the mixture appeared brown to violet in colour and the fat was visible on the surface. The Mojonnier tube was cooled in running water to RT, added with 30 mL of diethyl ether, stoppered and shaked vigorously for 30 s and kept

quiescent for phase separation. The top ethereal layer was carefully decanted into a dry round-bottom collecting flask. The extraction procedure was repeated at least three times to ensure that all fat was completely extracted from the vessel. The pooled solvents were removed from the vessel using a rotary evaporator. The oil left in the flask was dried in an oven at $102 \pm 2°C$ for 1 h, cooled in a desiccator. The dried oils were dissolved in a minimum amount of chloroform and collected in a pre-weighed flask. The chloroform washing was repeated twice and the resulting pooled chloroform was evaporated. The flask was dried in a $102 \pm 2°C$ oven for 1 h, cooled in a desiccator and weighed to the nearest 0.1 mg. The drying, cooling and weighing processes were repeated until a constant weight was attained. With each set of sample determination, a blank test was performed concurrently on 10 mL of distilled water instead of prepared sample using the same digestion method. When the blank exceeds 1 mg, the reagents should be checked and replaced.

Calculation of fat content of the test sample was followed according to:

$$\% \text{ Fat content } = \frac{(M_1 - M_2) - (M_3 - M_4)}{M_0} \times 100$$

Where M_0 = Mass (g) or volume (mL) of sample taken for analysis

M_1 = Constant mass of fat plus collecting flask of test sample (g)

M_2 = Mass of empty collecting flask of sample (g)

M_3 = Constant mass of fat plus collecting flask of blank (g)

M_4 = Mass of empty collecting flask of blank (g)

3.5.4 Determination of total solids

Total solids of set yoghurts were determined gravimetrically following AOAC (2000a) method 990.20 as described in section 3.3.5.

3.5.5   Colour measurement of set yoghurt

Colour measurement of yoghurt samples was examined using a portable Minolta Chroma Meter (Chuo-ku, Osaka, Japan) following the procedure in section 3.3.7 and reported as L^*, a^* and b^* values.

3.5.6   Large deformation measurement

Gel firmness of set yoghurts was determined using a TA-XT2 Texture Analyser (Stable Micro System Ltd., Godalming, UK) equipped with 5 kg load cell according to a method of Amatayakul *et al.* (2006a). A single compression test was performed with the Texture Expert Exceed software package (Stable Micro Systems 1995) using a 20 mm diameter cylindrical probe (P20). Test conditions were automatic trigger 0.1 N, pre-test and test speeds 1 mm s^{-1}, post-test speed 10 mm s^{-1} and distance 15 mm. Gel firmness was determined as the maximum force (N) on compression force-time curve. Four separate determinations were performed on each batch at 10°C.

3.5.7   Small deformation measurement

Rheological properties of yoghurt samples were determined on day 1, 7 and 28 of storage, using Haake RheoStress rheometer (Table 3.3) fitted with a cone and plate sensor (35 mm diameter 2° angle and 0.105 mm gap setting). Samples were stirred gently 20 times with a spatula and allowed to equilibrate to RT (20°C) before placing a 2 mL sample on the rheometer plate. For each sample, replicate measurements were taken independently and data processing was performed using a RheoWin Pro software package (Version 2.94, Thermo Haake, Karlsruhe, Germany). Rheological terms and corresponding parameters used in this study are summarised in Table 3.5.

Table 3.5   Summary of rheological terms and symbols

Terms	Symbols	Definitions
Stress (Pa)	σ	The applied force per unit area
Strain (dimensionless)	γ	The change in the size or shape of a body referred to its original size or shape due to the applied force
Shear rate (s^{-1})	$\dot{\gamma}$	The velocity gradient in a flowing material under shear force
Viscosity (Pa.s)	η	Ratio of σ to $\dot{\gamma}$ indicates the flow resistance of fluids
Storage or elastic or in-phase modulus (Pa)	$G'(\omega)$	Energy stored per deformation cycle during oscillatory test
Loss or viscous or out-phase modulus (Pa)	$G''(\omega)$	Energy dissipated per deformation cycle during oscillatory test
Complex viscosity (Pa.s)	$\eta*(\omega)$	Viscoelastic flow
Loss tangent (dimensionless)	Tan δ	Ratio of G'' to G' indicates the viscoelastic character of the material (e.g. more solid-like or liquid-like)

Source: Bourne (2002); Tunick (2000)
The SI units are presented in brackets.

Thixotropy tests were initially applied to characterise the flow behaviour of yoghurt samples. The representative sample of yoghurt was exposed to a shear rate of 500 s^{-1} for 60 s, followed by a 300 s equilibration as described by Purwandari *et al*. (2007). Shear stress was then recorded at increasing shear rates from 0 to 200 s^{-1} within 200 s (upward flow curve) followed by decreasing shear rates from 200 to 0 s^{-1} within 200 s (downward flow curve). Two types of plots were generated to demonstrate the flow behaviour of yoghurts i.e. flow curve and viscosity curve. To model flow behaviour, average data from the upward flow curve were fitted to Herschel-Bulkley ($\sigma = \sigma_o + K\dot{\gamma}^n$) model (Rohm 1993; Benezech and Maingonnat 1994), where σ = shear stress

(Pa), σ_o = yield stress (Pa), K = consistency index (Pa.s^n), $\dot{\gamma}$ = shear rate (s^{-1}) and n = flow behaviour index (dimensionless). Other rheological parameters considered were the area under the upward flow curve (A_{up}), the area difference under upward and downward flow curves (ΔA or hysteresis loop area) (Hassan *et al.* 2003) and apparent viscosity (η_{app}) evaluated as $\dot{\gamma} = 2$ s^{-1}.

Dynamic oscillation tests were subsequently conducted following the flow behaviour assessment to characterise the viscoelastic properties of yoghurt. Stress sweeps were elevated from 0.1 to 15 Pa to determine the linear viscoelastic range (LVR) of the yoghurt samples at a constant frequency of 1 Hz (6.28 rad s^{-1}). Frequency sweeps from 0.05 to 10 Hz were then performed within LVR at a constant shear stress of 1 Pa to obtain the dynamic complex viscosity ($\eta*$), the storage modulus (G′), the loss modulus (G″) and the loss tangent (tan δ) which represents the ratio of G″ to G′. When the material behaves more like a solid, the G′ dominates and consequently tan δ becomes < 1.0. The slope of log-log plots of G′ and G″ vs. oscillation frequency (ω) was also determined according to Hassan *et al.* (2003) using the RheoWin Pro software package (Version 2.94, Haake).

3.6 Microbiological analysis

3.6.1 Media preparation

3.6.1.1 Peptone water (For serial dilution)

Diluent peptone water (0.1%) was prepared by dissolving 2 g of buffered peptone medium (Code CM0509) in 1 L of distilled water. The pH of solution was adjusted to 7.0 ± 0.2, followed by autoclaving 90 mL and 9 mL portions at 121°C for 15 min.

3.6.1.2 de Man, Rogosa and Sharpe Agar (MRS agar)

MRS agar was prepared as recommended by the manufacturer (Bridson 1995) by suspending 62 g dehydrated medium (Code CM0361) in 1 L of distilled water. The suspension was warmed in a microwave oven to dissolve the agar, followed by autoclaving at 121°C for 15 min.

3.6.1.3 MRS-pH modified agar

MRS broth was prepared by suspending 52 g dehydrated medium (Code CM0359) in 1 L of distilled water and adjusting the pH to 5.2 using 0.1 M HCl. Twelve grams of agar powder (Code LP0011) were then added to MRS broth. The suspension was warmed in a microwave oven to dissolve the agar, followed by autoclaving at 121°C for 15 min (Ravula and Shah 1998).

3.6.1.4 M17 agar

The M17 agar was prepared as recommended by the manufacturer (Bridson 1995) by suspending 48.25 g dehydrated medium (Code CM0785) in 950 mL of distilled water. The suspension was autoclaved at 121°C for 15 min, cooled to 50°C before aseptically adding 50 mL of lactose solution (10%, w/v) which was sterilised by passing through Millipore HA (0.45 μm) membrane filters (Table 3.3).

3.6.1.5 *Lactobacillus casei* agar (LC agar)

This agar consists of 10 g bacteriological peptone (Code LP0037), 4 g Lab Lemco (Code LP0029), 1 g yeast extract (Code LP0021), 1 g casein hydrolysate (Code LP0041), 3 g sodium acetate, 1 g tri-ammonium citrate, 2 g KH_2PO_4, 0.2 g $MgSO_4$, 1 g Tween 80 and 0.05 g $MnSO_4$ (Table 3.1). To formulate media, all ingredients were weighted and dissolved in 1 L of distilled water. The pH of dissolved solution was

adjusted to 5.1 ± 0.1, followed by adding 6 mL of bromocresol green (0.1%) and 12 g of bacteriological agar (Code LP0011). The suspension was sterilised at 121°C for 15 min. A 10 mL aliquot of membrane-sterilised solution of 10% D-ribose was aseptically added per 90 mL of sterile media (Ravula and Shah 1998).

3.6.2 Enumeration of probiotic and starter cultures

Ten grams of each cultured milk were diluted with 90 mL of 0.1% sterile buffered peptone water and placed in stomacher for 2 min. Tenfold serial dilutions of 10^{-2}-10^{-8} were then prepared in 9 mL of 0.1% sterile peptone water and 1 mL of the three highest dilutions was pour-plated in duplicate. When sole starter was used for the fermentation, MRS agar as non-selective media was employed for enumeration of lactobacilli (LC-01 and LA-5) while for counting bifidobacteria (BB-12) filter sterilised 0.05% cysteine hydrochloride was added to MRS agar to create an anaerobic environment as suggested by Saxelin *et al*. (1999). In all cases, the plates were gently mixed clockwise and anti-clockwise to distribute the sample uniformly and allowed to set. All plates were incubated under anaerobic conditions (Gas-pack System, AN0025 AnaeroGen, Table 3.3) at 37°C for 72 h before enumerating the colonies. The numbers of Colony Forming Units (CFU) on plates containing 25 to 250 colonies were calculated per gram of sample as follows:

$$\text{CFU g}^{-1} = \frac{\text{Number of colonies} \times \text{Volume of dilute suspension}}{\text{Dilution factor}}$$

It is noted that when selective enumeration of LC-01 from cultured milk was required in the presence of mixed cultures of YC-380, LC agar, M17 agar and MRS-pH modified agar (pH 5.2) were used for isolation and determination of LC-01, ST and LB, respectively. Sets of MRS-pH modified plates and LC plates were incubated

anaerobically at 43°C for 72 h, and at 27°C for 72 to 96 h, respectively whereas M17 plates were incubated aerobically at 37°C for 24 h (Ravula and Shah 1998). Under these conditions, ST appeared as lenticular colonies with a diameter of 1-2 mm, LB formed lenticular star-shaped colonies 1-3 mm in diameter and LC-01 formed smooth white disc colonies measuring 1-2 mm in diameter.

3.7   Statistical analysis

The results obtained were statistically analysed using SPSS 15.0 software (SPSS Inc. 2006) unless described otherwise. The Duncan's multiple range test (DMRT) was applied for mean comparison when one-way analysis of variance (ANOVA) showed significant differences at the 95 and 99% confidence level. In addition, data were subjected to Student's t tests when mean comparison of two variables was required.

Chapter 4

Extraction of inulin-type fructans from Jerusalem artichoke[1]

4.1   Abstract

Inulin-type fructans were extracted from the tubers of Jerusalem artichoke using a hydrothermal extraction process, followed by clarification and concentration. The concentrated samples were treated with two different procedures to fractionate the high- and low-molecular-weight components. The optimum conditions for first process, the ethanol-mediated fractionation of inulins were established by response surface methodology and were found to be 32°B initial syrup concentration, ethanol-to-syrup ratio of 13 and temperature of 42°C. The results suggested that higher syrup concentration resulted in an increased inulin yield, but further increase in concentration up to 30°B had an opposite effect on average chain length and purity values. The second process involved cold storage of the inulin syrup where the insoluble heavier inulin fractions precipitated and were subsequently separated and spray-dried to obtain inulin powder. The precipitate yield of inulin showed a tendency to increase with the decreased storage temperature from 4 to −24°C and increased initial syrup concentration from 6 to 12°B.

4.2   Introduction

Inulin and oligofructoses are commercially manufactured for over a decade from chicory (*Cichorium intybus* L.) roots by European countries especially Belgium, the Netherlands and France (Franck 2000). Currently, Australia imports inulin products

---

[1] Based on the findings of this chapter a paper entitled "Process optimisation for fractionating Jerusalem artichoke fructans with ethanol using response surface methodology" was published in Food Chemistry 104 (2007) 73-80.

from Europe and China. Jerusalem artichoke (JA-*Helianthus tuberosus* L.) is an alternative source for inulin extraction as the tubers accumulate high levels of inulin-type fructans, instead of starch during their growth (Figure 4.1). On a dry weight basis, the tubers contain 68-83% inulins, 15-16% proteins, 13% insoluble fibre and 5% ash (Fleming and GrootWassink 1979). The degree of polymerisation (DP) of inulins in JA tubers is dependent on the cultivar, time of harvest and storage conditions (Baldini *et al*. 2004).

Jerusalem artichoke is produced in Australia, especially in Victoria's cool climate regions (Parameswaran 1996) only on a small scale for use as a vegetable in raw or cooked forms as the extraction technology of inulin is undeveloped. This plant is a low-requirement crop, suitable for Australian environment. The development of more commercial products from this plant could help Australian agriculture and economy by reducing the volume of imported inulin. One of the aims of this study was to evaluate the potential of JA tubers grown in Australia as raw material for inulin production.

Figure 4.1   Jerusalem artichoke (left) and its tubers (right)
Source: Christman (2003)

The conventional method in optimisation processes, the so-called change-one-factor-at-a-time approach needs a large number of experiments to describe the effect of individual factors on product characteristics or process conditions. This is not only

laborious and time-consuming, but also suffers from major drawbacks of giving unreliable results since it is unable to determine interactions among the factors. On the other hand, applying response surface methodology for optimisation studies has been shown to overcome those weaknesses (Montgomery 1996). The response surface methodology is a useful group of mathematical and statistical techniques, used for analysing the influence of several independent factors on one or more characteristics of the processes or products. These techniques have successfully been applied for optimising microbiological media composition, enzyme hydrolysis conditions and food processes (De Faveri *et al.* 2004).

Many researchers have studied the effects of different solvents for fractionation, and generally have used commercial standard grade inulin (Moerman *et al.* 2004). The technical details for inulin extract concentration are neither well-documented nor clearly understood. In addition, little is known about the combined effects of different process conditions during inulin precipitation by ethanol. There have also been no reports on the use of response surface methodology to optimise the precipitation conditions. Accordingly, the present work was undertaken to find the optimum conditions for ethanol fractionation of inulin from a concentrated JA extract. The objectives of the study were to obtain a high yield of inulins with high purity as well as high average chain length.

Several studies have reported the formation of inulin-rich precipitates as a crystalline or a pasty substance when the temperature of inulin extracts decreases in the range of 16°C to −24°C (Lopez-Molina *et al.* 2005; Silver 2003; Leite Toneli *et al.* 2007). Various processing factors affecting inulin precipitation i.e. cooling/freezing temperature, precipitation time, centrifugation velocity and time have already been investigated.

Lopez-Molina *et al.* (2005) and Leite Toneli *et al.* (2007) reported that the precipitate yield of inulin was highly dependent on the temperature but independent of centrifugation velocity and time. Consequently, the present study was aimed to examine the combined effects of initial concentration of JA inulin syrup (JAIS) and storage temperature on the precipitation yield of inulin.

4.3   Preparation of Jerusalem artichoke inulin (JAI)

4.3.1   Extraction of JAI

Two kilograms lots of peeled JA tubers (section 3.1.1) were chopped into fine pulp in 10 L of hot distilled water containing 100 ppm sodium metabisulphite ($Na_2S_2O_5$) in a Stephan Kettle (Table 3.2) at blade speed of 3,000 rpm, 95 to 98°C for 10 min. The presence of the antioxidant, and the high extraction temperature that immediately denatured the endogenous polyphenol oxidase (PPO) and inulases, resulted in a clear juice and prevented the degradation of the inulins during extraction. Moreover, the high temperature partially denatured the proteins (up to 5.5% fresh basis) (Praznik and Beck 1987; Rakhimov *et al.* 2003) and improved the solubility of oligosaccharides (Hansen and Madsen 1992). To avoid degradation of the inulin chain due to excessive shear force developed in the Stephan Kettle, the extraction time was limited to 10 min. This was an improvement on previous extractions that took 30 min to an hour. As most of the minerals are concentrated on the skin of the JA tubers (Mullin *et al.* 1994) the tubers were peeled prior to extraction to minimise the leaching of minerals into the resulting extract. This also decreased discolouration during the extraction.

The crude extract at 86°C was filtered through muslin cloth and the wet pulp was re-extracted with 5 L water at the same temperature range for 5 min, followed by hand-pressing, and *ca.* 3 kg crude extract with a soluble solids of *ca.* 2°B was collected.

Chapter 4

The pooled extracts were then concentrated to 50-60% of the original volume (*ca*. 5°B) using a single-stage climbing film evaporator (Table 3.2), operating under a vacuum of 68 kPa with steam supply at 138 kPa. The resulting concentrate was turbid due to the presence of particulate and colloidal matter, i.e. pectin, protein, and cell wall materials. To remove these impurities, the concentrate was mixed with a 5% slurry of calcium hydroxide at 50-60°C for 30 min, resulting in the formation of a flocculent precipitate and a brighter yellow supernatant. As a result of this technique, the pH rose from 5-6 in the original extract to 10-12 in the concentrate. After filtration under vacuum using paper filter (Whatman No. 4), 10% phosphoric acid (H_3PO_4) was added to the filtrate with vigorous stirring to precipitate excess calcium and coagulate organic material and to adjust the pH to *ca*. 8-9. The mixture was allowed to stand at 60°C for 2-3 h before re-filtering (Whatman No.4). The clarification process was repeated twice resulting in clarified, pulp-free juice with 6-7°B soluble solids. Activated carbon powder was added to the filtrates at 60°C and mixed for 15-30 min in order to remove coloured materials.

The treated juice was filtered (Whatman No.1) and the clear juice obtained was further concentrated by rotary evaporator (Table 3.2) at $\leq$ 70°C, to obtain syrups with soluble solids levels varying from 10°B to 40°B which were then stored at -20°C until further use. The various fractions obtained during processing stage, together with JA tubers were assayed for total fructans (section 3.3.1), total CHOs (section 3.3.2), total solids content (section 3.3.5) and ash (section 3.3.6). The processing scheme for the preparation of JAIS is shown in Figure 4.2.

Cleaned JA tubers | Na₂S₂O₅ | Ca(OH)₂ | H₃PO₄ | Activated carbon powder

△1 △1 △1 △1 △1

②2 Peel
②2 Weigh 100 ppm
②2 Prepare 5%, w/v
②2 Prepare 10%, v/v
②2 Weigh 1%, w/v

③3 Hot water extraction 95°C for 10 min

Crude extract

④4 Hot filter through muslin cloth

⑤5 Evaporate using climbing film evaporator

⑥6 Slaked lime (pH to 10-12) at 60°C & neutralise with acid (pH 8-9), repeated 2 times

Clarified juice

⑦7 Clarify using filter paper

⑧8 Decolourise ≥ 2 times at 60°C

Concentrate syrup

⑨9 Concentrate using rotary-evaporator at 70°C

▽10 Concentrate syrup (10-40°B)

Figure 4.2   Flow diagram for the preparation of JAIS on a laboratory scale

△ Incoming raw material   ◗ Delay

▽ Finish product   ⊡ Combined operations and inspection

○ Operations

79
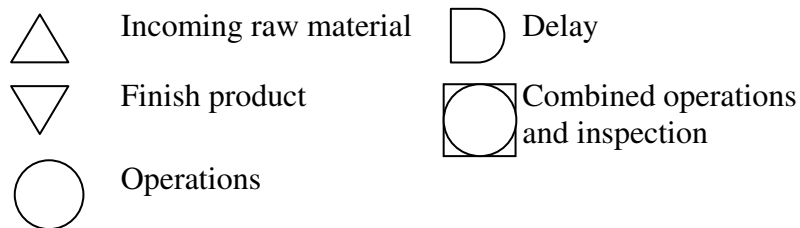
4.3.2 Solvent fractionation

A central composite rotatable design (CCRD) was adopted to evaluate the combined effects of dependant variables i.e. the initial syrup concentration, ethanol-to-syrup (E/S) ratio and precipitation temperature (Smits *et al*. 2001; Kunz *et al*. 1995; Moerman *et al*. 2004). Five levels of each variable were selected to cover the process conditions (Table 4.1). The axial distance ($\pm$ α) was $\pm$ 1.68 which made this design orthogonal. The complete designs consisted of 20 combinations i.e. 8 factorial points, 6 axial points and 6 centre points (Myers 1976; Montgomery 1996). Two replications were carried out for all design points except the centre points. The sequence of experiment was randomised to minimise the effects of uncontrolled factors.

Table 4.1   Independent variables and their levels used for this study

Variables	Symbols		Levels [1]				
	Coded	Un-coded	−1.68 (−α)	−1	0	1	+1.68 (+α)
Syrup concentration (°B)	X_1	A	9.9	16.0	25.0	34.0	40.1
E/S ratio	X_2	B	2.3	5.0	9.0	13.0	15.7
Temperature (°C)	X_3	C	3.1	15.0	32.5	50.0	61.9

[1] Levels are based on the central composite rotatable design.

To obtain inulin precipitate, aliquots (2 g) of JAIS at soluble solids content of 10, 16, 25, 34 and 40°B were individually weighed into pre-weighed test tubes and mixed with 2.3, 5, 9, 13 and 15.7 parts by weight of ethanol (abs. 99%), depending on the levels defined in the experimental design. Addition of ethanol to JAIS produced opalescence and yielded a heavy flocculent precipitate. The test tubes were vortexed and hermetically sealed before storage at temperatures 3, 15, 32.5, 50 and 62°C for 3 days. After storage, the supernatants were removed by siphoning and the precipitates were washed with 5 mL of ethanol, that was subsequently discarded and the tubes containing precipitates were placed in a hot air oven at 102°C for 30 min to remove excess solvent

and re-weighed. The precipitate, formed as a pasty substance, was then analysed for total fructans, total CHO, reducing sugar and dry matter content as described in sections 3.31-3.33 and 3.3.5, respectively. Each determination was performed in duplicate. To facilitate transfer of the precipitate from the test tube to drying dish and/or volumetric flask, it was mixed with small amount of warm distilled water (60°C).

The following equations were used to calculate dependant variables or responses i.e. the fructans yield, average chain length, and purity value of fructans.

The fructans yield was calculated according to the Equation 1:

$$Y_1 = \frac{F_P}{F_M} \times 100 \quad (1)$$

where Y_1 is fructans yield (%), F_P is the amount of fructans contained in the precipitate (g) and F_M is the amount of fructans in JAIS utilised for precipitation (g).

The average chain length was calculated according to the Equation 2:

$$Y_2 = \frac{TC}{RS} \quad (2)$$

where Y_2 is average chain length, TC is total amount of CHO (g) and RS is total amount of reducing sugar (g).

The purity value was calculated according to the Equation 3:

$$Y_3 = \frac{F_P}{DM} \times 100 \quad (3)$$

where Y_3 is purity value and DM is the dry matter content of the precipitate (g).

The least square regression methodology by SPSS 15.0 for Windows (SPSS Inc. 2006) was used to fit the data to the second-order equations:

$$Y = b_o + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 \quad (4)$$

where Y = dependent or response variable; b_0 = intercept; b_1, b_2, b_3 = linear coefficients; b_{11}, b_{22}, b_{33}, = quadratic coefficients; b_{12}, b_{13}, b_{23} = interaction coefficients; and X_1, X_2 and X_3 = independent variables (Myers 1976). The Student's t-test was employed to evaluate the statistical significance of regression coefficients. Non-significant terms ($P > 0.05$) were deleted from the second-order polynomial and a new polynomial was recalculated to obtain a predictive model for each dependent variable (De Faveri *et al.* 2004). The quality of the fit of each equation was expressed by the coefficient of determination (R^2), and its statistical significance was checked by the Fisher's test.

The coded variables were transformed into the actual experimental variables (un-coded variables) using the following equation:

$$X_i = \frac{x_i - (x_{high} + x_{low})/2}{(x_{high} - x_{low})/2} \quad (5)$$

where X_i is the coded variable, x_i is the actual variable, x_{high} and x_{low} are the high and low values of the actual variable, correspondingly.

The response surface plots were drawn using STATISCA version 5.0 for Windows (StatSoft Inc. 1995). One independent factor was kept constant at a centre point and the other two factors were varied within the experimental range. The optimum set of conditions was searched using Design Expert software (trial version 7.0.2, Stat-Ease Inc., Minneapolis, MN, USA). The validity of the mathematical models obtained was verified by comparing between the experimental values and values calculated from the model.

4.3.3   Cold fractionation

A 2^2 factorial design was applied and four experimental runs were performed for studying the combined effects of two independent variables i.e. initial syrup concentration and temperature on the precipitation of inulin at low temperature. In the course of this study, tubers of JA harvested during May-June 2007 were used to prepare JA inulin syrup (JAIS) following the method given in section 4.3.1. The syrups with soluble solids 6 and 12°B were separately warmed to 60°C and aliquots of *ca*. 50 mL of each sample were transferred into duplicate conical screw cap centrifuge tubes and stored at 4 and −24°C overnight (approx.16 h). The freezing and thawing times, as well as centrifugation time and velocity were not independent variables and thus were kept constantly. The frozen samples were then thawed at the RT which caused the precipitation of inulin appearing as a dense white substance at the bottom of the tubes that was recovered by centrifugation at 2,700 *g* and 4°C for 10 min. Samples stored at 4°C were centrifuged under the same conditions. The supernatants of all samples were gently removed by siphoning and the centrifuge tubes containing the precipitates were weighted. The precipitation yield was calculated as the percentage ratio of total precipitated mass of inulin to the mass of the syrup placed in the centrifuge tubes, on fresh weight basis. All treatments were performed in triplicate.

In order to produce a stable ingredient for long-term storage, similar to commercially available inulin powder, a Niro laboratory spray drier with a vane type rotary atomiser (Table 3.2) which was employed to transform the precipitate into a dry powder. The feed stock for spray drying was prepared by re-dissolving the precipitate by placing the centrifuge tubes in a water bath at 60°C prior to spray drying with an inlet temperature of 120°C, outlet temperature of 80°C and air pressure of 5 kg cm^{-2}.

Chapter 4

4.4   Results and discussion

4.4.1   Development of extraction processes

Table 4.2 summarises the chemical compositions of JA tubers and various fractions obtained during extraction process. Tubers of JA, contained *ca*. 26 g solids per 100 g on fresh basis which mainly consisted of 13.2 g total fructans accounting for 85% of total CHO, sucrose (2.1 g), hexoses (0.1 g) and small amount of ash (1.1 g). The balance is made up of insoluble fibre, protein and fat (Frese 1993). This composition puts the JA as a promising source for preparation of inulins.

Various extraction procedures were primarily tested in the laboratory to study their efficiency for CHO release. Tubers of JA (*ca*. 100 g) were either thinly sliced (2-3 mm thickness) or cut into stripes, placed in universal bottles, and mixed with 1.5-2 parts warm water and kept in a water bath at 60, 70 or 80°C with constant stirring for 1 h. The resulting extract showed only 5-8°B. Upon slicing the JA tubers discoloured due to the action of PPO present and resulted in a dark brown extract. Therefore, the extraction process involving the inactivation of the PPO was approached. Blanching the whole tubers in hot water for 5-10 min or with steam at 98°C for 4-12 min prior to slicing and hot water extracting was found to minimise the discolouration of the extract. However, the processes needed optimisation as the set up was time and labour intensive and not suitable for achieving high throughput, and seemed to be only suitable for small scale extraction.

Table 4.2   Chemical compositions of JA tubers and various fractions obtained during extraction process[1]

Compositions (%, w/v)	Type of samples							
	JA tuber	n	Crude extract[2]	n	Clarified juice[3]	n	Conc. syrup (25°B)	n
Total solids	26.43 ± 0.78[4]	6	4.81 ± 0.56	6	5.39 ± 0.29	6	26.39 ± 1.62	6
Ash	1.12 ± 0.15	6	0.40 ± 0.12	6	0.25 ± 0.04	6	1.51 ± 0.36	6
Total CHO	15.50 ± 1.08	6	3.29 ± 0.70	6	4.57 ± 0.38	6	25.65 ± 0.32	6
Total fructans	13.23 ± 0.54	6	-		3.95 ± 0.31	6	17.86 ± 0.40	6
Sucrose	2.08 ± 0.01	2	-		-		7.50 ± 0.28	2
Free glucose	0.02 ± 0.00	2	-		-		0.08 ± 0.01	2
Free fructose	0.08 ± 0.01	2	-		-		0.34 ± 0.03	2

n = Number of observations.
[1] Performed in 2005.
[2] After hydrothermal extraction and filtration through muslin cloth.
[3] After lime and carbonation treatments.
[4] Values are mean ± SD.

In the adopted optimised process, the resulting juice with soluble solids concentration of 6-7°B was clear (no absorption at 420 nm), with negligible protein content (no absorption at 280 nm), but still containing 0.25% ash since it was not subjected to ion-exchangers. In addition to inulins, the resulting juice contained some mono- and disaccharides. The concentrated thick syrup at 25°B, for example comprised of 7.5% sucrose, 0.34% fructose and 0.08% glucose (w/v) (Table 4.2). These sugars mainly originated from the tubers but small amounts could also have been formed during the extraction and clarification steps.

Table 4.3 presents the yield and soluble solids of the JA extract in each processing step. The hydrothermal extraction process developed in this study produced 13.2% (w/v) total fructans from 2 kg of tubers that resulted in *ca*. 3.5 kg of clarified juice (7°B) containing 4.0% (w/v) total fructans. This is a yield of *ca*. 30% based on the amount of inulins in the tubers which is rather low, probably due to short extraction time, filtration

inefficiency, incomplete recovery of juice from the pulp and high ratio (5:1, v/w) of distilled water to tubers used. However, in the study by Leite Toneli *et al*. (2007), a ratio of 2:1 (v/w) was employed, and a comparable yield (*ca*.1.7 kg) and solid content (*ca*. 8°B) was obtained after the extraction in a Stephani multiprocessor at 80°C for 1 h. Satisfactory yield and the solids content could be achieved if a countercurrent extractor, similar to that used in conventional sugar beet extraction was employed (Berghofer *et al*. 1993; Kunz 1995; Silver 2003).

Table 4.3   Yield of JA extract at several stages of preparation

Process steps	Tuber mass (kg)	Water mass (L)	Mass yield (kg)	Soluble solids (°B)
1st extraction	2	10	7	4
2nd extraction	–	5	3	2
Concentration	–	–	6	5
Lime-carbonation treatment	–	0.5	3.6	6-7
Activated carbon treatment	–	–	3.5	6-7

4.4.2   Solvent fractionation

In this part of the study, ethanol (abs. 99%) was employed to precipitate the inulin from the concentrated syrup. The experiments were performed according to a response surface methodology design, in a syrup concentration range of 10 to 40°B with varying ethanol-to-syrup (E/S) ratios from 2.3 to 15.7 and a temperature range of 3 to 62°C. The combined effects of the three dependent variables on fructans precipitation yield, average chain length and purity are presented in Table 4.4.

Table 4.4   The central composite rotatable design with the effects of independent variables on three dependent variables

Run[a]	Independent variables						Dependent variables[e]		
	Coded levels			Un-coded levels					
	X_1[b]	X_2[c]	X_3[d]	A[b]	B[c]	C[d]	Y_1	Y_2	Y_3
1	−1	−1	−1	16.0	5.0	15.0	44.41	11.87	67.78
2	−1	−1	+1	16.0	5.0	50.0	34.86	10.50	63.63
3	−1	+1	−1	16.0	13.0	15.0	45.60	11.80	60.50
4	−1	+1	+1	16.0	13.0	50.0	46.48	16.42	68.28
5	+1	−1	−1	34.0	5.0	15.0	67.61	22.65	71.64
6	+1	−1	+1	34.0	5.0	50.0	64.42	21.90	74.92
7	+1	+1	−1	34.0	13.0	15.0	67.93	19.31	71.97
8	+1	+1	+1	34.0	13.0	50.0	67.81	21.66	75.12
9	−1.68	0	0	9.9	9.0	32.5	33.52	12.01	61.09
10	+1.68	0	0	40.1	9.0	32.5	61.56	19.67	63.53
11	0	−1.68	0	25.0	2.3	32.5	34.73	14.94	74.30
12	0	+1.68	0	25.0	15.7	32.5	63.10	19.68	75.99
13	0	0	−1.68	25.0	9.0	3.1	54.31	17.59	62.74
14	0	0	+1.68	25.0	9.0	61.9	51.79	17.93	64.88
15	0	0	0	25.0	9.0	32.5	59.41	25.10	70.39
16	0	0	0	25.0	9.0	32.5	59.35	23.93	71.44
17	0	0	0	25.0	9.0	32.5	55.54	20.13	71.60
18	0	0	0	25.0	9.0	32.5	55.04	21.85	70.19
19	0	0	0	25.0	9.0	32.5	58.12	20.89	70.86
20	0	0	0	25.0	9.0	32.5	55.15	21.05	70.03

[a] Does not correspond to order of processing.
[b] X_1 and A, initial syrup concentration (°B).
[c] X_2 and B, ethanol-to-syrup ratio.
[d] X_3 and C, precipitation temperature (°C).
[e] Y_1, Y_2 and Y_3 represent fructans yield (%), average chain length and purity value (%), respectively.

The independent and dependent variables in Table 4.4 were fitted to the second-order equations by the least square technique where their statistical significances were judged by Student's t-tests at a probability of 0.001, 0.01 or 0.05. The coefficients of variables in the equations developed are presented in Table 4.5. The table also summarises the

statistical parameters, namely the determination coefficient (R^2) and F-test probability, both of which are used for measuring the correlation and significance of the models. Results of the R^2 values showed a good agreement between experimental data and predicted data for all regressions (0.87, 0.89 and 0.82 for Y_1, Y_2 and Y_3, respectively). The results of F-test showed a statistically significant relationship between the variables within 95% confidence interval.

Table 4.5 **Regression coefficients, R^2 and F-test probability for three dependent variables**

Regression coefficients[a]	Y_1	Y_2	Y_3
b_0 (constant)	56.925[*]	22.147[*]	70.676[*]
b_1	10.513[*]	3.501[*]	2.750[**]
b_2	4.703[***]	0.750	0.054
b_3	−1.187	0.397	1.000
b_1^2	−2.221	−2.158[**]	−2.485[***]
b_2^2	−1.735	−1.638[***]	2.050[***]
b_3^2	−0.273	−1.479[***]	−1.955[***]
b_{12}	−1.137	−1.179	0.395
b_{13}	0.670	−0.206	0.350
b_{23}	1.687	1.136	1.475
R^2	0.87	0.89	0.82
F-test probability	0.003[**]	0.001[*]	0.010[***]

[a] Expressed according to Equation (4); subscripts: 1 = syrup concentration; 2 = ethanol-to-syrup ratio; 3 = precipitation temperature.
* Significant at 0.001 level.
** Significant at 0.01 level.
*** Significant at 0.05 level.

After rejecting non-significant terms ($P > 0.05$) from the second-order polynomial and recalculating, newly predicted models, expressed in terms of coded and un-coded variables for each dependent variable were developed that are summarised in Table 4.6.

Table 4.6   **Final equations in terms of coded and un-coded variables for the prediction of three response variables**

Response variables[a]	Coded equations (Un-coded equations)[b]
Y_1	$= 54.037 + 10.513 X_1 + 4.703 X_2$
	$(= 14.252 + 1.168 A + 1.176 B)$
Y_2	$= 22.147 + 3.501 X_1 + 0.750 X_2 + 0.397 X_3 - 2.158 X_1^2 - 1.638 X_2^2 - 1.479 X_3^2$
	$(= -20.048 + 1.721 A + 2.030 B + 0.337 C - 0.027 A^2 - 0.102 B^2 - 0.005 C^2)$
Y_3	$= 70.676 + 2.750 X_1 + 0.054 X_2 + 1.000 X_3 - 2.485 X_1^2 + 2.050 X_2^2 - 1.955 X_3^2$
	$(= 39.139 + 1.839 A - 2.293 B + 0.865 C - 0.0307 A^2 + 0.128 B^2 - 0.0124 C^2)$

[a] Y_1, Y_2, Y_3 represent fructans yield (%), average chain length and purity value (%), respectively.
[b] Transformation of variables from coded to un-coded could be calculated according to Equation (5) with the following terms: $X_1 = (A-25)/9$, $X_2 = (B-9)/4$ and $X_3 = (C-32.5)/17.5$.
X_1 and A, initial syrup concentration (°B).
X_2 and B, ethanol-to-syrup ratio.
X_3 and C, precipitation temperature (°C).

The results of F-test for all new regressions showed a high statistical significance at 99.9% confidence interval. The R^2 values of the models dropped to 0.80, 0.83 and 0.77 for Y_1, Y_2 and Y_3, respectively.

4.4.2.1 Effects of precipitation conditions on fructans precipitate yield

The results presented in Table 4.5 indicate that the initial syrup concentration had a strong linear effect on fructans precipitate yield, followed by the linear effect of E/S ratio. Analysis of regression coefficients also indicated that temperature had no significant effect on the yield ($P > 0.05$). After rejecting the statistically insignificant terms, the predictive model for the yield was found to be a first-order equation (Table 4.6). As a result, the response surface plot generated for the fructans yield showed flat areas and no maximum or minimum responses were present (Figure 4.3). It was observed that the precipitate yield increased linearly as the initial syrup concentration increased. However, in order to achieve more than 50% fructans yield from the precipitate, a syrup with a minimum concentration of 16°B should be utilised.
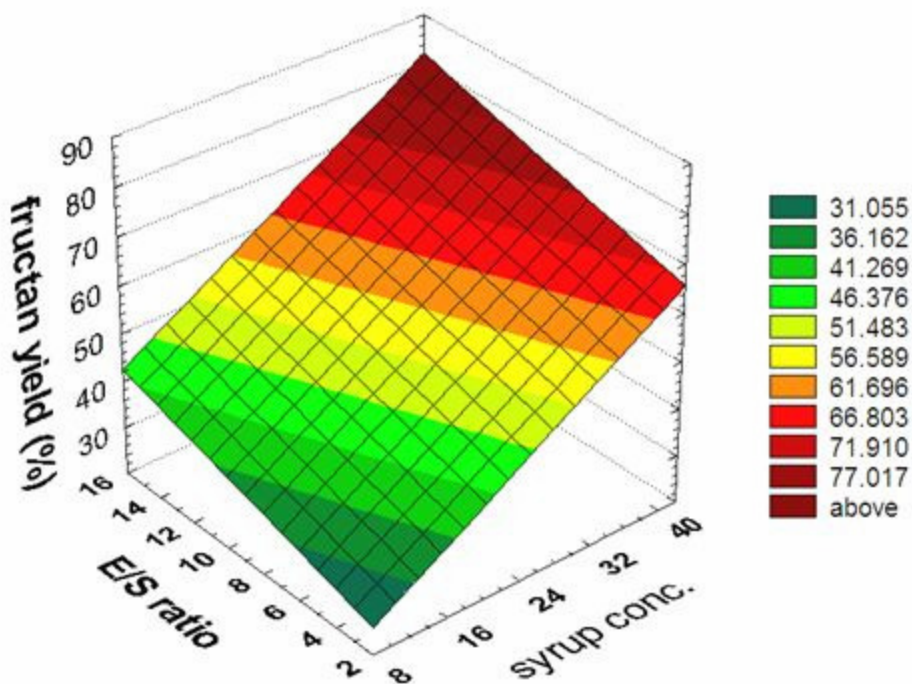
Figure 4.3 **Response surface graphs of fructans precipitation yield (%) as a function of initial syrup concentration (°B) and E/S ratio**

4.4.2.2 Effects of precipitation conditions on average chain length

The average chain length was calculated by dividing the amount of total CHO present in the precipitate by the amount of reducing end groups. It is considered as an index of the precipitate since it is closely related to the functional properties when used in food applications. The average chain length also indicates the ability of the extraction process to cause undue hydrolysis of poly- or oligosaccharides.

The concentration of syrup was the most significant determinant of the average chain length in precipitate since its linear and quadratic effects mainly contributed to the total variation (Table 4.5). The relationship between three independent variables on average chain length is demonstrated in Table 4.6 and Figure 4.4. The behaviour of response surface graphs (Figures 4.4a & 4.4b) indicated that increasing syrup concentration up to 32°B had a positive effect on average chain length in the precipitate. However, there seemed to be less effect of further increases in syrup concentration beyond 32°B on the

chain length. As can be seen in Figures 4.4a & 4.4b, a similar average chain length was obtained from syrups with concentrations between 24 and 40°B. The effects of E/S ratio and temperature on the average chain length were similar to the effect of syrup concentration (Figure 4.4c). It was found that the optimum precipitation should be carried out at 33°C with E/S ratio of 10:1 (v/v) to yield the highest average chain length and any further increase beyond these values resulted in lower average chain length. The results of this study were in agreement with those of Moerman *et al*. (2004) who found that the average chain length of commercial grade chicory inulin and dahlia inulin decreased with increased level of added solvent (ethanol, acetone or methanol) beyond a solvent to solution ratio of 1:2 (v/v). It is believed that at the initial stage, any increase in solvent ratio leads to increased average chain length due to the precipitation of longer-inulin chains, but adding excess solvent beyond certain ratio precipitates the shorter-chain CHOs thus reducing the average chain length of the precipitate (Moerman *et al*. 2004; Ku *et al*. 2003).
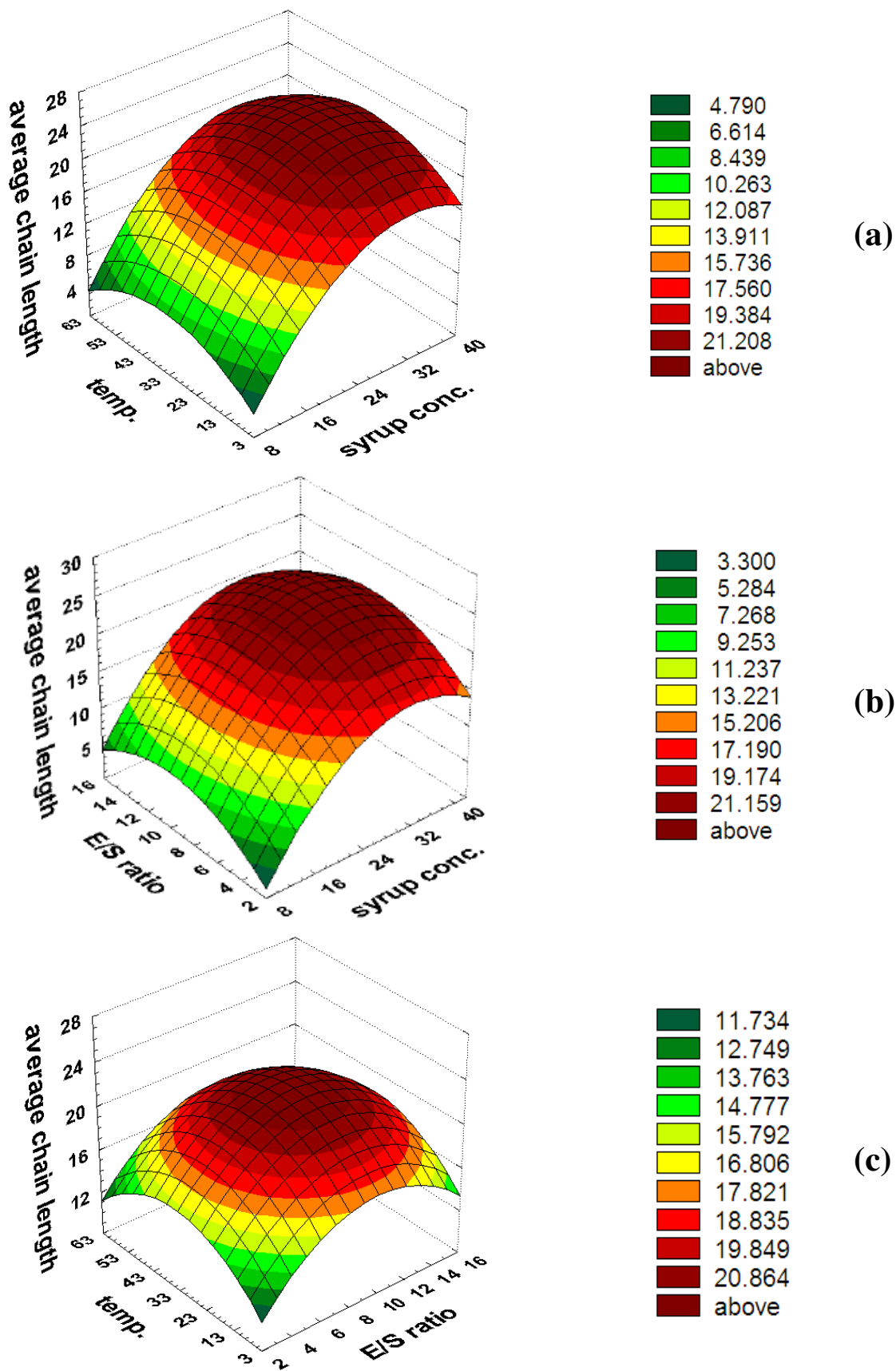
Figure 4.4   Response surface graphs of average chain length at (a) E/S ratio of 9:1, (b) temperature of 32.5°C and (c) syrup concentration of 25°B

4.4.2.3 Effects of precipitation conditions on the purity of inulins

Purity represents an index of the amount of inulins in the precipitate obtained. High value means that inulins are accounted as the main components of the precipitate. Low purity value, on the other hand, indicates the high contents of mono- and disaccharides, and/or non-sugar substances such as ashes in the precipitate. Table 4.6 and Figure 4.5 describe the dependence of purity value on syrup concentration, E/S ratio and temperature. With the increase in syrup concentration, the purity of precipitate increased gradually, but decreased after the concentration reached a maximum at 30°B (Figures 4.5a & 4.5b). The effects of E/S ratio and temperature were not profound in comparison to syrup concentration. At constant temperature of 32.5°C, the precipitates formed in syrup with concentration range of 8 to 24°B showed a high purity at intermediate E/S ratios (8:1-10:1, v/v). At higher syrup concentrations of 24 to 36°B, either low E/S ratio (less than 7:1, v/v) or high E/S ratios (more than 11:1, v/v) was required (Figure 4.5a). Figure 4.5c presents the combined effects of temperature and E/S ratio on the purity of inulin at syrup concentration of 25°B. It shows that moderate temperatures between 23 and 43°C resulted in higher purity values, particularly when using either extremely low or high ratios of E/S. The maximum response was obtained at 37°C.
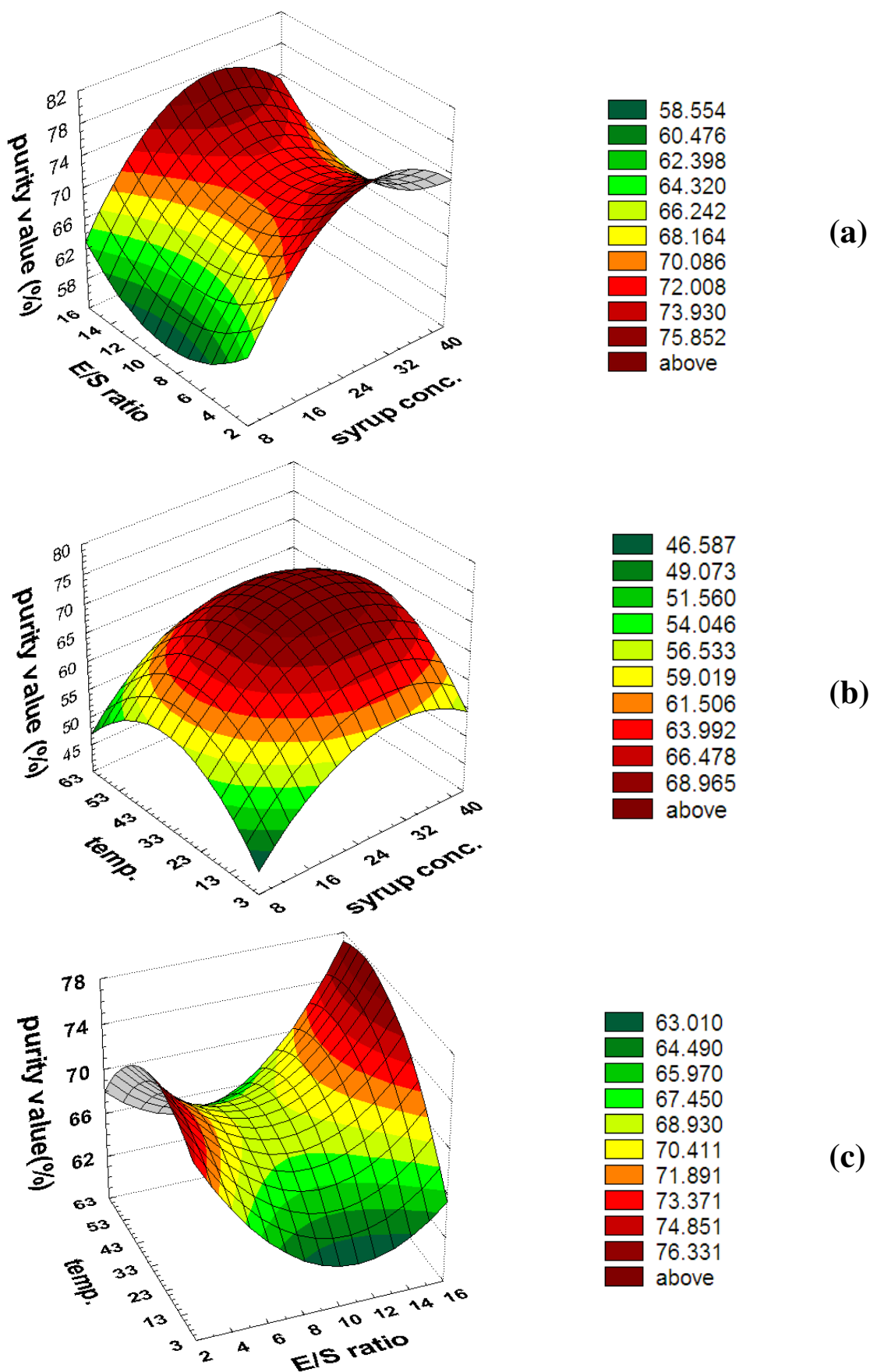
Figure 4.5 **Response surface graphs of purity value (%) at (a) temperature of 32.5°C, (b) E/S ratio of 9:1 and (c) syrup concentration of 25°B**

4.4.2.4 Optimisation of precipitation conditions and verification of the results

The optimum precipitation conditions for concentrated JAIS were achieved by means of graphical technique using design expert software (De Faveri *et al*. 2004). Three contour plots generated from the predicted equations were superimposed to achieve the experimental region giving desired values of the responses. In this study, the independent variables would be considered optimum if all dependent variables were as high as possible. Therefore, the criteria applied for the graphical optimisation were 68% > Y_1 > 50%, Y_2 > 20 and 76% > Y_3 > 70%. Figure 4.6 shows the overlaid plot of the three dependent variables. The white area represents the region produced by the criteria outlined above and the flag depicted within the same area indicates the optimised point. The best combination of process variables was found to be the initial syrup concentration of 32°B, E/S of 13:1 and temperature of 42°C. Under these conditions, the model gave predicted values of Y_1, Y_2 and Y_3 to be 66, 22 and 75%, respectively.
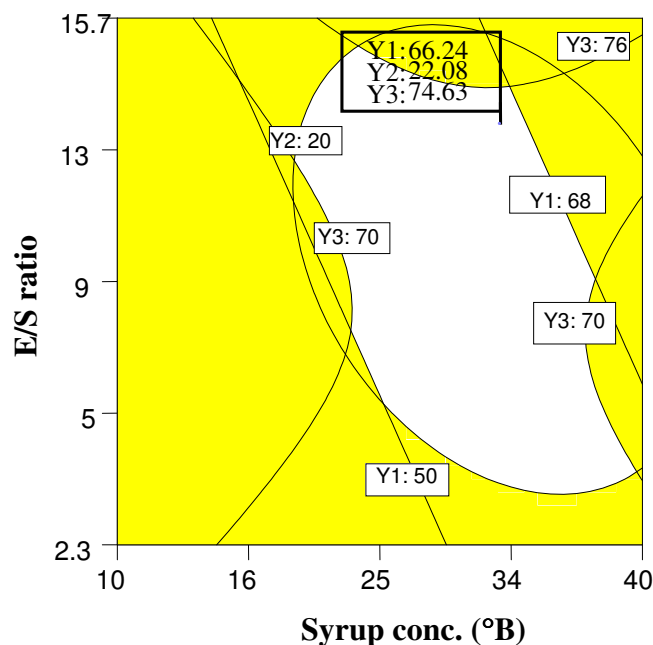
Figure 4.6   Optimum region identified by the overlaid plot of the three responses: fructans yield (Y_1), average chain length (Y_2), purity value (Y_3)

Chapter 4

Verification experiments were carried out to confirm the adequacy of the models for predicting the values of dependent variables. Under the selected conditions from the response surface methodology optimisation, the experimental results and the predicted values are listed in Table 4.7. A fructans precipitate yield of 65% with an average chain length of 21 found in the experiments confirms the closeness of fit between the model and the experimental results. The prediction of the purity value (73%) did not closely match the experimental value (68%), possibly due to low R^2 value of the model (0.77), indicating that a high proportion of variability was not explained by the model.

Table 4.7 **Experimental and predicted values for response variables at optimum conditions[a]**

Dependent variables[b]	Experimental values[c]	Predicted values
Y_1	64.85 ± 0.76	66.92
Y_2	21.40 ± 0.35	21.86
Y_3	68.30 ± 1.04	72.83

[a] Optimum reaction conditions: JAIS concentration 32°B, E/S 13:1 and temperature 42°C.
[b] Y_1, Y_2, Y_3 represent fructans yield (%), average chain length and purity value (%), respectively.
[c] Results shown are mean ± SD for three experiments each with two replications.

4.4.3 Cold fractionation

The solubility of inulin in water is temperature dependant, varying from 6% at 10°C to 35% at 90°C (Silva 1996). Low solubility at low temperatures is a useful property which can be employed to separate high-MW inulin fractions from aqueous solutions. As the temperature decreases, the heavier-MW inulins tend to settle at the bottom of the container and push the low-MW inulins and mono- and disaccharides upward (Moerman *et al*. 2004).

Table 4.8 shows the precipitate yield of inulin as a function of cooling temperature and initial syrup concentration. No precipitation was observed at 4°C with syrup concentration of 6°B and it was necessary to raise initial syrup concentration to 12°B to

achieve the precipitation. The precipitate yield of inulin obtained varied from *ca.* 14% to 36%. The best result was associated with the highest syrup concentration (12°B) and the lowest temperature (−24°C). For frozen samples at −24°C, upon thawing at RT, phase separation was evident and their precipitate yield increased proportional to the concentration of the syrup.

Table 4.8   Precipitate yields of inulin corresponding to combined effects of initial syrup concentration and precipitation temperature

Run	Factor		Precipitate yield (%, w/w)
	Initial syrup concentration (°B)	Precipitation temperature (°C)	
1	6	4	NP
2	12	4	14.1 ± 0.9^c
3	6	-24	18.2 ± 0.6^b
4	12	-24	35.6 ± 0.9^a

Results shown are mean ± SD for three experiments each with two replications.
Different letters on the value denote significant difference at $P \leq 0.05$ by DMRT.
NP = no precipitate.

After cold fractionation, the precipitates were separated by siphoning the supernatants, re-dissolved by warming in a water bath and spray-dried. The powder contained *ca.* 96.9 ± 1.1% (w/w) solids, comprising *ca.* 77.1% inulin, 6.3 % ash and 0.1% fat. It appeared as an off-white powder with average colour values of $L^* = 81.0 \pm 3.5$, $a^* = -1.3 \pm 0.8$ and $b^* = -1.7 \pm 1.0$. The powder showed total plate count of < 2,000 CFU g^{-1} and total yeast and mould counts of < 30 CFU g^{-1}. No viable *E. coli* was found in the powder (data not shown).

4.5   Comparison of the fractionation techniques

In the work presented here, two different techniques i.e. solvent and cold fractionation were adopted to fractionate the high- and low-MW fractions of inulin extract. The syrups used as starting material for each fractionation process were diverse in their

initial CHO composition due to the difference in cultivation year of the tubers. Therefore, the efficiency of the two procedures could not be compared directly. However, a comparison was made on the improvement of total fructans in final products of each fractionation process as presented in Table 4.9.

Table 4.9 Improvement of total fructans in samples obtained by two fractionation techniques

Compositions (%w/w)	Total fructans (%)	Total CHO (%)	Total fructans /total CHO (%)	Differences (%)
Ethanol fractination[1]				
Concentrate[3]	65.4	94.5	69.2	
Precipitate[4]	69.0	80.3	86.0	16.8
Cold fractionation[2]				
Original powder[5]	73.0	85.6	85.3	
Powder from fractionated syrups[6]	77.1	78.9	97.8	12.5

[1,2] Performed in 2005 and 2007, respectively.
[3] Concentrate syrups employed for ethanol fractionation.
[4] Precipitates isolated after ethanol fractionation.
[5] Spray-dried inulin powder obtained from inulin syrups without cold fractionation.
[6] Spray-dried inulin powder obtained from inulin syrups with cold fractionation.

The precipitates obtained from ethanol fractionation contained nearly 17% more fructans content than that of concentrate syrups employed as starting material. The cold fractionation seemed less satisfactory. The fructans content in spray-dried powders prepared from fractionated inulin syrups increased by nearly 13% compared to those prepared without cold fractionation. However, ethanol fractionation is rather complex. The process involves large volumes of solvent which is problematic for safety and environment. In contrast, cold fractionation is a more economical and environmentally friendly process. The inulin precipitate can easily be converted into powder. For these reasons, in further investigations, cold fractionation was employed for inulin fractionation from JAIS.

4.6 Conclusions

The tubers of JA grown in Australia contain several types of water extractable carbohydrates, predominantly inulin-type fructans. Response surface methodology which combines factorial designs and regression analysis is a useful tool for optimising process conditions for ethanol fractionation of inulin from JA concentrate. The models developed allowed identification of the optimum reaction conditions (32°B syrup concentration with 13:1 ethanol-to-syrup ratio at 42°C) and can be used for predicting the quality of inulin precipitate. A satisfactory agreement was found between the values predicted and the values determined experimentally, particularly for the fructans precipitation yield and the average chain length. As another process, cooling of JA concentrate below freezing temperature and/or leaving in the refrigerator also resulted in the precipitation of inulin as a pasty substance. The results showed that initial syrup concentration and precipitation temperature affected the precipitate yield of inulin. Within the range of conditions studied, inulin syrup with 12°B gave the highest precipitate yield of *ca.* 36% by weight when frozen to −24°C.

Chapter 5

Lactulose production from milk concentration permeate using calcium carbonate-based catalysts[1]

5.1 Abstract

Milk concentration permeate (MCP), a low-value by-product of ultrafiltration plants and calcium carbonate-based catalysts were used for lactulose production. With the optimum reaction conditions of 12 mg per mL catalyst loading, isomerisation time of 120 min at 96°C, a maximum yield of 18-21% lactulose was achievable as measured by HPLC. The results obtained showed the suitability of oyster shell powder (OSP) for lactose isomerisation as a replacement for egg shell powder (ESP). Removal of protein contaminants from MCP by acidification prior to isomerisation improved lactulose formation at an earlier stage, but did not significantly increase the yield. The brown colour of the lactulose-enriched MCP was partially removed by treatments with activated carbon (5 g per 100 mL) or with 17.5% hydrogen peroxide (10 mL per 100 mL) without significant loss of lactulose.

5.2 Introduction

A large volume of MCP is produced in Australia by dairy processing plants as a consequence of the increased utilisation of ultrafiltration (UF) for preconcentration of milk. Permeate is not suitable for human consumption and has little commercial value. The solid content of MCP consists predominantly of 4.5-4.8% lactose and 0.44-0.47% mineral salts which is similar to that of skim milk, except for lacking the proteins

---

(Suarez *et al.*2006). Having a high biological oxygen demand (BOD), the MCP needs appropriate treatment before disposal as a waste which is commercially disadvantageous to manufacturers. The existing practices are to use MCP for feeding farm animals, milk standardisation, blending with other dairy liquids and recovering lactose for use in food formulation or other industrial uses. One novel approach is to use it to isomerise lactose into non-absorbable lactose-derivatives e.g. lactulose, lactitol and lactobionic acid (Harju 2001).

Theoretically, lactulose is produced from an alkaline lactose solution via the Lobry de Bruyn-Alberda van Ekenstein molecular rearrangement (Aider and de Halleux 2007). Although there have been a number of studies for lactulose isomerisation using different catalytic systems (section 2.1.4.4), little is known about the suitability of calcium carbonate-based catalysts. In a recent study by Montilla *et al.* (2005), the feasibility of using ESP for isomerisation of MCP has been addressed with a lactulose yield of 1.18 g per 100 mL of MCP.

In the present study, the suitability of other calcium carbonate-based catalysts, including OSP and limestone (pure calcium carbonate) as a replacement to ESP was examined for lactose isomerisation in MCP. A preparation of 4% lactose solution was used as control. The OSP was chosen due to the fact that oyster shells are a waste product from mariculture, causing unpleasant smells in shell-harvesting areas. Apart from the use of oyster shells as a fertiliser and as a mineral source in chicken feed and fish pellets, there is a need for the food industry to find alternative ways for using the shells. The OSP has comparable chemical composition to ESP and can be easily removed after the isomerisation by centrifugation without a need for sophisticated techniques such as chromatographic purification. Therefore, this study was aimed at turning a large surplus

of MCP and oyster shells as by-products into a high value-added product through enrichment of MCP with lactulose. The optimum process condition *viz.* the amount of catalyst, isomerisation temperature and time to achieve maximum isomerisation were determined.

5.3   Isomerisation method

An aliquot of 100 mL MCP (section 3.1.2) was transferred into a 250-mL round-bottom flask. The selected catalyst (section 3.1.3) was added to the vessel and mixed well with magnetic stirrers at room temperature. The flask was then placed in a glycerol bath, and refluxed while stirring at constant temperatures for 120 to 150 min. The zero time of process was taken when the mixture reached the required temperature. Aliquots of 10 mL were taken every 30 min and rapidly placed in an ice bath to stop the reaction. At the completion of heating, the catalyst was removed from the aliquots by centrifugation at 3,600 *g* and 20°C for 10 min. The collected supernatant was stored at 4°C and its sugar content (lactose and lactulose), pH and brown colour (as b^* value and/or absorbance at 420 nm) were determined within 24 h as described in sections 3.41 and 3.43-3.44.

Based on sugar content, the conversion of lactose to lactulose (Y_1, %) and the degradation of lactose (Y_2, %) were calculated as follows:

$$Y_1 = \frac{L_F}{L_B} \times 100 \text{ and}$$

$$Y_2 = \frac{(L_B - L_A)}{L_B} \times 100$$

where L_F is the amount of lactulose formed in the mixture (g per 100 mL), L_B is the amount of lactose before isomerisation (g per 100 mL) and L_A is the amount of residual lactose after the isomerisation (g per 100 mL).

5.4   Treatments

5.4.1   Effect of the catalyst loading on lactose isomerisation

The effect of catalyst loading on lactose isomerisation was studied using ESP at a final concentration of 0, 6, 12, 20 and 30 mg per mL. The isomerisation was performed in triplicate at 98°C for 150 min, following the procedure described in 5.3.

5.4.2   Effect of isomerisation temperature and time on lactose isomerisation

The effect of isomerisation temperature and time on lactose isomerisation was determined in duplicate samples of MCP with 12 mg per mL ESP loading. The isomerisation temperature was set to 90, 96 or 100°C. Samples were taken every 30 min up to 180 min.

5.4.3   Effect of the catalyst type on lactose isomerisation

Limestone, OSP and ESP were compared for their catalytic power in MCP and the control lactose solution. The isomerisation was performed in triplicate with catalyst loading of 12 mg per mL at 96°C for 150 min. Blank trials (without catalyst addition) were also conducted in both solutions, following the procedure described in 5.3.

5.4.4   Effect of de-proteination on lactose isomerisation

Before isomerisation, the proteins contaminants in MCP were removed by acidification and mild heat treatment. The pH of MCP was adjusted to 4.5 with 1% lactic acid. To prevent the hydrolysis of lactose, the acidified MCP was heated for 2-3 min at < 60°C, followed by filtration through Whatman No.1 filter paper, resulting in a green-yellowish clear liquid. The pH was then re-adjusted to 6.7 with 0.1 M NaOH. The de-proteinated MCP was then isomerised in quadruple using 12 mg per mL ESP at 96°C for 150 min, following the procedure described in 5.3.

5.4.5 Colour removal from lactulose-enriched MCP

Aliquots of 100 mL of the lactulose-enriched MCP were treated with 10 mL of 17.5% H_2O_2 at 95°C for 10 min according to the method described by de Haar and Pluim (1991). In another trial, 100 mL lactulose-enriched MCP was treated with 5 g activated carbon powder at 60°C for 10 min and then filtered through Whatman No. 1 filter paper. Each trial was repeated three times. The changes in colour were determined as absorbance values (420 nm) within 24 h after the treatment.

5.5 Results and discussion

Under the experimental conditions employed in this study, heating a mixture of MCP and calcium carbonate-based catalysts resulted in a loss of lactose in favour of lactulose formation. Heating under reflux kept the soluble solids of MCP mixture relatively unchanged at *ca*. 4-5°B. Any extension of heating time beyond maximum isomerisation resulted in the rapid decomposition of lactulose leading to formation of reaction by-products e.g. galactose. In addition, a drop in solution pH and darkening of solution colour were evident.

5.5.1 High-Performance Liquid Chromatography (HPLC) of isomerised MCP

Figure 5.1 shows HPLC-RID chromatograms of carbohydrate fractions of MCP treated with 12 mg per mL ESP before and after isomerisation at 96°C for 120 min. The chromatogram of MPC before isomerisation (Figure 5.1a) shows solvent and lactose peaks with the retention times of 4.19 and 17.61 min, respectively while after isomerisation two new peaks were detected with the retention times of 9.96 min and 15.98 min (Figure 5.1b). The first peak was assigned as galactose which was not always detected unless heating time was extended beyond 90 min and when its concentration was lower than 0.05% of the total sugars, while the second peak was identified as

lactulose. The present HPLC-RID method achieved the separation of the three types of carbohydrates confirming that the isomerisation routes involved both the conversion of lactose into lactulose and decomposition of lactulose into galactose. However, peaks corresponding to lactose and lactulose were not completely resolved and therefore reported concentrations of sugars were taken from peak heights as these provide a suitable means of estimating the amounts (Brian 1992). The concentration of by-product saccharides was not reported in the current study because of the poor sensitivity associated with refractometry which did not allow the determination of epilactose. The peak assigned to galactose may also include glucose due to their close retention times.

(a)   **(b)**
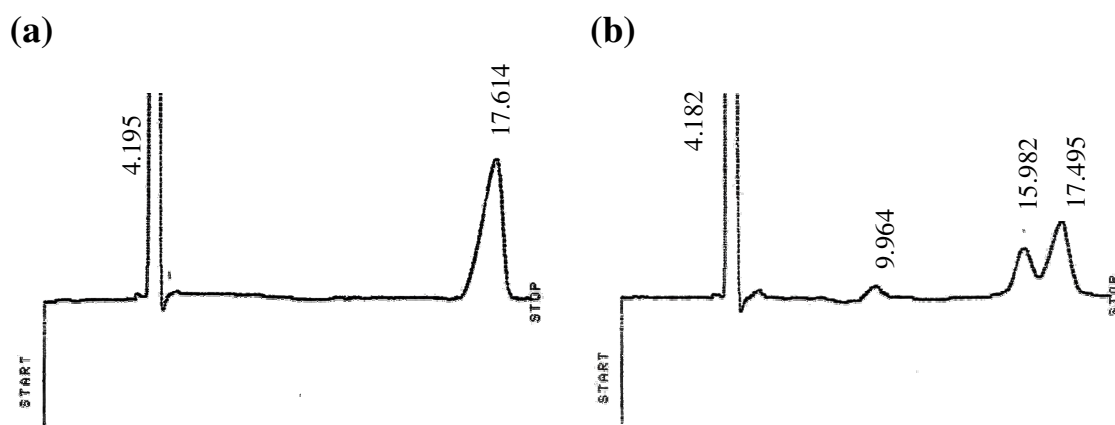
Figure 5.1   **HPLC-RID chromatograms of MCP treated with 12 mg per mL ESP (a) before and (b) after isomerisation with at 96°C for 120 min**
The peaks correspond to: solvent (4.19 & 4.18), lactose (17.61 & 17.49), galactose & glucose (9.96) and lactulose (15.98).

5.5.2   Effect of the catalyst loading on lactose isomerisation

The ESP was employed to determine the appropriate amount of catalyst needed for lactose isomerisation. Figure 5.2 shows variation of lactulose content and pH during the isomerisation of MCP in the presence of 0, 6, 12, 20 and 30 mg per mL of ESP at 98°C for 150 min. In the absence of catalyst (blank) the isomerisation rate was very slow and only 0.12 ± 0.02 g lactulose per 100 mL was detected in MCP after 150 min.
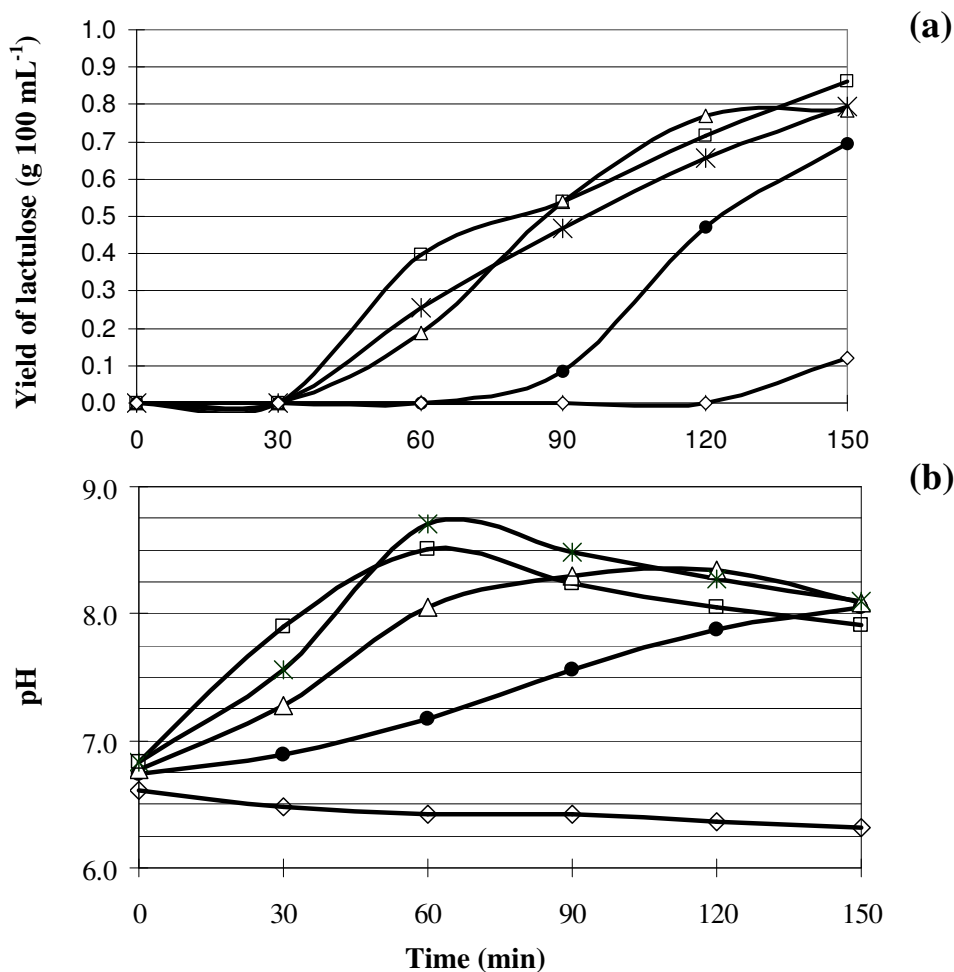
Figure 5.2 **Changes in lactulose content (a) and pH (b) with time in MCP treated with 6 (——●——), 12(——△——), 20 (——✳——) and 30 (——□——) mg ESP per mL, and without catalyst (Blank ——◇——) at 98°C**
Data points are mean of three different experiments.

In the presence of catalyst, a general trend of increased lactulose formation with time was observed although lactulose was not formed in any of the samples during the first 30 min of treatment (Figure 5.2a). After 60 min, the yield of lactulose increased significantly in samples containing ≥ 12 mg ESP per mL while no lactulose was formed in samples containing 6 mg ESP per mL. The yield of lactulose ranged from as high as 0.40 ± 0.07 g per 100 mL with a catalyst loading of 30 mg per mL to as low as 0.18 ± 0.05 g per 100 mL with 12 mg per mL. After 150 min heating, all samples showed higher lactulose yield, samples containing 6 mg ESP per mL consistently showed lower yield (max. 0.69 ± 0.06 g per 100 mL), while the highest yield (0.86 ± 0.02 g per 100 mL) was

achieved in samples with 30 mg ESP per mL. Catalyst loadings of 12 and 20 mg per mL gave close yields of lactulose at 0.77 ± 0.13 and 0.79 ± 0.05 g per 100 mL, respectively. This could be due to the fact that the reaction had reached a catalyst independent stage, therefore, an excess loading of catalyst did not result in higher yield of lactulose. A higher catalyst loading would however affect the isomerisation time. For example, with 6 mg per mL ESP loading the time needed to obtain the maximum yield was 150 min while the similar yield could be attained within 120 min by increasing ESP loading to 12-30 mg per mL.

Figure 5.2b shows the effects of catalyst loading on the pH variations of MCP during isomerisation for 150 min at 98°C. The initial pH of MCP was similar to that of fresh milk and in all cases the change of pH was marginal upon the addition of ESP (*ca*. 6.7-6.8). At a catalyst loading of 20 and 30 mg per mL, the pH reached > 7.5 after 30 min of treatment, then rose to > 8.5 after 60 min and dropped back marginally to 7.9-8.1 after 150 min. The observed pH drop at the final stages of isomerisation could be due to the formation of small amounts of organic acids i.e. isosaccharinic acid (Moreno *et al*. 2003) and formic acid (Berg and van Boekel 1994), derived from degradation of lactulose with prolonged heating. The pH changes were less pronounced in treatments containing 6 mg ESP per mL (max. pH 8.1 at 150 min) and 12 mg per mL (max. pH 8.4 at 120 min).

Although a higher yield of lactulose could be achieved with increasing the catalysts loading, it may lead to the formation of a higher level of coloured by-products as reflected by increased b^* value (data not shown). Therefore, a catalyst loading of 12 mg per mL was considered sufficient for optimum conversion level and minimum formation of degradation by-products, and this loading level was adopted in subsequent investigations.

5.5.3   Effect of isomerisation temperature and time on lactose isomerisation

Table 5.1 shows changes in lactulose, pH and $b*$ values of MCP treated with 12 mg ESP per mL at the various temperatures (90, 96 and 100°C) and times. No lactulose was formed at 90°C for 150 min, and even when the process was prolonged to 180 min only *ca.* 0.06% of initial lactose was isomerised to lactulose (0.24 g per 100 mL). A slight variation in $b*$ values and a slow rise in pH were observed during this process, confirming the very slow rate of lactose conversion.

The yield of lactulose was significantly enhanced at elevated temperatures between 96 and 100°C. Both treatments gave statistically similar amounts of lactulose formed ($P > 0.05$) showing the rapid increase after 60 min (0.24-0.28 g per 100 mL), further rise between 90 and 120 min (0.51-0.76 g per 100 mL) and levelling off thereafter (0.73-0.81 g per 100 mL). Concurrently a drop in pH and a rise in $b*$ values were observed. The maximum amount of lactulose formed in MCP heated at 96 and 100°C was reached after 150 min (0.81 and 0.79 g 100 per mL). The extension of treatment time from 120 to 150 and 180 min, however, was deemed unnecessary as the lactulose yield at the three isomerisation times was statistically similar ($P > 0.05$).

Table 5.1 Average values of lactulose, pH and $b*$ during the isomerisation of MCP at various temperatures and times

Value	Temperature (°C)	Isomerisation time (min)						
		0	30	60	90	120	150	180
Lactulose	90	ND	ND	ND	ND	ND	ND	0.24 ± 0.04
	96	ND	ND	0.24 ± 0.04[c]	0.57 ± 0.06[b]	0.75 ± 0.07[a]	0.81 ± 0.06[a]	0.73 ± 0.25[a]
	100	ND	ND	0.28 ± 0.02[c]	0.51 ± 0.07[b]	0.76 ± 0.02[a]	0.79 ± 0.06[a]	0.79 ± 0.02[a]
pH	90	6.7 ± 0.0[e]	6.8 ± 0.0[e]	7.1 ± 0.1[d]	7.2 ± 0.1[c]	7.3 ± 0.0[c]	7.8 ± 0.1[b]	8.4 ± 0.1[a]
	96	6.7 ± 0.0[e]	7.2 ± 0.1[d]	8.2 ± 0.0[a]	8.3 ± 0.0[a]	8.3 ± 0.1[a]	8.0 ± 0.0[b]	7.9 ± 0.1[c]
	100	6.7 ± 0.0[d]	7.3 ± 0.1[c]	8.1 ± 0.2[b]	8.4 ± 0.1[a]	8.1 ± 0.0[b]	8.0 ± 0.1[b]	7.9 ± 0.1[b]
b*	90	-4.4 ± 0.3[c]	-4.2 ± 0.3[bc]	-4.4 ± 0.2[bc]	-4.3 ± 0.3[bc]	-4.3 ± 0.1[c]	-3.9 ± 0.1[b]	-3.2 ± 0.0[a]
	96	-4.3 ± 0.3[f]	-3.9 ± 0.2[f]	-1.8 ± 0.2[e]	-0.2 ± 0.0[d]	1.6 ± 0.2[c]	2.5 ± 0.3[b]	3.8 ± 0.2[a]
	100	-4.4 ± 0.2[g]	-4.0 ± 0.0[f]	-2.0 ± 0.1[e]	-0.5 ± 0.1[d]	2.4 ± 0.3[c]	3.1 ± 0.1[b]	4.2 ± 0.1[a]

[1] Values are mean ± SD of two different experiments.
[2] Results of sugar content are expressed in g per 100 mL.
[3] Different letters in the same row differ significantly at $P \leq 0.05$ by DMRT.
[4] ND = non-detectable (below detection limit).

5.5.4 Effect of the catalyst type on lactose isomerisation

Figure 5.3 shows the conversion (%) of lactose into lactulose from MCP and control lactose solution using limestone, OSP and ESP. In blank, only *ca.* 3% conversion was detected in MCP after 150 min, and in control lactose solution after 120 min. In the presence of 12 mg catalyst per mL, irrespective of type, a significantly higher level of conversion was achieved in both solutions and at earlier stages of heating, i.e. within 60 min *ca.* 13% conversion was detected in control lactose solution but only *ca.* 3-6% in MCP. As the treatment time progressed, the conversion in both solutions reached 12-15% at 90 min, rising to 18-20% after 120 min, but dropped back to 16-18% after 150 min of heating.

Changing catalyst type had only a small effect on the conversion level. It was found that limestone gave 3% less conversion than ESP and OSP (2.8% cf. 5.9-6.1%) in MCP after 60 min (Figure 5.3a), and 2% less in the control lactose solution (9.6% cf. 11.6-12.0%) after the first 30 min (Figure 5.3b). However, all catalysts showed similar conversion rates between 90 and 150 min in both solutions. These findings support the effectiveness of OSP or limestone for lactose isomerisation as a replacement to ESP. Interestingly, it was found in a preliminary study that calcium carbonated-based catalysts could be re-used at least twice without significant loss of their catalytic ability which is an advantage over the soluble catalysts such as NaOH and $Ca(OH)_2$.

The lower level of lactulose formation in MCP at the start of process compared to control lactose solution could be attributed to the buffering effect of residual proteins and/or other miscellaneous constituents present in MCP used in this study. As shown in Figure 5.4, upon the addition of catalysts to control lactose solution the pH rose from the adjusted initial value of 6.7 to *ca.* 9.0 before dropping back to < 8.0 with reaction

time. Under these conditions, lactulose was formed within 30 min of heating (*ca.* 9.6% conversion).
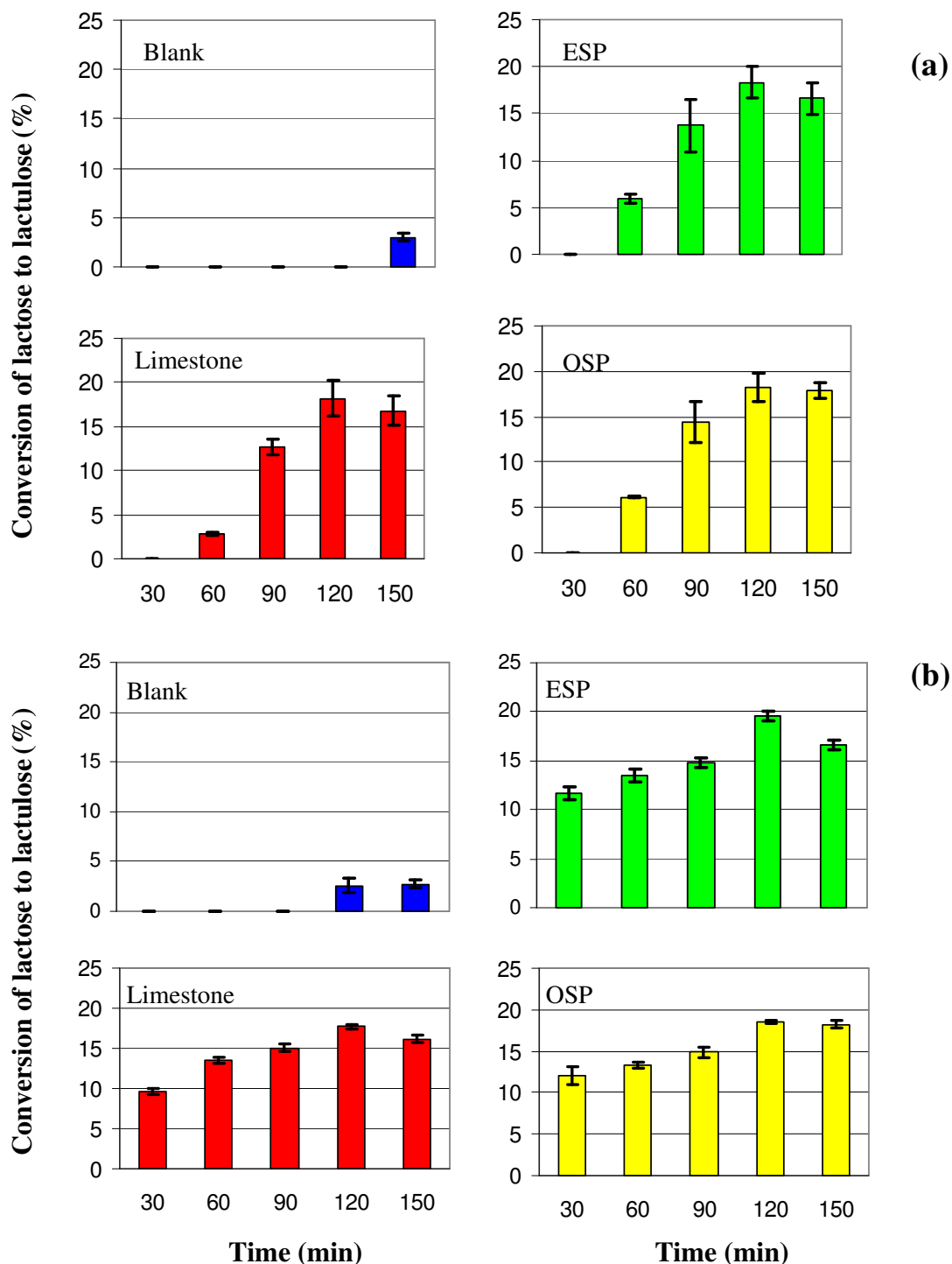
Figure 5.3   Conversion of lactose to lactulose from (a) MCP and (b) control lactose solution using different catalysts and without catalyst (Blank)
Data represent the mean ± SD of three different experiments.

On the other hand, the pH of the MCP did not change much upon catalyst addition and after 60 min heating reached > 8.0, but stayed < 8.5 after 90 min of heating, probably due to heat denaturation of residual proteins and loss of their buffering capacity. This is in agreement with previous reports by Olano *et al.* (1987) and Claeys *et al.* (2002) who found that lactulose did not form at the earlier stages of heating when the pH was close to that of fresh milk, but formed faster when the pH of reaction mixture was near or above 8.0. Furthermore, the residual proteins in MCP could form a complex with lactulose to produce lactosyl-amino compounds, thus lowering lactulose yield (O'Brien 1997).
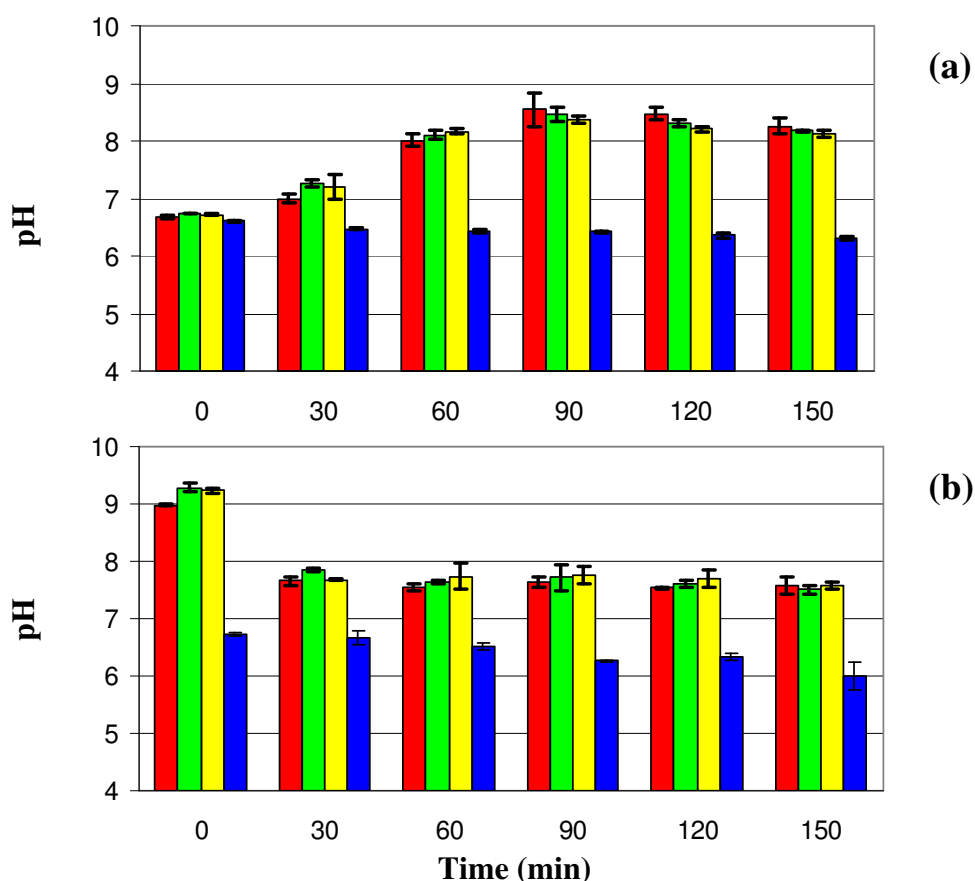
Figure 5.4 Changes in pH during isomerisation of (a) MCP and (b) control lactose solution using limestone (■), ESP (■) and OSP (□); and without catalyst (Blank ■)
Data represent the mean ± SD of three different experiments.

Figure 5.5 shows the degradation of lactose during isomerisation of MCP and control lactose solution using different calcium carbonate-based catalysts and the blank. In the absence of catalyst, while no lactulose was formed, lactose content diminished up to 10% in MCP after 120 min, and 3.3% in control lactose solution after 90 min. The higher loss of lactose in MCP could be ascribed to partial condensation of lactose with residual proteins via the Maillard reaction. According to Berg and van Boekel (1994) 80% of lactose in heated milk undergoes Lobry de Bruyn-Alberda van Ekenstein transformation, but 20% enters the Maillard reaction. In the presence of all catalysts, a considerable loss of lactose in favour of lactulose formation was observed in both solutions. The maximum net degradation of lactose after subtraction of that of blank was *ca*. 36% of the initial mass of lactose, and after deduction of the maximum yield of lactulose obtained (*ca*. 20% of initial lactose), it was assumed that the rest of degraded lactose was converted to other compounds e.g. monosaccharides and organic acids. Montilla *et al*. (2005) reported that *ca*. 9% of lactose degraded into by-products (0.34 g per 100 mL galactose, 0.09 g per 100 mL epilactose and 0.02 g per 100 mL organic acids) under isomerisation conditions similar to the present study.

One of the by-products of lactose isomerisation is the formation of brown coloured compounds e.g. melanoidins (van Boekel 1998). This can be measured using spectrophotometric method, however, because of its turbidity the brown colour of MCP was assessed using a colorimeter and reported as b^{*} value which is directly correlated to yellowness.
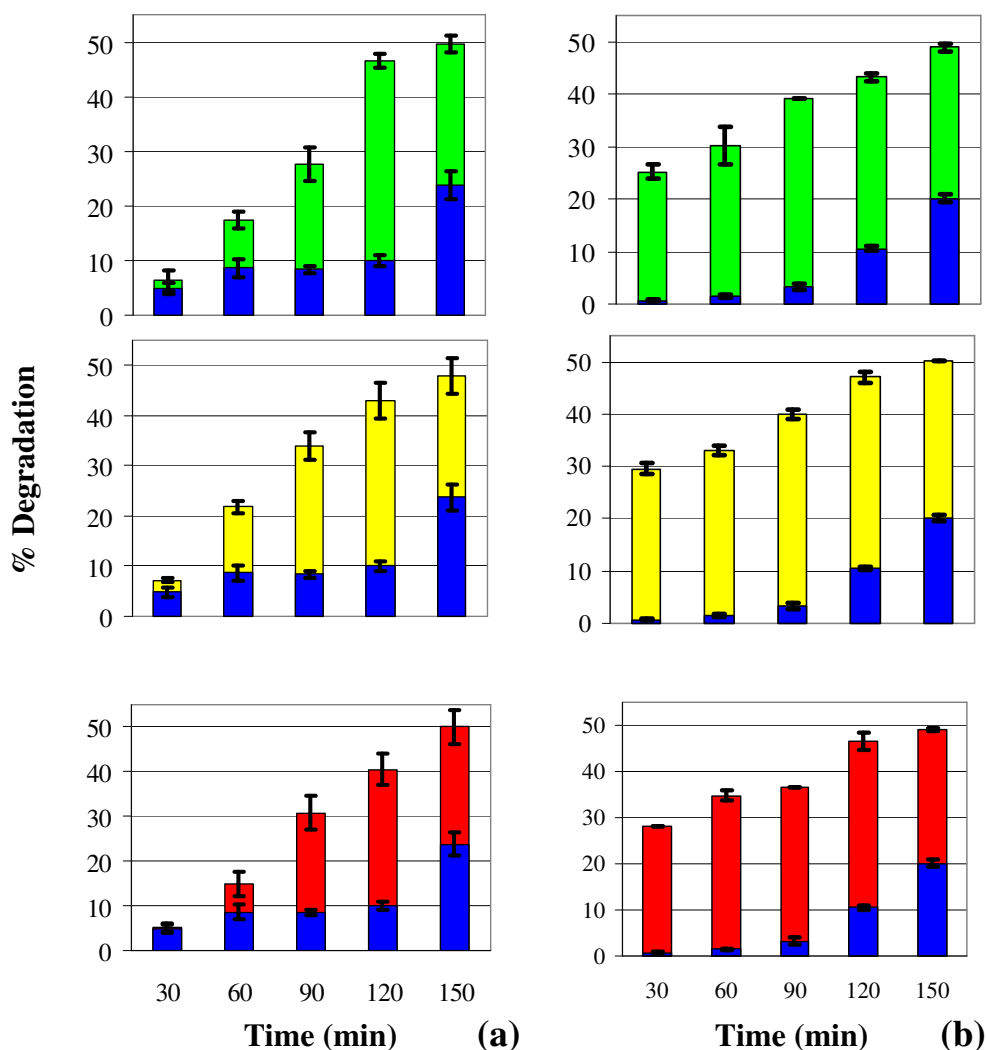
Figure 5.5 **Degradation of lactose in (a) MCP and (b) control lactose solution using limestone (■), ESP (■) and OSP (■); and without catalyst (Blank ■)**
Data represent the mean ± SD of three different experiments.

Figure 5.6 shows changes in colour during the isomerisation of MCP measured as the b^* values and of control lactose solution designated as absorbance values at 420 nm. The increase development of the brown colour was independent of catalyst type. In both solutions, there was a small variation in the colour of blanks but a noticeable increase in the intensity of brown colour in all catalyst-treated samples.
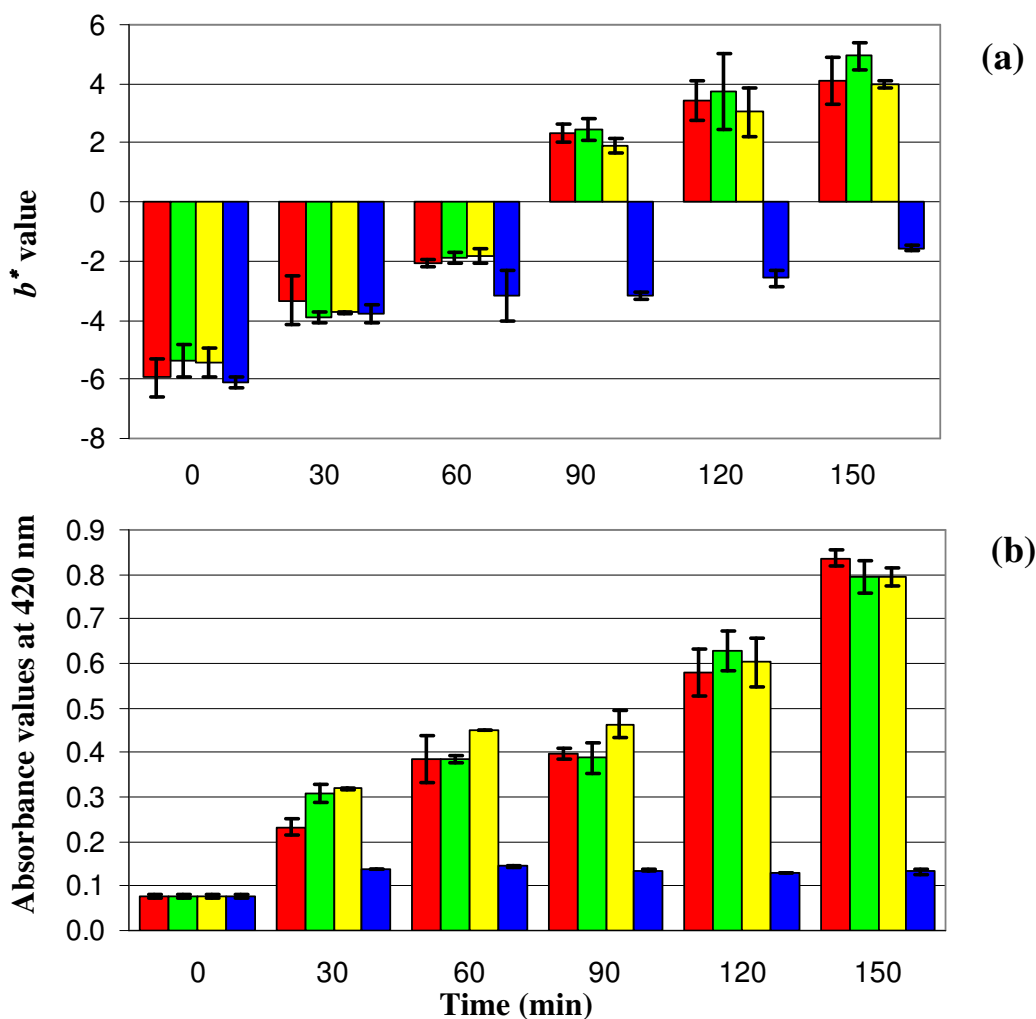
Figure 5.6 **Increase in b^* and absorbance values at 420 nm during isomerisation of MCP (a) and control lactose solution (b) using limestone (■), ESP (■) and OSP (□); and without catalyst (Blank ■)**
Data represent the mean ± SD of three different experiments.

In this study, the OSP gave comparable yield of lactulose to ESP and limestone. Undoubtedly, the use of ESP and OSP is economical due to their origin being waste materials, and is beneficial to the environment by alleviating shell disposal problems. However, both catalysts may have variable chemical composition compared to pure limestone. Oyster shells come from the sea therefore its mineral content cannot be controlled, while the constituents of egg shell depend closely on the formulation of the feed.

5.5.5 Effect of de-proteination on lactose isomerisation

The usefulness of ESP as catalyst for lactose isomerisation has been reported by Montilla *et al.* (2005) who achieved the maximum lactulose conversion of 25.3% in MCP after 90 min of treatment with 4 mg per mL ESP at 98°C. In the present study, however, lactulose yield did not exceed 21% of the initial lactose content during heat treatment with 12 mg per mL ESP at 96°C for 120 min. The higher yield of lactulose reported in study by Montilla *et al.* (2005) can be attributed to the variation in MCP compositions, isomerisation conditions and a more sensitive detection method (GC). The MCP used in the current study was contaminated with *ca*. 0.5% protein.

So far, little is known about the effects of protein on the formation of lactulose in the catalytic systems. The majority of published data are on the lactulose formation in heated milk (without catalyst addition) and are contradictory. Greig and Payne (1985) and Olano *et al.* (1989) reported that the addition of amino group (L-lysine) into model milk solution or into 5% lactose-buffer solution dropped the amount of lactulose formed. Andrews and Prasad (1987) also suggested that increasing protein concentration of milk reduced lactulose formation. On the other hand, Martinez-Castro *et al.* (1986) observed less lactulose formation in simulated milk ultrafiltrate (SMUF) than in milk under the similar conditions. These effects were ascribed to the buffering action of the proteins in milk.

In the earlier stage of the current study (sections 5.5.2-5.5.4), the effectiveness of calcium carbonate-based catalysts for the isomerisation were examined in MCP contaminated with protein. The buffering action of protein contaminants resulted in the pH stability of catalyst-treated MCP at the initial stage of heating and was responsible for slower rate of lactulose formation as compared to control lactose solution (Figure

5.4). To overcome the adverse effect of protein contaminants in MCP on lactose isomerisation, the MCP was de-proteinated by acidification under mild heat treatment and filtration to remove proteins prior to isomerisation.

Figure 5.7 shows changes in lactose and lactulose contents and pH of de-proteinated MCP during the isomerisation at 96°C for 150 min. Lactulose was formed rapidly in de-proteinated MCP within the first 30 min of isomerisation (0.38 ± 0.04 g per 100 mL) which doubled after 60 min (0.69 ± 0.15 g per 100 mL) and reached a maximum (0.90 ± 0.12 g per 100 mL) equivalent to 21.9% of initial lactose content, within 120 min (Figure 5.7a). Contrary to the expectation that the removal of protein contaminants would eliminate the Maillard reaction and thus would lead to a significant increase in lactulose yield, the results revealed that de-proteinated MCP gave only *ca.* 0.15 g per 100 mL more lactulose yield than the original MCP under the same isomerisation conditions, and nearly identical yield to control lactose solution. These findings indicated that the presence of protein contaminants in original MCP did not adversely affect the maximum yield of lactulose nor the time required for isomerisation, possibly as a result of the retarding effect of protein on pH drop of solution during heating (O'Brien 1997). According to Aider and de Halleux (2007), the maintenance of high pH favours lactulose formation and also delays the formation of degradation by-products.

An interesting observation was that upon the addition of catalysts the pH of the de-proteinated MCP did not rise (Figure 5.7b). This indicated that besides the buffering action from the residual proteins in MCP, other miscellaneous constituents e.g. citrates and phosphates may also help maintain the pH at the start of the process. However, within 30 min of heating the pH rose significantly from *ca.* 6.8 to 8.7 ± 0.1 before dropping back to 8.4 ± 0.2 after 60 min and stabilising to *ca.* 7.8 after 90 and 150 min.
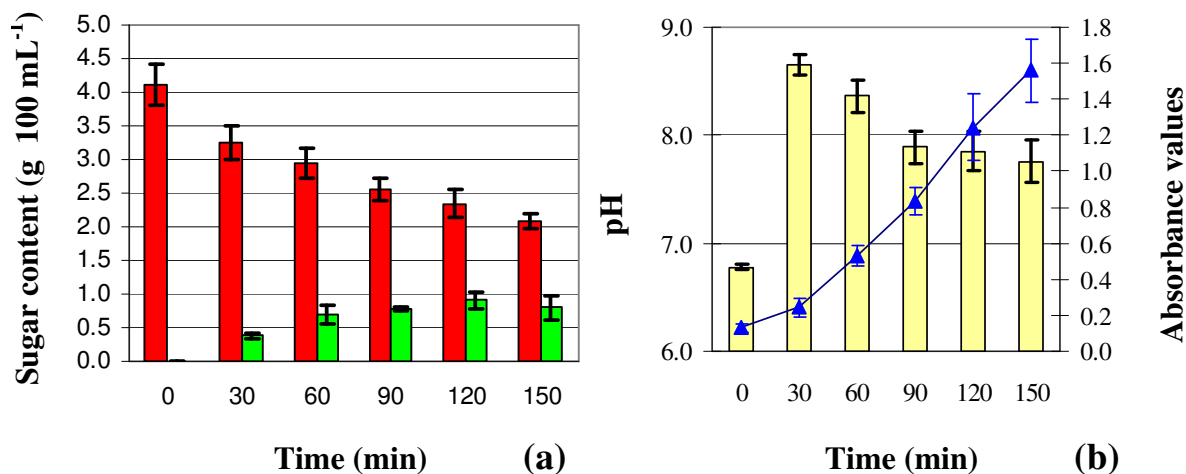
Figure 5.7 **Changes of lactose (■), lactulose (■), pH (□) and absorbance values (–▲–) during the isomerisation of de-proteinated MCP added with 12 mg per mL ESP at 96°C for 150 min**
Data represent the mean ± SD of four different experiments.

Figure 5.7b also shows a noticeable increase in undesirable brown colour development measured at 420 nm during the course of treatment. The highest absorbance value of 1.6 ± 0.2 was attained in the samples heated for 150 min, similar to those reported by Montilla *et al*. (2005). The brown colour development was more pronounced in de-proteinated MCP than in control lactose solution (Figure 5.6b) under the similar isomerisation conditions. With 120 min, for instance, the absorbance values of de-proteinated MCP and control lactose solution were 1.2 ± 0.2 and 0.6 ± 0.1, respectively. Similar trends were observed in the earlier study by Mahran *et al*. (1995).

5.5.6 Effect of colour removal on quality of lactulose-enriched MCP

Figures 5.8 and 5.9 show changes of lactulose content and the reduction in colour of lactulose-enriched MCP after treatments with H_2O_2 and activated carbon. The lactulose-enriched MCP originally had a golden-yellow colour with the absorbance value (420 nm) of 0.5 ± 0.1. By H_2O_2 treatment (10 mL per 100 mL of 17.5% H_2O_2), excess colour was effectively removed within 10 min at 95°C (Figure 5.9b), resulting in a five-fold drop in absorbance values. Similarly, addition of 5 g per 100 mL of activated carbon

resulted in an 84% reduction in absorbance values within 10 min (Figure 5.9c). Analysis

of sugar contents showed that decolourisation had a small but statistically insignificant

effect ($P > 0.05$) on lactulose content (Figure 5.8b). Lactulose content dropped from

0.84 ± 0.07 to 0.77 ± 0.02 as a result of H_2O_2 treatment and to 0.72 ± 0.07 g per 100 mL

by treatment with activated carbon.
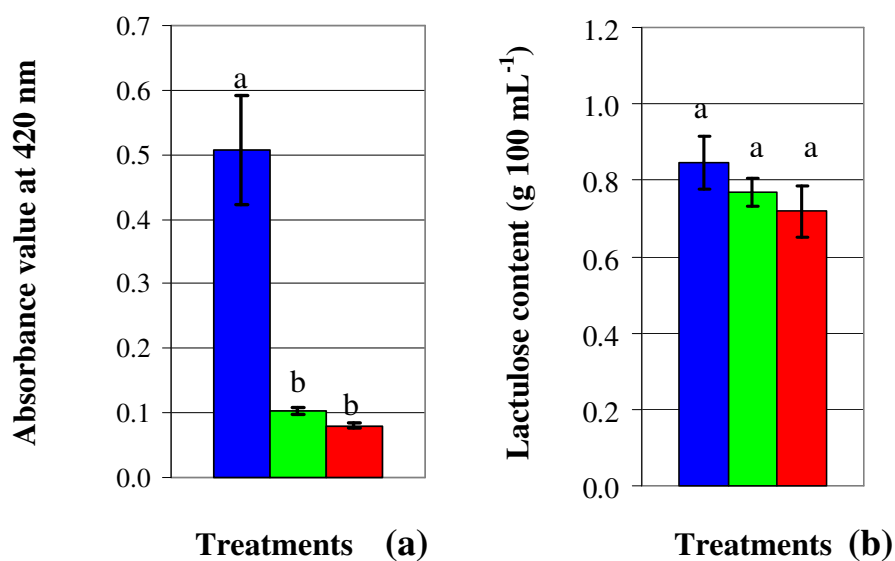
Figure 5.8 Brown colour and lactulose content of lactulose-enriched MCP before (■) and after treatment with H_2O_2 (■) and/or activated carbon (■)
Data represent the mean $\pm$ SD of three different experiments.
Bars with different letters are significantly different at $P \leq 0.05$ by DMRT.
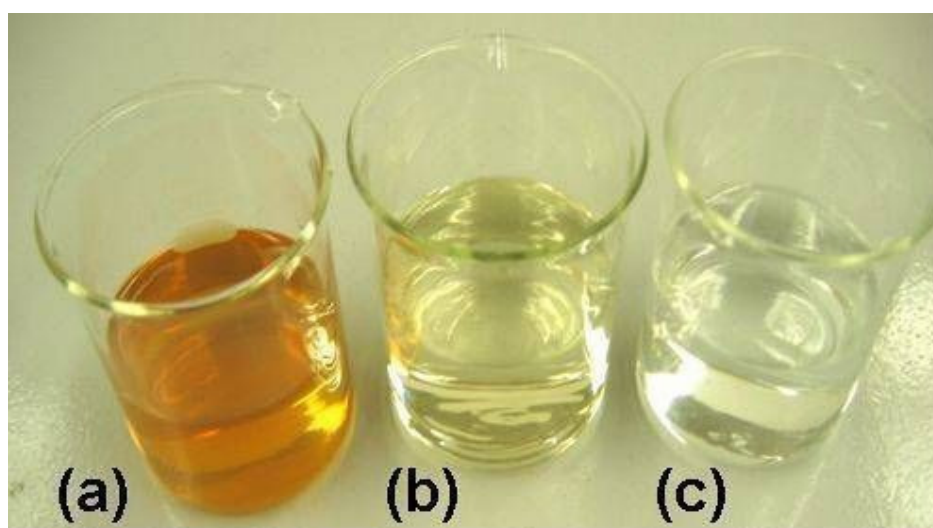
Figure 5.9 Colour of lactulose-enriched MCP before (a) and after treatment with H_2O_2 (b) or activated carbon (c)

Chapter 5

5.6 Conclusions

A low-value waste stream of MCP can serve as a cheap and readily available lactose source for lactulose production. Proteins if present in MCP can affect the isomerisation rate only in the earlier stages without adverse effect on yield of lactulose at longer heating periods. Other calcium carbonate-based catalysts i.e. OSP and limestone were shown to be equally effective as catalysts for lactulose production as ESP. They are insoluble reagents which could be easily removed from the reaction mixture for subsequent re-use. Isomerisation of MCP with OSP or ESP offers an alternative way to use those waste materials. A catalyst loading of 12 mg per mL was considered sufficient for optimum conversion level as increasing catalyst loading caused insignificant increase in yield of lactulose. Calcium carbonate was shown to possess the catalytic activity in the temperature range between 96 and 100°C with the optimum isomerisation time of 120 min. In addition to formation of organic acids and monosaccharides, the formation of pigmented by-products directly depended on heating time, temperature and catalyst loading. The brown colour developed can efficiently be removed by treatment with either activated carbon or H_2O_2.

The optimised isomerisation conditions were determined as: carbonate-based catalyst loading of 12 mg per mL of de-proteinated MCP and isomerisation time of 120 min at 96°C for preparing lactulose-enriched MCP with acceptable lactulose yield of 22%. The bifidogenic power of lactulose-enriched MCP syrup (40°B) is examined in the following chapter.

Chapter 6

Bifidogenic effects of JAI and lactulose-enriched MCP syrup on probiotic bacteria

6.1   Abstract

The prebiotic effects of experimentally-prepared prebiotic compounds, *viz.* lactulose-enriched MCP syrup and Jerusalem artichoke inulins (concentrate and powder) on the growth and acid production by three probiotic organisms, namely *Bifidobacterium bifidum* BB-12, *Lactobacillus acidophilus* LA-5 and *Lactobacillus casei* LC-01 were studied in reconstituted skim milk (RSM) and modified MRS broth. The growth-sustaining ability of JAIP was compared with two commercial chicory inulin powders on *Lactobacillus casei* LC-01. Changes in pH, titratable acidity and bacteria counts were monitored on day 1 and during storage of yoghurts at 4°C for 4 weeks. The results showed that by the end of the storage period the numbers of LC-01 in JAIP-supplemented yoghurts were *ca*. 7 log CFU g^{-1} which gave them the status of functional ingredients.

6.2   Introduction

Yoghurt is currently manufactured and consumed in many countries around the world (Chandan and O'Rell 2006). Recent developments in yoghurt production are incorporating probiotic organisms for therapeutic benefits (Holzapfel and Schillinger 2002) and exopolysaccharide-producing starters for texture improvement (Amatayakul *et al*. 2006a, 2006b). Interest in incorporating probiotic organisms in yoghurt dates back to late 1970s (Huges and Hoover 1991) and the market for this product is still rising. In Australia, yoghurt consumption has increased steadily from 5.3 kg per capita per year

in 2000 to 6.8 kg in 2007 (Dairy Australia 2007), and the probiotic yoghurt constitutes the largest share in the probiotic dairy foods market, representing 82% of the total market volume (Anon 2003).

One of the most important requirements for manufacturing and marketing of the probiotic yoghurt is to maintain a high number of probiotic organisms ≥ 6 log CFU g^{-1} at the point of consumption (Kurman and Rasic 1991; Lourens-Hattingh and Viljoen 2001), in order to achieve the claimed health benefits. However, various probiotic lactobacilli and bifidobacteria show a decline in their viability during product's shelf life (Hull *et al*. 1984; Medina and Jordano 1994; Schillinger 1999). Several factors are responsible for the viability of these organisms e.g. the strains used, culture conditions, antagonism among cultures present, storage time and temperature, initial counts, hydrogen peroxide and oxygen contents in the medium, and the amount of organic acids in the product (Shah 2000; Medina and Jordano 1994). Probiotic organisms especially bifidobacteria grow slowly in milk due, in part, to their lack of proteolytic activity (Klaver *et al*. 1993), thus requiring the incorporation of essential growth factors such as peptides and amino acids to enhance their growth (Elli *et al*. 1999). However, they do not attain the same high numbers as common yoghurt bacteria (Champagne *et al*. 2005).

Considerable studies have been conducted to stimulate the growth of probiotic bacteria during yoghurt fermentation and to improve their survival until the use-by-date, by supplementing yoghurt milk with growth factors such as vitamin-enriched protein hydrolysate, amino nitrogen, whey protein concentrate and oxygen scavengers (Klaver *et al*. 1993; Dave and Shah 1998; Kailasapathy and Supriadi 1996; Oliveira *et al*. 2001; Akalin *et al*. 2007; Dave and Shah 1997b; Amatayakul *et al*. 2006a, 2006b). Use of proteolytic yoghurt bacteria i.e. *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) and

Streptococcus thermophilus (ST) in probiotic yoghurt manufacture also enhances the growth and viability of probiotics and helps to reduce fermentation time (Samona and Robinson 1994; Saxelin *et al*. 1999; Dave and Shah 1998).

A further step to enhance probiotic growth and survival is to incorporate prebiotic substrates (Bruno *et al*. 2002; Akalin *et al*. 2004; Shin *et al*. 2000; Desai *et al*. 2004). Products containing a combination of prebiotics and probiotics are known as synbiotics (Gibson and Roberfroid 1995; Ziemer and Gibson 1998). It is important to select appropriate prebiotics for improving the viability retention of probiotics in fermented dairy products and for sustaining their growth in the colon (Bielecka *et al*. 2002; Crittenden *et al*. 2001). The most studied substances are chicory-derived inulin-type fructans (Roberfroid 1998) and to a certain degree, the lactulose (Strohmaier 1998).

The two previous chapters described development of protocols for inulin extraction from JA tubers and lactulose isomerisation from MCP. The aim of this study was to evaluate the prebiotic power of the developed ingredients to support the growth and survival of probiotic bacteria in fermented milk. Consequently, the objectives of this study were to:

1. Examine the growth and acid production of three probiotic strains in the presence of lactulose-enriched MCP syrup or JAI (concentrate and powder). The comparison was made in both basal media and reconstituted milk with non-supplemented samples as well as commercial prebiotic products.
2. Select the probiotic strain that shows preference to JAI for subsequent development of synbiotic yoghurt.

Chapter 6

3. Determine the effects of inulin powder addition (JAIP and chicory inulins) on the acidifying activities and survival of selected probiotic (LC-01) during 28 days of yoghurt shelf life.

6.3 Materials and methods

6.3.1 Experimentally-prepared prebiotic compounds and working cultures

Lactulose is normally sold in the syrup form because of difficulties in its crystallisation and drying. In this study, therefore, the lactulose-enriched MCP prepared under optimum conditions reported in section 5.3.5 was further concentrated using a rotary evaporator at $\leq 70°C$ to obtain lactulose-enriched MCP syrups with 40°B soluble solids.

For JA-based inulins, JAIS at 40°B was prepared following the method given in 4.3.1 while spray-dried JAIP was prepared under optimum conditions for cold fractionation reported in 4.4.3.

Freeze-dried cultures (LC-01, BB-12 and LA-5) (section 3.1.4) were activated by adding 10 mg of each freeze-dried culture into 100 mL of MRS broth, incubated overnight at 37°C and stored at 4°C until required for use. Prior to each trial, 10 mL of this bulk cultures was sub-cultured in triplicates into 90 mL of MRS broth and incubated at 37°C for 12-16 h to give working culture.

6.3.2 Determination of the prebiotic effect of lactulose-enriched MCP syrup

6.3.2.1 Fermentation in broth

The carbohydrate-free MRS broth was prepared as a basal growth medium by dissolving 10 g peptone from casein, 4 g yeast extract, 8 g Lab-Lemco powder, 2 g tri-ammonium citrate, 1 mL Tween 80, 2 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.4H_2O$ and 5 g sodium acetate (Table 3.1) in distilled water and making the

volume to 1 L (Kneifel *et al*. 2000). The medium was sterilised at 121°C for 15 min and aliquots of 8.75 mL were aseptically distributed into sterile test tubes which were supplemented with 0.25 mL of filter-sterilised (0.45 μm) CHO sources (40°B) and inoculated in triplicate with 1 mL of probiotic working culture, so that a final concentration of lactulose-enriched MCP syrup was 1%. For comparison, lactulose-free concentrated MCP (40°B) and 40% lactulose solution (Analytical grade, Sigma®) were used as control and reference substrates, respectively. The media tubes containing LA-5 were incubated aerobically overnight at 37°C while those containing LC-01 and BB-12 were transferred into anaerobic jars containing AnaeroGenTM 2.5 L sachets and incubated overnight at 37°C followed by enumeration (section 3.6.2).

6.3.2.2   Fermentation in RSM

The suitability of the lactulose-enriched MCP syrup as a prebiotic supplement was determined by supplementing RSMs at 12, 13, 14 and 15% solids content with different levels (4, 3, 2 and 1%) of MCP syrup to final solids content of 16% (Table 6.1). The control (non-supplemented RSM, 16%, w/v) and lactulose-supplemented RSMs were heat-treated at 90°C for 10 min with constant stirring and cooled to 37°C. In this study, direct inoculation with freeze-dried probiotic cultures (LC-01 and LA-5) was chosen for milk fermentation. Based on the culture manufacture's instruction, 25 mg of each of the two freeze-dried probiotics was individually inoculated into 500 mL RSMs, completely suspended in milk then transferred aseptically into 100 mL plastic containers, tightly covered and incubated at 37°C overnight. Duplicate determinations of bacterial counts (section 3.6), titratable acidity (TA) and pH (sections 3.5.1-3.5.2) were conducted on duplicate samples taken from each batch after overnight incubation.

Table 6.1   Ingredients used for yoghurt preparation

RSM solids content (%)	Supplementation level (%)	Ingredient		
		LHSMP[a] (g)	Syrup at 40°B[b] (mL)	Distilled water (mL)
16	0	16.3	-	83.7
15	1	15.3	2.5	82.2
14	2	14.3	5.0	80.7
13	3	13.3	7.5	79.2
12	4	12.2	10.0	77.8

[a] Low-heat skim milk powder contains a total solid of 98%.
[b] Lactulose-enriched MCP syrup or JAIS

6.3.3   Determination of the prebiotic effect of JAIS

6.3.3.1   Effect of JAIS supplementation level

Prebiotic properties of JAIS at 40°B (section 6.3.1) were assessed with LC-01, BB-12 and LA-5, at the same supplementation levels as used for MCP lactulose in RSMs (0, 1, 2, 3 and 4%, Table 6.1). Fifteen experiments were conducted in triplicate by a 3×5 factorial design (three cultures × five supplementation levels) under the same conditions described in section 6.3.2.2. Yoghurt samples thus produced were analysed for probiotic counts, pH and TA after overnight incubation.

6.3.3.2   Effect of culture compositions

In probiotic yoghurt manufacturer, to provide the typical yoghurt flavour and to speed-up the fermentation process, the use of yoghurt starters is essential (Chandan and O'Rell 2006). However, as they grow faster than probiotic bacteria during fermentation and produce acids that could adversely affect the viability of probiotic, the initial fermentation with probiotic cultures and completion of fermentation with yoghurt cultures is recommended (Lankaputhra and Shah 1997). Hence, the objective of this part of the study was to evaluate the effect of traditional yoghurt starters (YC-380, section 3.1.4) on the growth of LC-01 in RSMs containing JAIS (40°B) and to establish a suitable method for incorporating the two groups of cultures in yoghurt milk.

Two litres of JAI-supplemented RSM at a final solids content of 16% were prepared by dissolving 244 g LHSMP (98% total solids) in 1,556 mL distilled water, followed by adding 200 mL JAIS at 40°B (Figure 6.1) to give a final inulin concentration of 4%. The mixture was then heat-treated at 90°C for 10 min, cooled to 37°C and divided into four equal batches (500 mL each). Two batches were fermented in a single-stage process (Figure 6.1), one batch (Trial A) with LC-01 (0.02%) as the sole culture and the other batch (Trial B) with mixed cultures of YC-380 and LC-01 (0.01% each) at 37°C until the desired pH of 4.5. The remaining two batches were made with mixed cultures using two-stage fermentation. One batch (Trial C) was initially inoculated with 0.01% of freeze-dried YC-380 and incubated at 37°C for 4 h, followed by secondary inoculation with 0.01% LC-01 and incubation at 37°C to reach pH 4.5. The same procedure was repeated for the last batch (Trial D), except that LC-01 was inoculated before YC-380 (Figure 6.1). The length of fermentation time of 4 h was determined by estimating from the growth curve of LC-01 that the culture should be at the late lag phase growth times. All trials were repeated three times. Bacterial counts (section 3.6), TA and pH measurement (sections 3.5.1-3.5.2) were carried out post-inoculation, post-incubation and overnight refrigeration (day 1) and on after 7 days of storage at 4°C.

244 g LHSMP + 200 mL JAIS 40°B + 1,556 mL distilled water

↓

Supplemented-RSM 16% total solids

↓

Heat treatment (90°C 10 min)

↓

Cooling (37°C)

↓

Inoculation

(A)	(B)	(C)	(D)
LC-01 0.02%	YC-380 + LC-01 (0.01% each)	YC-380 0.01%	LC-01 0.01%
↓	↓	↓	↓
Filling	Filling	Incubation (37°C 4h)	Incubation (37°C 4h)
↓	↓	↓	↓
Incubation (37°C until pH ~ 4.5)	Incubation (37°C until pH ~ 4.5)	Inoculation LC-01 0.01%	Inoculation YC-380 0.01%
↓	↓	↓	↓
Cooling & storage (4°C)	Cooling & storage (4°C)	Filling	Filling
		↓	↓
		Incubation (37°C until pH ~ 4.5)	Incubation (37°C until pH ~ 4.5)
		↓	↓
		Cooling & storage (4°C)	Cooling & storage (4°C)

Figure 6.1   Flow diagram for yoghurt production trials

6.3.4   Determination of the prebiotic effect of JAIP

The objectives of this study were to gain a better understanding of prebiotic effect of
JAI and to verify the capability of selected probiotics on JAI utilisation. The growth
promoting ability of spray-dried JAIP (section 6.3.1) on a range of probiotics (LA-5,
LC-01, BB-12) in basal media was compared with three commercial chicory powders
with varying degree of polymerisation (DP) i.e. Raftilose® P95, Raftiline® GR and

Raftiline® HP and two sugars i.e. lactose and glucose (section 3.1.5), all added at 4% level. Control batches contained no prebiotics or sugars.

Carbohydrate-free MRS broth was prepared from individual ingredients as described in section 6.3.2.1. To maintain uniform growth conditions throughout the experiments, the sterilised media was made in large batch and divided into 18 pre-sterilised universal bottles (80 mL each). Four grams of each of the six carbohydrates were dissolved with distilled water and made up to 20 mL, and after filter sterilisation the solutions were separately added to a series of cooled sterile media bottles (n = 3) containing 80 mL growth media to make a final concentration of 4% (w/v). Non-supplemented medium (100 mL) was used as control.

One millilitre of each of the activated cultures (section 6.3.1) was added into each media bottle and incubated under aerobic condition for LA-5, and an anaerobic atmosphere for BB-12 and LC-01 at 37°C for 16 h. The turbidity of the growth media was examined at the end of incubation period by measuring their optical density (OD). The incubated bottles were vortexed for 20 s, and the homogenised media were transferred into optical cuvettes for absorbance readings at 600 nm (n = 3) against the non-supplemented, non-cultured basal medium (Kneifel *et al*. 2000).

6.3.5 Effects of inulin addition on the survival of probiotic and lactic acid bacteria in yoghurt during refrigerated storage

The effectiveness of supplementing JAIP in improving the viability of probiotic and yoghurt cultures was assessed in comparison to chicory inulin powders over 28 days of cold storage (4°C). Three experimental yoghurts, labelled as JAY, INY and OFY were made with 12% RSMs supplemented with 4% of JAIP, chicory inulin (Raftiline® GR)

and oligofructose (Raftilose® P95), respectively. A batch of control non-fat yoghurt (16% total solids), labelled as NFCY, was also prepared. The set yoghurts were prepared as described in section 6.3.3.2, using mixed cultures of LC-01 and YC-380 (0.01% each). The experiments were replicated three times. The sampling schedule for testing was immediately after the addition of starter culture into milk (Day 0), after overnight incubation (day 1), 24 h-post fermentation (day 2) and at weekly intervals (day 7, 14, 21 and 28). The analyses were performed on triplicate samples in triplicate for TA and pH (sections 3.5.1-3.5.2), and in duplicate for bacterial counts (section 3.6). The viability of each culture in different samples was calculated as follows (Bruno *et al*. 2002):

$$\% \text{ Viability} = \frac{\text{CFU after 4 week of storage}}{\text{initial CFU}} \times 100$$

6.4 Results and discussion

Three sets of trials were conducted to determine the prebiotic properties of lactulose-enriched MCP syrup, JAIS and JAIP. The probiotic strains, comprising *L. acidophilus* LA-5, *L. casei* LC-01 and *B. bifidum* BB-12 were selected on the basis of their common use as probiotic cultures in yoghurt manufacturers in Australia. Depending on the experiment, these cultures were used as direct vat inoculation or as fresh cultures after activation of freeze-dried cultures.

6.4.1 Prebiotic effect of lactulose-enriched MCP syrup

Lactulose plays a beneficial role in human gut environment as a prebiotic by promoting the growth of probiotic organisms. To evaluate the prebiotic power of the lactulose-enriched MCP syrup (40°B) developed in this project and to determine its possible

application in dairy products, the growth of the different probiotic strains in modified

MRS media and in RSM containing lactulose-enriched MCP syrup were studied.

Comparative viable counts of probiotics in modified MRS broths supplemented with

1% of either lactulose-enriched MCP syrup (40°B), lactulose-free concentrated MCP

(40°B, control), and reference lactulose solution (40%) is summarised in Table 6.2.

Counts of LA-5 and LC-01 were maximal in media containing reference lactulose

solution, followed by those in lactulose-enriched MCP syrup and the control. The

capacity of lactulose-enriched MCP to enhance the growth of both strains was lower

than that of reference lactulose solution possibly due to the presence of isomerisation

by-products. Montilla *et al*. (2005) using isomerisation conditions similar to this study

reported *ca*. 9% lactose degradation into by-products (0.34% galactose, 0.09%

epilactose and 0.02% organic acids). The reference lactulose solution prepared from

lactulose powder with 95% purity would also have higher quantity of lactulose

compared to lactulose-enriched MCP syrup that had an estimated quantity of lactulose

content of *ca*. 20% (w/v).

The comparable bacterial counts between lactulose-enriched and lactulose-free MCP

syrup additions could be ascribed by the similarity in their sugar and mineral contents.

The differentiation would have been noticeable if the supplementation level of

lactulose-enriched MCP syrup was increased or non-supplemented MRS broth was used

as the control.

Addition of lactulose-enriched MCP syrup or reference lactulose solution to broths, did

not significantly improve the counts of BB-12 (8.7 and 9.0 log CFU mL^{-1} cf. 8.9 log

CFU mL^{-1} in control). These findings are in accordance with those of Kontula *et al*.

Chapter 6

(1998) and Kneifel *et al*. (2000) whereby *L. acidophilus* strains (including LA-5) and members of *L. casei* (including LC-01) grew well on lactulose-containing broth and were shown to utilise lactulose pronouncedly. No report is found in the literature on the utilisation of lactulose by BB-12, however, lactulose was found to promote the growth of *B. longum* (BB-46) in broth, but not *B. lactis* (Kneifel *et al*. 2000; Crittenden *et al*. 2001). Similarly, Bruno *et al*. (2002) reported that the probiotic action of lactulose was strain-dependent, and that lactulose was effective in stimulating the growth of *B. longum*, *B. animalis* and *B. pseudolongum* grown in RSM but not *B. infantis*. According to Smart *et al*. (1993) bacterial strains with higher β-galactosidase or phospho-β-galactosidase activity had more potential to utilise lactulose.

Table 6.2   **Probiotics growth in modified MRS broth containing lactulose-free MCP, lactulose-enriched MCP syrup and reference lactulose solution**

Probiotic	Carbons source	Bacterial count (log CFU mL^{-1})
LA-5	Lactulose-free concentrated MCP	8.60 ± 0.35^{b}
	Lactulose-enriched MCP syrup	8.71 ± 0.27^{ab}
	Reference lactulose	8.92 ± 0.31^{a}
LC-01	Lactulose-free concentrated MCP	9.63 ± 0.28^{b}
	Lactulose-enriched MCP syrup	9.77 ± 0.42^{ab}
	Reference lactulose	10.21 ± 0.38^{a}
BB-12	Lactulose-free concentrated MCP	8.88 ± 0.45
	Lactulose-enriched MCP syrup	8.74 ± 0.51
	Reference lactulose	8.99 ± 0.18

Results shown are mean ± SD for three experiments each with three replications.
Different letters in the same column of each probiotic differ significantly at *P* ≤ 0.05 by DMRT.

Table 6.3 shows the viable counts and acidifying activity of LA-5 and LC-01 grown in RSMs containing various levels of lactulose-enriched MCP syrup after overnight incubation at 37°C. The probiotic BB-12 was not included in this part of the study since this strain show no response to lactulose addition in the earlier experiment. Overall, the

growth and activity of LA-5 increased as the level of lactulose increased whereas the corresponding values for LC-01 were not affected by lactulose level.

The higher counts of LA-5 (*ca*. 8.5 log CFU g^{-1}) were achieved with 3 and 4% lactulose-enriched MCP syrup compared to *ca*. 7.8-7.9 log CFU g^{-1} with 1 and 2% addition and *ca*. 7.9 log CFU g^{-1} with control (Table 6.3). At the rates of 3 and 4% lactulose-enriched MCP syrup addition, a significant drop in yoghurt pH (4.2-4.3) and a slightly higher level of acid production by LA-5 (0.80-0.85%) was observed compared to control (pH 4.5, TA 0.78%). Similar results were reported by Ozer *et al*. (2005) who found that lactulose powder when added to yoghurt at 0.25% and 2.5% promoted the counts of LA-5 to a great extent.

Table 6.3   Counts of LA-5 and LC-01 and acid production in reconstituted skim milk supplemented with lactulose-enriched MCP syrup (40°B)

Probiotic strain	Concentration (%)	Bacterial count (log CFU g^{-1})	pH	TA (% lactic acid)
LA-5	0 (control)	7.94 ± 0.17^b	4.5 ± 0.1^{ab}	0.78 ± 0.01
	1	7.91 ± 0.12^b	4.5 ± 0.2^a	0.79 ± 0.06
	2	7.78 ± 0.25^b	4.5 ± 0.2^a	0.79 ± 0.11
	3	8.52 ± 0.06^a	4.3 ± 0.0^{bc}	0.80 ± 0.06
	4	8.50 ± 0.19^a	4.2 ± 0.0^c	0.85 ± 0.01
LC-01	0 (control)	8.48 ± 0.18	5.7 ± 0.0	0.49 ± 0.04
	1	8.28 ± 0.06	5.6 ± 0.0	0.46 ± 0.08
	2	8.16 ± 0.28	5.6 ± 0.1	0.47 ± 0.01
	3	8.38 ± 0.72	5.7 ± 0.1	0.46 ± 0.04
	4	8.10 ± 0.28	5.7 ± 0.1	0.48 ± 0.02

Results shown are mean ± SD for two experiments each with two replications.
Different letters in the same column of each probiotic differ significantly at $P \leq 0.05$ by DMRT.

In the case of LC-01, the growth (*ca*. 8.1-8.4 log CFU g^{-1}) and acidification in the presence of 1-4% lactulose-enriched MCP syrup were similar to those obtained in the

control ($P > 0.05$). Lactulose-enriched MCP syrup did not promote the growth of LC-01 in RSM, as it did in broths (Table 6.2), possibly due to the effect of aerobic incubation. Saarela *et al*. (2003) suggested anaerobic incubation of milk supplemented with lactulose powder for the growth of *Lactobacillus* strains.

6.4.2   Prebiotic effect of JAIS

Two separate experiments were conducted to evaluate the prebiotic power of the JAIS in RSMs and to determine suitable culture compositions for use in the manufacture of yoghurts supplemented with JAIS: (i) trials with single strains of probiotics and (ii) trials with mixed cultures of probiotic and yoghurt cultures.

Table 6.4 shows variations in the growth and acid production of probiotic organisms affected by the level of supplement with 40°B JAIS and the strains used. No stimulating effect of JAIS on the growth of BB-12 was observed and their viable counts remained between 8.4 and 8.6 log CFU g^{-1} ($P > 0.05$) when syrup level was increased from 0 to 4%. In contrast, the growth of LC-01 was significant ($P \leq 0.05$) at supplementation levels of 3-4%, reaching 8.6-8.8 log CFU g^{-1}, while supplementation with 1-2% JAIS resulted in 7.9-8.0 log CFU g^{-1} that was not noticeably different to the control (8.3 log CFU g^{-1}). Strains of LA-5 also showed a positive response to JAIS addition. Increasing syrup levels from 1% to 3 or 4% resulted in a significant increase in the viable counts of LA-5, reaching a maximum count of 8.3 log CFU g^{-1} at 4% supplementation level.

Besides bacterial numbers, the lowering of pH and increase in acidity of milk were used as indices of the fermentability of JAIS by probiotic bacteria. The pH of RSMs inoculated with LA-5, LC-01 BB-12 dropped from *ca*. 6.4 ± 0.1 to 4.5-4.8, 4.7-5.1 and 5.2-5.4, respectively (Table 6.4). Acid production showed a good correlation with pH

values. The BB-12 produced less acid (TA 0.46-0.48%) than LC-01 (0.49-0.63%) and LA-5 (0.70-0.76%).

Table 6.4   **Counts of LA-5, LC-01 and BB-12 and acid production in reconstituted skim milk supplemented with 40°B JAIS**

Probiotic strain	Concentration (%)	Bacterial count (log CFUg^{-1})	pH	TA (% lactic acid)
LA-5	0 (control)	7.91 ± 0.34^b	4.5 ± 0.0^b	0.76 ± 0.04
	1	7.90 ± 0.34^b	4.8 ± 0.1^a	0.71 ± 0.05
	2	8.11 ± 0.15^{ab}	4.7 ± 0.1^a	0.70 ± 0.04
	3	8.27 ± 0.19^a	4.6 ± 0.1^b	0.73 ± 0.07
	4	8.31 ± 0.16^a	4.6 ± 0.1^b	0.75 ± 0.03
LC-01	0 (control)	8.33 ± 0.30^b	5.1 ± 0.4^a	0.49 ± 0.08^b
	1	7.91 ± 0.30^b	4.9 ± 0.2^{ab}	0.51 ± 0.09^b
	2	8.02 ± 0.41^b	4.8 ± 0.1^b	0.52 ± 0.06^b
	3	8.59 ± 0.31^a	4.7 ± 0.2^b	0.56 ± 0.0^{5b}
	4	8.75 ± 0.09^a	4.7 ± 0.2^b	0.63 ± 0.05^a
BB-12	0 (control)	8.51 ± 0.21	5.2 ± 0.1	0.46 ± 0.04
	1	8.60 ± 0.21	5.4 ± 0.2	0.46 ± 0.03
	2	8.57 ± 0.16	5.3 ± 0.2	0.46 ± 0.03
	3	8.42 ± 0.34	5.3 ± 0.2	0.48 ± 0.05
	4	8.35 ± 0.21	5.3 ± 0.1	0.47 ± 0.05

Results shown are mean ± SD for three experiments each with three replications.
Different letters in the same column of each probiotic differ significantly at $P \leq 0.05$ by DMRT.
Incubation conditions: 37°C, overnight (18 h).

Considering for maximum numbers of probiotics achieved, strain of LC-01 could be the desired probiotic organism for fermentation of milk supplemented with JAIS at an optimal supplementation level of 4%. This addition level also means that a typical serving size of yoghurt (100-150 mL) would have provided 4-6 g of inulin which is the recommended daily intake of inulin and oligofructose (Rao 2001). However, one of the main concerns of using probiotic LC-01 alone is low acid production. Under the

conditions used in this study, the pH of milks was still above 4.7 at the end of fermentation (18 h) while according to Australia-New Zealand Food Standard 2.5.3 (ANZFA 2003) yoghurt must have a maximum pH of 4.5. To accelerate acidification process and thus shorten the fermentation time, the LC-01 was combined with traditional yoghurt starter cultures (LB and ST) (Tamime *et al*. 2005). The two cultures were either inoculated together at the start or in two-stages (section 6.3.3.2).

Table 6.5 shows the effects of culture compositions and fermentation style on acid production and bacterial counts of LC-01, ST and LB in RSMs supplemented with 4% JAIS (40°B). The presence of yoghurt starters reduced the incubation time considerably. Yoghurt made with only LC-01 (Trial A) took *ca*. 18 h at 37°C to drop pH to 4.5 ± 0.2, compared to *ca*. 12 h in combination with yoghurt cultures (Trials B-D) (data not shown). Combining yoghurt cultures and LC-01 (Trials B-D) also enhanced the acid production (TA 1.03-1.11%) more significantly ($P \leq 0.05$) than LC-01 alone (TA 0.84%). On day 1, viable counts of LC-01 and yoghurt cultures in all RSMs increased by 2 log cycles (Table 6.5), however, the significant higher number of LC-01 was observed in RSMs inoculated only with LC-01 (Trial A, 8.6 log CFU g^{-1}) than those with mixed cultures (Trials B-D), probably due to longer incubation period and higher initial inoculum size.

In two-stage process, incorporating probiotic strain before or after yoghurt starters after 4 h (Trials C and D) had no major influence on acidification and growth stimulation, since comparable levels of acid (1.07-1.11%) and probiotic counts (8.0-8.2 log CFU g^{-1}) were found at the end and the differences were statistically insignificant ($P > 0.05$). The results obtained also indicated no differences in growth of LC-01 and milk acidification between single- and two-stage fermentation processes using the same culture

compositions (Trial B vs. C, D). These findings contradict the results of Lankaputhra and Shah (1997), who reported 4-5 times increase in probiotic numbers with the application of two-stage fermentation process. In their studies, the cultured products were prepared by initially growing probiotic bacteria for 2 h until they reached the final stage of lag phase or initial stage of log phase followed by adding yoghurt cultures. The differences between results of two studies may be due to the individual nature of the strains tested.

Table 6.5 **Effects of culture compositions and fermentation style on acid production and viable counts of LC-01, ST and LB in reconstituted skim milk supplemented with 4% JAIS (40°B)**

Trials	A	B	C	D
(1) Before incubation				
LC-01 count	6.08 ± 0.07^a	5.86 ± 0.05^b	5.88 ± 0.04^b	5.84 ± 0.07^b
ST count	-	5.57 ± 0.14	5.48 ± 0.23	5.50 ± 0.24
LB count	-	5.46 ± 0.17	5.51 ± 0.16	5.44 ± 0.23
(2) Day 1				
%TA	0.84 ± 0.02^b	1.03 ± 0.06^a	1.07 ± 0.18^a	1.11 ± 0.14^a
LC-01 count	8.60 ± 0.19^a	8.12 ± 0.14^b	8.02 ± 0.14^b	8.17 ± 0.22^b
ST count	-	8.26 ± 0.13^a	8.02 ± 0.09^b	7.98 ± 0.14^b
LB count	-	8.19 ± 0.12	8.05 ± 0.08	8.15 ± 0.13
(3) Day 7				
%TA	0.92 ± 0.04^b	1.26 ± 0.11^a	1.15 ± 0.05^a	1.25 ± 0.17^a
LC-01 count	8.56 ± 0.11^a	8.07 ± 0.11^b	8.04 ± 0.15^b	8.20 ± 0.21^b
ST count	-	8.21 ± 0.04^a	8.06 ± 0.16^b	7.99 ± 0.15^b
LB count	-	7.88 ± 0.12	8.02 ± 0.08	7.98 ± 0.14

A: RSMs fermented with LC-01 (single-stage fermentation)
B: RSMs fermented with LC-01 and YC-380 (single-stage fermentation)
C: RSMs fermented with YC-380 followed by LC-01 (two-stage fermentation)
D: RSMs fermented with LC-01 followed by YC-380 (two-stage fermentation)
Results shown are mean ± SD for three experiments each with two replications.
Different letters in the same row differ significantly at $P \leq 0.05$ by DMRT.
Initial pH and TA values of inoculated RSMs containing JAIS were approx. 6.4 and 0.24% respectively.
Final pH of RSMs was approx. 4.3 (on day 1)

Several workers have reported that yoghurt starters can suppress the growth and metabolic activity of probiotic organisms during shelf life due to injury from high rate of acid production (Shah and Jelen 1990; Talwalker and Kailasapathy 2004) and incompatibility between bacterial combinations (Radke-Mitchell and Sandine 1984; Zarate *et al*. 2000). However, according to Vinderola *et al*. (2002) yoghurt bacteria do not commonly exert any negative effect on the growth of probiotic bacteria. Conversely, some strains of lactobacilli, often *L. acidophilus* strains (but not *L. casei*) weakly inhibited the growth of some strains of LB. In the current study, the presence of traditional yoghurt cultures and their higher acid production had no adverse effect on the viability of probiotic LC-01 as their numbers remained stable > 8.0 log CFU g^{-1} in all samples after 7 days of refrigerated storage. Small changes in numbers of two yoghurt cultures were also observed in most cases. This could mean that the combinations of LC-01 and the two yoghurt cultures used allowed good survival of probiotic strains.

6.4.3   Prebiotic effect of JAIP

A question that needs answering is whether the high numbers of probiotic strains found in RSMs containing JAIS was due to the inulin and sugars present in the syrup or due to the presence of background levels of lactose in milk. A trial was therefore designed to compare the utilisation of inulins of JAIP (which is made from the heavy fraction of JAIS), in comparison with commercial inulin powders (Raftilose® P95, Raftiline® GR and Raftiline® HP) and simple sugars (glucose and lactose), in modified MRS broth containing LA-5, LC-01 and BB-12. All ingredients were added at 4% level to MRS broth except the control that contained no carbon source. The utilisation level was measured by measuring OD at 600 mm with a spectrophotometer.

Figure 6.2 shows the capability of utilising various CHOs by three probiotic strains, expressed as the OD at 600 nm. All strains preferred glucose and lactose to inulin powders. In contrast to the studies by Hopkins *et al*. (1998) and Crittenden *et al*. (2001), growth rates of various bifidobacteria strains were higher on non-digestible oligosaccharides (e.g. OF and inulin) than on simple sugars (e.g. glucose, fructose and galactose). This phenomenon suggests that cultures tested in this study may not have an efficient mechanism for transporting inulin substrates.

Of the three probiotic strains studied, the strain of LC-01 could utilise inulin powders the best, followed by BB-12. On the other hand, the growth of LA-5 was minimal in the presence of all inulin powders, showing ODs at 600 nm of *ca*. 0.7-0.8 compared with *ca*. 0.9-2.2 by LC-01 and 1.1-1.6 by BB-12.

The growth of LC-01 was highest in broths containing oligofructose (Raftilose® P95), showing comparable ODs to those of glucose and lactose, and lowest in broth containing long-chain inulin (Raftiline® HP). The JAIP stimulated the growth of LC-01 to a similar level as medium-chain inulin (Raftiline® GR). Although growth and activity of BB-12 in RSM was not enhanced considerably by the addition of JAIS (Table 6.4), it showed efficiency in utilising JAIP and chicory powders, especially OF (Figure 6.2). This finding did not agree with several studies reporting that the majority of *B*. *bifidum* strains were not able to grow on OF and low DP inulins (Hidaka *et al*. 1986; Bielecka *et al*. 2002). This may be explained by the differences in strain used.
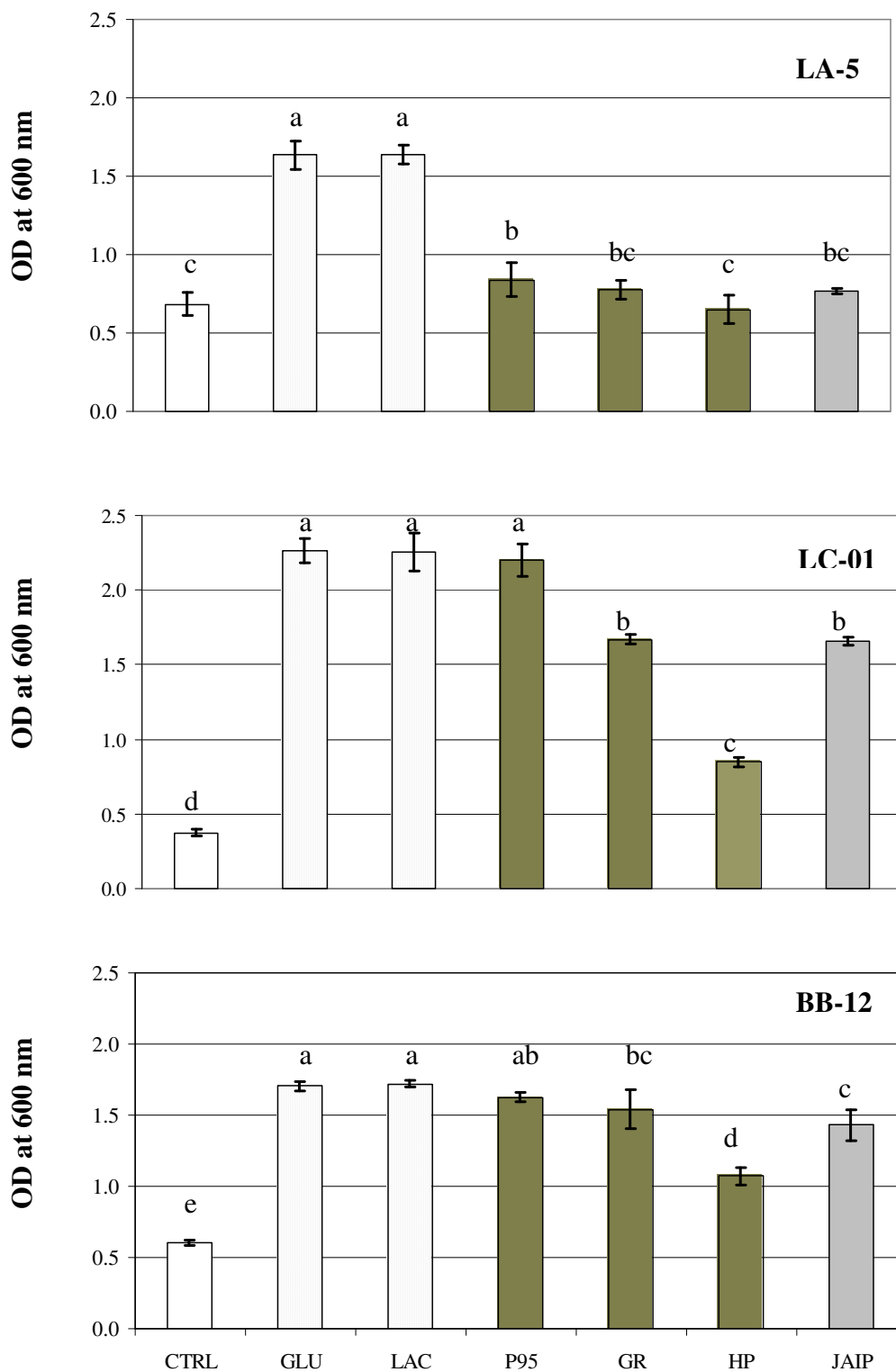
Figure 6.2 **Growth rates of LA-5, LC-01 and BB-12 in media containing six different carbon sources**

Results are mean ± SD of three replicates.

Bar followed by different letters differ significantly at $P \leq 0.05$ by DMRT.

CTRL: control; GLU: glucose; LAC: lactose; P95: oligofructose; GR: medium-chain inulin; HP: long-chain inulin; JAIP: Jerusalem artichoke inulin powder.

According to Voragen (1998), variations in chemical structure of saccharides (linear or branched), DP, composition of monomer units and water solubility affect their utilisation by micro-organisms. In the current study, Raftilose® P95 was the best utilised powder by all three probiotic organisms because of its short-chain length, unbranched nature and high water solubility, while the growth of probiotic strains in the presence of higher DP inulins was poor.

These findings are in good agreement with findings of Tashiro *et al.* (1997) and Roberfroid *et al.* (1998) who concluded that short-chain inulin (DP < 10) was the most fermentable substrate, being fermented twice as quickly as longer-chain inulin. In addition to the DP of inulin chains, the utilisation of inulin by probiotic organisms depends on the purity of the preparations where less purified inulins were fermented more preferably than the highly purified inulins (Biedrzycka and Bielecka 2004). Raftilose® P95 used in this study contained readily available mono- and disaccharides which are better and often not selectively utilised by the bacteria. The ability of JAIP in enhancing the growth of the three probiotics was comparable to medium-chain inulin, but not as efficiently as OF. The 4% supplementation level of JAIP resulted in greater growth rates of LC-01 than BB-12 and LA-5. These results correlated with those of RSMs supplemented with 40°B JAIS (section 6.4.2), confirming the preference of LC-01 for JAI substrates.

6.4.4 Assessment of chemical and microbiological qualities of yoghurt during fermentation and refrigerated storage

During the refrigerated storage, the viability of probiotic organisms in yoghurts is required to remain high in order to allow their survival and multiplication in big numbers in human GIT to exhibiting the expected health-promoting effects (O'Sullivan

2001). In this study, a synbiotic yoghurt was manufactured to study the effects of inulin powder addition on the survival and acidifying activities of probiotic and yoghurt cultures during fermentation and refrigerated storage at 4°C. The probiotic LC-01 was chosen as this strain showed the best growth in the presence of inulin in earlier experiments.

Figure 6.3 shows variations in pH and acidity of experimental and control yoghurts during refrigerated storage for up to 28 days. The addition of inulin powders regardless of the type used did not affect the initial pH (6.6-6.7) and TA (0.20-0.27%) of yoghurt milk. There were no significant differences ($P > 0.05$) in the pH and TA values of supplemented and non-supplemented control yoghurt (NFCY) throughout the storage period. Evidence of this trend was also reported by Guven *et al*. (2005) and Zhu (2004).
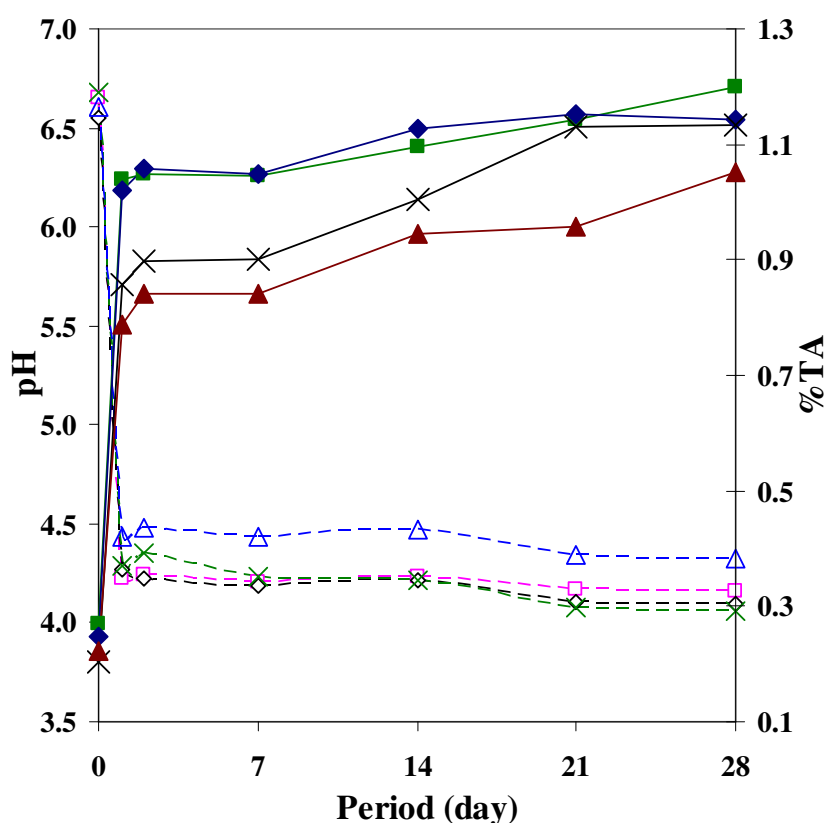
Figure 6.3   Changes in pH (dotted lines) and TA (solid lines) of four yoghurts during fermentation and storage at 4°C
Triangle: INY; Cross: OFY; Diamond: JAY; Square: NFCY.

On day 1, the acid production in JAIP-supplemented yoghurt (JAY, pH 4.2, TA 1.02%) was higher, but not statistically significant to those of chicory oligofructose (OFY, pH 4.4, TA 0.86%) and inulin-containing yoghurts (INY, pH 4.5, TA 0.79%). Yoghurts containing inulin had a stable pH and acidity over time, showing maximum of 0.2 pH unit drops and a 0.28% increase in TA at the end of storage. On day 28, the pH of the yoghurts averaged from 4.1 (for OFY and JAY) to 4.3 (for INY). A small post-production acidification in these yoghurts could be attributed to the type of probiotic and yoghurt starters used. Based on the information provided by the culture manufacturer, they are mild acid-producing cultures (Chr. Hansen 2004).

A variation in viable counts of LC-01, ST and LB in yoghurts with and without inulin supplementation during fermentation and over shelf life period of 28 days is presented in Table 6.7. Overall, the retention of viability of ST was better than those of LB and LC-01. Compared to the control, the addition of all inulin powders did not influence the survival of ST and LB, but significantly improved the viability of LC-01. Of the three inulin powders tested, the best retention of LC-01 numbers was observed with Raftiline® GR (7.4 log CFU g^{-1}) following by Raftilose® P95 (7.3 log CFU g^{-1}) and JAIP (7.1 log CFU g^{-1}).

The initial counts of ST in all inoculated milks ranged from 5.3 to 5.5 log CFU mL^{-1} and increased by *ca*. 3 log cycle after overnight (15 h) incubation. No marked difference in the counts of ST was observed between non-supplemented and supplemented yoghurt batches for each storage day ($P > 0.05$). This was supported by Kaplan and Hutkins (2000) who reported that several strains of *L. casei* and *L. acidophilus* were able to ferment OF well, but not most of the LB and ST strains.

Table 6.7   The viability of LC-01, ST and LB in yoghurts with and without inulin addition during fermentation and storage at 4°C

Culture	Period	NFCY	JAY	OFY	INY
LC-01	0	5.26 ± 0.08^e	5.31 ± 0.09^e	5.28 ± 0.08^e	5.44 ± 0.04^e
	1	8.01 ± 0.22^a	8.26 ± 0.13^a	8.34 ± 0.14^a	8.21 ± 0.15^{ab}
	2	7.98 ± 0.12^a	8.20 ± 0.11^{ab}	8.34 ± 0.18^a	8.28 ± 0.07^a
	7	7.86 ± 0.46^a	8.21 ± 0.14^{ab}	8.20 ± 0.19^{ab}	8.20 ± 0.13^{ab}
	14	7.52 ± 0.46^b	8.10 ± 0.16^b	8.15 ± 0.16^b	8.14 ± 0.12^b
	21	7.11 ± 0.13^c	7.42 ± 0.16^c	7.54 ± 0.14^c	7.89 ± 0.06^c
	28	6.06 ± 0.31^d	7.10 ± 0.15^d	7.30 ± 0.20^d	7.37 ± 0.07^d
	% Viability	75.66^C	85.96^B	87.53^{AB}	89.75^A
ST	0	5.32 ± 0.08^d	5.46 ± 0.05^d	5.42 ± 0.11^c	5.46 ± 0.12^c
	1	8.29 ± 0.09^a	8.36 ± 0.14^a	8.38 ± 0.12^a	8.29 ± 0.16^a
	2	8.27 ± 0.08^{ab}	8.35 ± 0.07^a	8.31 ± 0.11^a	8.29 ± 0.11^a
	7	8.26 ± 0.09^{ab}	8.26 ± 0.16^{ab}	8.34 ± 0.10^a	8.29 ± 0.09^a
	14	8.14 ± 0.19^{abc}	8.21 ± 0.09^{ab}	8.28 ± 0.16^a	8.20 ± 0.21^{ab}
	21	8.09 ± 0.15^{bc}	8.12 ± 0.11^{bc}	8.12 ± 0.16^b	8.13 ± 0.14^{ab}
	28	8.02 ± 0.30^c	8.01 ± 0.33^c	8.13 ± 0.14^b	8.07 ± 0.17^b
	% Viability	96.74^A	95.81^A	97.02^A	97.35^A
LB	0	5.29 ± 0.12^e	5.36 ± 0.16^f	5.27 ± 0.08^e	5.44 ± 0.12^f
	1	8.18 ± 0.10^a	8.13 ± 0.35^a	8.15 ± 0.16^a	8.16 ± 0.11^a
	2	8.16 ± 0.11^a	8.13 ± 0.21^a	8.13 ± 0.20^a	8.16 ± 0.16^a
	7	7.67 ± 0.19^b	7.71 ± 0.24^b	7.72 ± 0.13^b	7.84 ± 0.16^b
	14	7.00 ± 0.16^c	7.03 ± 0.15^c	7.02 ± 0.16^c	7.02 ± 0.14^c
	21	6.33 ± 0.23^d	6.49 ± 0.08^d	6.36 ± 0.34^d	6.64 ± 0.09^d
	28	6.20 ± 0.13^d	6.20 ± 0.16^e	6.23 ± 0.28^d	6.39 ± 0.09^e
	% Viability	75.79^A	76.26^A	76.44^A	78.31^A

Data are means $\pm$ SD of three experiments, and each experiment was examined in duplicate.
[a-f] Means in the same column with different letters differ significantly at $P \leq 0.05$ by DMRT.
[A-C] Means in the same row with different letter differ significantly at $P \leq 0.05$ by DMRT.
% viability = (CFU g^{-1} after 4 week storage/initial CFU g^{-1}) $\times$ 100.
NFCY = non-fat control yoghurt; JAY = yoghurt supplemented with 4% JAIP; OFY = yoghurt supplemented with 4% oligofructose (Raftilose®P95); INY = yoghurt supplemented with 4% inulin (Raftiline®GR).

The experimental and control yoghurts showed ability in sustaining high numbers of ST after 14 days of storage ($P > 0.05$) and only a marginal decline occurred in the following 14 days. After 28 days, all yoghurts contained > 8.0 log CFU g^{-1} of ST, decreasing from the initial counts by only 2.7-4.2%. This reflected the high stability of ST in the products. These observations were consistent with the findings of Medina and Jordano (1994), Dave and Shah (1997a), Vinderola *et al*. (2000), Akalin *et al*. (2004) and Ozer *et al*. (2005) who reported higher stability of ST than LB and bifidobacteria in probiotic yoghurts during storage time.

After overnight incubation, the initial counts of LB in all yoghurts were comparable to those of LC-01 and ST (*ca*. 8.1 log CFU g^{-1}). Supplementation with inulin powders did not help the viability of LB as their numbers in all supplemented yoghurts dropped by *ca*. 1 log after 14 day of storage, similar tendency to those of the controls. An ongoing decline in the numbers of LB was observed until the end of the storage period wherein the final counts were *ca*. 6.2-6.4 log CFU g^{-1} (76-78% of the initial counts). Previously Ozer *et al*. (2005) also reported the decline of viable counts of LB by 2.5 to 4.2 times in inulin-supplemented yoghurts during 14 days of storage. Several workers report that low numbers of LB would have benefited the survival of probiotic organisms due to lesser risks of post-acidification by LB (Holcomb and Frank 1991; Laroia and Martin 1991; Shah 1995).

The addition of inulin powders was helpful in improving the growth of LC-01 during fermentation and their survival during storage time. The results of this study showed a high initial counts of LC-01 ($P \leq 0.05$) in all inulin-supplemented yoghurts (*ca*. 8.3 log CFU g^{-1}) than that in control without inulin (*ca*. 8.0 log CFU g^{-1}) prior to the storage.

These results are consistent with the findings of Aryana and McGrew (2007) who reported a marked increase in *L. casei* counts with the addition of inulin powders. During cold storage, although the counts of LC-01 for each trial declined with time which became significant on day 7 ($P \leq 0.05$), the rate of reduction was slower in the presence of inulin powders. In all inulin-supplemented yoghurts, viable counts of LC-01 retained above 8.0 log CFU g^{-1} up to 14 days of storage which gradually dropped to 7.0 log CFU g^{-1} by the 28th day of storage. As shown in Table 6.7, the highest viability of LC-01 on day 28 was noticed in INY, followed by OFY, recorded at 90 and 88% viability, respectively whereas the lowest viability was observed with NFCY at 6 log CFU g^{-1} (76%). The JAIP had a comparable effect on the viability retention of LC-01 to oligofructose (Raftilose® P95), with average values of 86%, but was slightly less effective than that of inulin (Raftiline® GR). These results seemed to corroborate the findings by Aryana *et al*. (2007) and Lankaputhra *et al*. (1996) who found higher counts of probiotic bacteria in yoghurts with medium- and long-chain inulins than those with OF at the end of storage. This phenomenon was ascribed to the larger amounts of acid developed in yoghurts with OF.

The utilisation of inulin powders by various probiotic organisms has been reported earlier. In accordance with the findings of the present study, Shin *et al*. (2000), Bruno *et al*. (2002), Akalin *et al*. (2004) and Varga *et al*. (2003) reported a significant improvement in the viability retention of bifidobacteria in yoghurts containing prebiotics (inulin/OF) during cold storage compared with the control. Similarly, Desai *et al*. (2004), Capela *et al*. (2006), Aryana *et al*. (2007) and Donkor *et al*. (2007) observed that chicory-based inulins were favoured carbon source for *Lactobacillus* strains, hence increasing the growth performance and sustaining the viability during storage. However, some other literatures by Bozanic *et al*. (2002) and Ozer *et al*. (2005) reported that "inulin did not

support the growth and survival of *L. acidophilus* in fermented bovine milk and acidophilus-bifidus yoghurts". The inconsistencies reported here could be attributed mainly to strain-dependant response of probiotics to prebiotic supplementation.

The mechanism by which inulin improves the viability of the probiotic organisms during cold storage is still unclear. The two possible mechanisms proposed so far state that inulins provide additional nutrients for promoting culture growth (Makras *et al*. 2005) and that they protect probiotic cells from acid injury (Desai *et al*. 2004).

6.5   Conclusions

The three strains of probiotic organisms, namely *B. bifidum* BB-12, *L. acidophilus* LA-5 and *L. casei* LC-01 showed different preference for lactulose-enriched MCP syrup, JAIS and JAIP. Preliminary studies in modified MRS broths indicated that lactulose-enriched MCP syrup (40°B) could stimulate the growth LA-5 and LC-01, but not BB-12. The addition of lactulose-enriched MCP syrup to RSM in sufficiently high concentration (3-4%) appeared to have positive effect on the growth and acid production by LA-5. Compared to reference lactulose solution the growth-sustaining capacity of lactulose-enriched MCP syrup was lower. Therefore, a purification step using chromatography appears to be necessary to obtain high purity lactulose syrup. For JAIS and JAIP, a pronounced response was observed only with LC-01 in both RSM and modified MRS broth at supplementation levels of 3-4%. Strain of LA-5 showed the preference to JAIS in RSM but not to JAIP in broth, and *vise versa* for BB-12. To reduce fermentation time and achieve higher acid production, the use of mixed cultures of LC-01 with ST and LB was necessary.

Chapter 6

The addition of inulin, particularly high DP inulin powder improved the survival of probiotic LC-01 during shelf life at 4°C, showing *ca*. 1 log cycle order higher number than non-supplemented yoghurt at the end of day 28. The JAIP had the effect on sustaining the viability of LC-01 comparable to that of OF. There was no improvement in the growth and survival of yoghurt bacteria in the presence of inulin. The numbers of ST in the experimental and control yoghurts were stable with > 8.0 log CFU g^{-1} throughout the storage time while the numbers of LB decreased below 8.0 log CFU g^{-1} from week 1 onwards. The post-acidification was found minimal in all yoghurts during refrigerated storage. The initial pH prior to storage ranged from 4.2 to 4.5 and these dropped to 4.1-4.3 after 28 days.

Chapter 7

Effects of inulin-type fructans on rheological properties of set yoghurt[1]

7.1 Abstract

Effect of inulin-type fructans addition on rheology of non-fat yoghurt was studied by the large (Texture Analyser) and small (dynamic oscillatory rheometry) deformation tests. Reconstituted skim milk (12% solids) was supplemented with Jerusalem artichoke inulin powder (JAIP) and three commercial chicory inulin powders with different chain lengths at 4% level and inoculated with mixed cultures (1:0.5:0.5) of *Lactobacillus casei* LC-01, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* overnight at 37°C to a final pH of 4.5 ± 0.2. Two non-supplemented yoghurt samples were prepared from reconstituted (16% total solids) whole milk and skim milk powders and used as controls. Rheological tests were conducted after overnight storage at 4°C and during the shelf life of 28 days. All inulin-containing yoghurts in comparison with the non-supplemented control were characterised by lower values of firmness, storage and loss moduli, apparent viscosity, yield stress and complex viscosity. Non-fat yoghurt supplemented with long-chain inulin demonstrated rheological behaviour closer to that of the control full-fat yoghurt. The optimal effect was achieved when incorporating long-chain inulin at 3-4% level. The effect of JAIP on the rheological properties of non-fat yoghurt was comparable to those obtained by adding short- and medium-chain commercial inulins.

---

[1] Based on the findings of this chapter a paper entitled "Rheology and texture of set yoghurt as affected by inulin addition" has been submitted for publication in Journal of Texture Studied (In press).

7.2 Introduction

Yoghurt is a fermented dairy product with textural and rheological properties that are important attributes for consumer acceptability. Texture of yoghurt is influenced by various factors e.g. quality and composition of milk and its fat and total solid content, heat treatment of milk, combination of the lactic acid bacteria used, acidification rate of milk, and storage time (Sodini *et al*. 2004; Dello Staffolo *et al*. 2004; Purwandari *et al*. 2007). In recent years manufacturers have responded to consumers' demand for low-fat and non-fat dairy products, however these type of products somewhat lack the textural and sensory attributes of their full-fat counterparts. Attempts have been made to improve low-fat yoghurt gel attributes by incorporating different additives, and one such approach is the use of inulins as fat replacers.

Several investigations have been conducted into the effect of inulin addition on the sensorial and rheological properties of low-fat dairy products such as ice-cream (Schaller-Povolny and Smith 2001; El-Nagar *et al*. 2002; Akin *et al*. 2007), milk beverages (Villegas and Costell 2007), starch-based dairy desserts (Tarrega and Costell 2006) and fresh cheeses (Koca and Metin 2004). There are also several publications on the quality of yoghurt gels supplemented with inulin products. Dello Staffolo *et al*. (2004) found no difference in viscosity and acceptability of stirred low-fat yoghurt supplemented with 1.3% inulin compared with non-supplemented full-fat yoghurt. Guven *et al*. (2005) found that at higher levels inulin negatively affected the physical properties e.g. whey separation, consistency (using a penetrometer) and organoleptic scores of set-type low-fat yoghurt. Kip *et al*. (2006) reported that inulin improved the creamy mouthfeel of stirred low-fat yoghurts and that increasing inulin DP 23 concentration improved the viscosity of the product but the effect was less pronounced

with inulin DP 9. Recently, Aryana *et al*. (2007) reported no significant effect of inulin chain length on the viscosity of set yoghurts.

In view of these conflicting reports, the current study was undertaken to:

1. Characterise the rheological properties of non-fat set yoghurts supplemented with chicory inulins using large (Texture Analyser) and small (dynamic oscillatory rheometry) deformation measurements,

2. Examine the effects of different chain lengths and supplementation levels on the rheological properties of non-fat set yoghurt,

3. Evaluate the suitability of JAIP as a fat replacer in non-fat yoghurt system.

7.3   Yoghurt preparation

Two separate studies were conducted to evaluate the effects of inulin chain length and supplementation level on the rheology of non-fat yoghurt.

- **Study 1**

The objective of this study was to determine the rheological characteristics of non-fat yoghurt when different types of inulin powders were used as a fat-mimic. The tested inulin powders included the spray-dried JAIP (prepared under optimum conditions reported in 4.4.3) and three commercial chicory inulins: long (Raftiline® HP), medium (Raftiline® GR) and short (Raftilose® P95) chain lengths (section 3.1.5). These inulins were incorporated into 12% reconstituted skim milk (section 3.1.4) at a supplementation level considered to have a prebiotic effect (4%, w/v). Two non-supplemented reconstituted milk samples (16%, w/v) were also prepared as controls: one with WMP (full-fat control yoghurt-FFCY) and another with LHSMP (non-fat control yoghurt-

Chapter 7

NFCY). Batch coding and the list of ingredients used are shown in Table 7.1. The statistical analyses (ANOVA) of results were conducted as described in section 3.7.

Table 7.1　Yoghurt production protocol

Code	Supplement	Ingredients (%)				Protein content (%)
		WMP	LHSMP	Inulin	Distilled water	
FFCY	–	16	0	0	84	4.2
NFCY	–	0	16	0	84	5.4
JAY	JAIP	0	12	4	84	4.1
OFY	Raftilose® P95	0	12	4	84	4.1
MCIY	Raftiline® GR	0	12	4	84	4.1
LCIY	Raftiline® HP	0	12	4	84	4.1

WMP: whole milk powder, LHSMP: low-heat skim milk powder.
FFCY: full-fat control yoghurt; NFCY: non-fat control yoghurt; JAY: JAIP-containing yoghurt; OFY: oligofructose-containing yoghurt; MCIY: medium-chain inulin-containing yoghurt; LCIY: long-chain inulin-containing yoghurt.

- **Study 2**

The objective of this study was to measure the degree of difference in rheological characteristics between the non-fat yoghurts with different supplementation of inulin and non-supplemented full-fat sample. Non-fat yoghurts were prepared from RSMs at 12, 13, 14 and 15% solid content supplemented with 4, 3, 2 or 1% of long-chain inulin (Raftiline® HP) as shown in Table 7.2. The statistical difference was determined using Student's t-tests at 95% confidence level.

Table 7.2　Yoghurt supplementation levels

Code	Addition levels	Ingredients (%)			Protein content (%)
		LHSMP	Inulin	Distilled water	
LCIY 1	1% HP	15	1	84	5.1
LCIY 2	2% HP	14	2	84	4.8
LCIY 3	3% HP	13	3	84	4.4
LCIY 4	4% HP	12	4	84	4.1

LHSMP: low-heat skim milk powder.
LCIY: long-chain inulin-containing yoghurt.
HP: Raftiline® HP

All reconstituted milk samples were heat-treated at 90°C for 10 min prior to cooling to 37°C and inoculation with 0.01% (w/v) of freeze-dried cultures *Lactobacillus casei* LC-01 and YC-380 (section 3.1.4). After slow agitation for 10-15 min under aseptic conditions to distribute the cultures evenly, the inoculated milk samples were aseptically transferred into 100 mL plastic containers, tightly sealed, incubated at 37°C overnight until the desired pH of 4.5 developed and then transferred to fridge at 4°C on the following day.

Large and small deformation measurements were performed at least in duplicate on duplicate samples taken on day 1, and after 7 and 28 days of storage, following the methods described in sections 3.5.6 and 3.5.7. Titratable acidity (% lactic acid) and pH were determined on day 1 and day 28, following the procedure described in sections 3.5.1 and 3.5.2, respectively. Fat and total solid contents, together with colour of yoghurt were determined only on day 1 samples using the methods described in sections 3.5.3-3.5.5.

7.4   Results

7.4 .1   Influence of inulin chain lengths

In this study the 4% supplementation level of inulin was elected on the basis of preliminary trials which found maximum activity of probiotic *L. casei* LC-01 at 4% level of addition (Table 6.4, section 6.6.2). Table 7.3 presents the physico-chemical properties of six yoghurt batches produced. As expected, FFCY showed significantly higher fat content (*ca*. 3.5%) than all non-fat yoghurts (*ca*. 0.1%). No significant differences in pH values (4.2-4.5) and titratable acidity (0.8-1.0%) were found among all yoghurt samples on day 1. After 28 days of storage at 4°C, the acidity of all samples increased to 1.0-1.2% and pH dropped marginally to 4.1-4.3. This indicated ongoing metabolic and enzymic activities of mixed cultures during low temperature storage.

Table 7.3   Physico-chemical properties of yoghurts with and without inulin supplementation (4%)

Properties	Yoghurt batches					
	FFCY	**NFCY**	**JAY**	**OFY**	**MICY**	**LCIY**
Total solids (% f.m.)	15.3 ± 0.5^a	14.8 ± 0.3^a	15.6 ± 0.3^a	15.4 ± 0.9^a	16.0 ± 0.6^a	15.3 ± 0.1^a
Fat (% f.m.)	3.5 ± 0.1^a	0.1 ± 0.0^b	0.1 ± 0.0^b	0.1 ± 0.0^b	0.1 ± 0.0^b	0.1 ± 0.0^b
$L*$	4.3 ± 1.0^a	82.5 ± 1.0^b	79.4 ± 2.1^d	80.3 ± 1.7^{cd}	81.1 ± 0.7^c	80.3 ± 1.7^{cd}
$a*$	-1.5 ± 0.2^a	-1.9 ± 0.1^b	-2.2 ± 0.2^c	-1.8 ± 0.2^b	-2.1 ± 0.2^c	-2.2 ± 0.2^c
$b*$	2.4 ± 0.3^a	1.3 ± 0.3^b	0.2 ± 0.2^d	0.3 ± 0.1^d	0.6 ± 0.2^c	1.3 ± 0.4^b
pH at day 1	4.3 ± 0.2^a	4.2 ± 0.0^a	4.3 ± 0.0^a	4.4 ± 0.1^a	4.5 ± 0.1^a	4.3 ± 0.1^a
TA (%) at day 1	1.0 ± 0.1^a	1.0 ± 0.1^a	1.0 ± 0.2^a	0.9 ± 0.1^a	0.8 ± 0.1^a	1.0 ± 0.1^a
pH at day 28	4.1 ± 0.1^a	4.2 ± 0.1^a	4.1 ± 0.1^a	4.1 ± 0.1^a	4.3 ± 0.1^a	4.3 ± 0.3^a
TA (%) at day 28	1.1 ± 0.1^a	1.2 ± 0.2^a	1.1 ± 0.1^a	1.1 ± 0.1^a	1.0 ± 0.2^a	1.1 ± 0.3^a

FFCY: full-fat control yoghurt; NFCY: non-fat control yoghurt; JAY: JAIP-containing yoghurt; OFY: oligofructose-containing yoghurt; MCIY: medium-chain inulin-containing yoghurt; LCIY: long-chain inulin-containing yoghurt.

[a, b, c, d] Different letters on the value in the same row denote significant difference at $P \leq 0.05$ by DMRT.

All yoghurt samples appeared white and shiny to human eye, however, upon instrumental colour measurement, samples containing inulin showed lower L^* value (a loss of lightness) while a^* and b^* values shifted to higher intensity of green and lower intensity of yellow, respectively (except for b^* value of LCIY).

- **Flow behaviour**

Figure 7.1 shows the flow curves of four inulin-containing yoghurts and two control yoghurts on day 1 by plotting shear rate vs. shear stress. All flow curves showed hysteresis loops and the lack of linear characteristic indicated shear thinning behaviour (pseudoplasticity) as described by Rohm (1993). The same trend was also observed in day-7 and day-28 samples for all batches. In agreement with Jaros *et al*. (2002), the NFCY showed higher slope of flow curves than other yoghurts, reflecting their higher resistance to shear forces. It was also observed that the addition of JAIP, OF (Raftilose® P95) or medium-chain inulin (Raftiline® GR) resulted in yoghurts with lower value of shear stress at maximum shear rate compared to both NFCY and FFCYs. However, LCIY gave nearly identical shear stress to FFCY.

Flow behaviour can also be presented from the effect of shear rate on flow resistance (Braun and Rosen 1999). Figure 7.2 shows the viscosity curves for all yoghurt samples on day 1. The viscosity curves of inulin-containing yoghurts were similar to that of FFCYs, particularly for LCIY and MCIY. In all samples, a rapid breakdown of the structure occurred on initial shearing ($\dot{\gamma}$ = 2-20 s^{-1}) followed by much slower changes at higher shear rates. The viscosity of samples decreased considerably with increasing shear rate between 0 and 200 s^{-1}. At the shear rate of *ca*. 30-40 s^{-1}, the viscosity levelled off between 0.4 and 0.7 Pa.s and then decreased very slowly to 0.1-0.2 Pa.s at the maximum applied shear rate of 200 s^{-1}. Such behaviour confirmed a

shear thinning characteristic and indicated that the yoghurts have an apparent

viscosity (η_{app}).
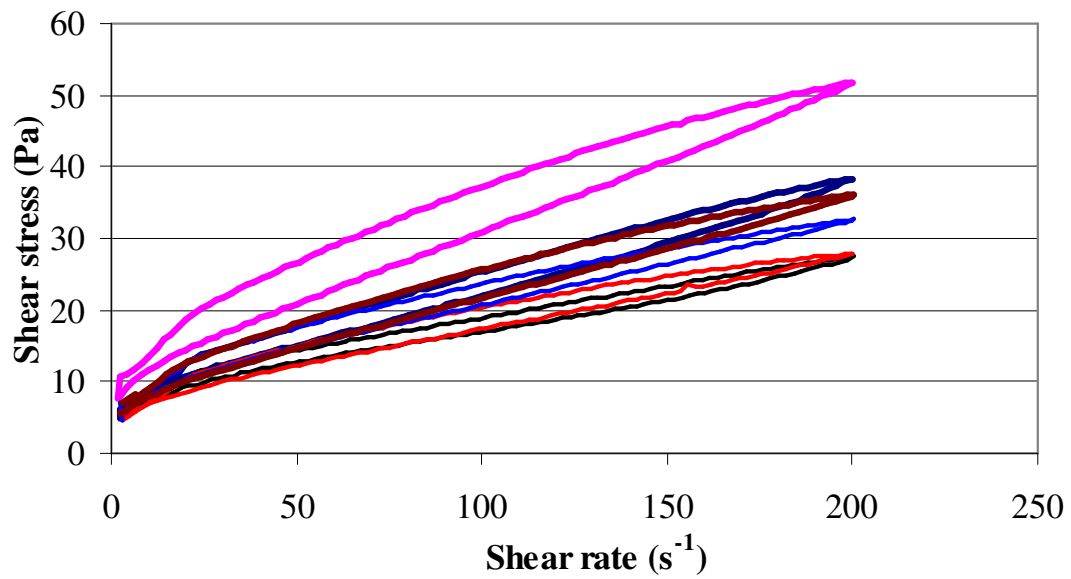
Figure 7.1   Flow curves for inulin-containing and control yoghurts on day 1
Shear rate was first increased and then decreased. Measurement temperature was 20°C.
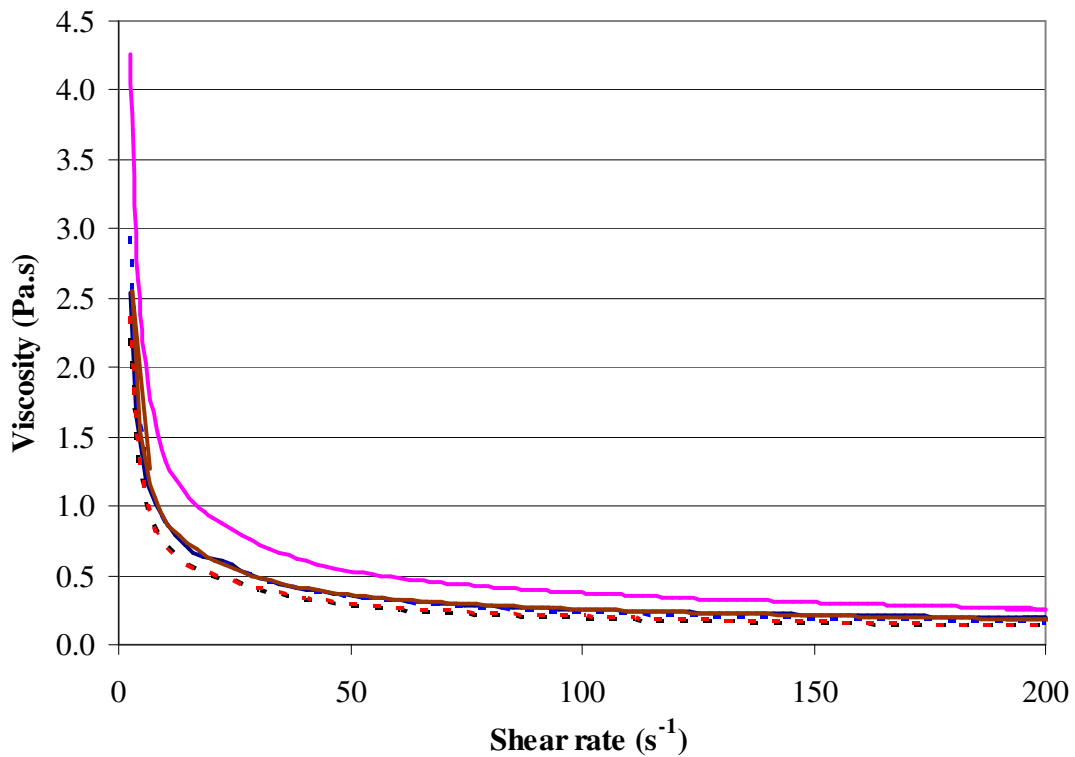
Figure 7.2   Viscosity decay with time for inulin-containing and control yoghurts on day 1

Table 7.4 shows the values of the rheological parameters obtained by fitting in the Herschel-Bulkley model. The model satisfactorily fitted the experimental data for each sample, showing a good correlation coefficient ($0.98 \leq r \leq 0.99$). The NFCY was characterised by the highest average values of consistency coefficient (K), followed by yoghurts containing medium- and short-chain inulins and JAIP, while LCIY achieved similar K values to FFCY ($P \leq 0.05$). The flow index (exponent n) as a measure of deviation from Newtonian flow is $0 < n < 1$ for shear thinning fluids whereas for Newtonian fluids $n = 1$ (Steffe 1992). As expected the values of n in all tested samples were below 1. Yoghurts containing JAIP, medium- and short-chain inulins ($n = 0.52$-0.65) had slightly higher deviations from Newtonian flow (lower n) than LCIY ($n = 0.64$-0.73) and the controls ($n = 0.66$-0.83). When fitted to the Herschel-Bulkley model, yield stress (σ_o) became evident which represented the minimum shear stress required to trigger flow of yoghurt. Again, yoghurts containing JAIP, medium- and short-chain inulins exhibited significantly lower σ_o (3.3 to 4.3 Pa) than LCIY (4.4-5.1 Pa), FFCY (4.4-6.4 Pa) and NFCY (7.5-8.7 Pa).

The effect of inulin addition on the apparent viscosity (η_{app}), upward curve area (A_{up}) and the area of hysteresis loop (ΔA) are also shown in Table 7.4. The ΔA is an indication of yoghurt structural breakdown and rebuilding (a degree of thixotropy) during shearing (Benezech and Maingonnat 1994; Hassan *et al*. 2003). The NFCY showed the highest ΔA and A_{up} followed by LCIY and FFCY, indicating that more structural breakdown and better re-structuring (reversibility) took place during shearing. Yoghurts containing OF and medium-chain inulin showed similar ΔA sizes to JAY ($P > 0.05$) but were significantly different from both controls. Throughout the storage time, the highest apparent viscosity at $\dot{\gamma} = 2$ s^{-1} was observed for the NFCY

(4.15-4.39), twice that of the other samples. All inulin-containing samples had comparable consistency to FFCY, ranging from 2.28 to 2.96.

Table 7.4   **Rheological parameters of inulin-containing and control yoghurts during storage at 4°C obtained from thixotropy tests**

Day	Batch codes[1]	Parameters					
		η [Pa.s][2]	A_{up} [Pa.s^{-1}][3]	ΔA [Pa.s^{-1}][4]	σ_o [Pa][5]	K [Pa.s^n][5]	n [-][5]
1	FFCY	2.53^{b,A}	4916^{b,A}	469^{b,A}	5.79^{b,AB}	0.54^{c,A}	0.83^{a,A}
	NFCY	4.26^{a,A}	7045^{a,A}	885^{a,A}	7.54^{a,A}	1.04^{a,A}	0.70^{bc,AB}
	JAY	2.39^{b,A}	3534^{c,A}	380^{b,A}	3.43^{c,A}	0.73^{ab,B}	0.63^{c,A}
	OFY	2.37^{b,A}	3870^{c,A}	430^{b,A}	3.80^{c,A}	0.78^{ab,B}	0.65^{bc,A}
	MCIY	2.96^{b,A}	3937^{c,A}	377^{b,A}	3.85^{c,A}	0.90^{ab,A}	0.64^{bc,A}
	LCIY	2.56^{b,A}	4882^{b,A}	571^{b,A}	5.11^{b,A}	0.66^{c,B}	0.73^{b,A}
7	FFCY	2.56^{b,A}	5048^{b,A}	461^{bc,A}	6.44^{b,A}	0.42^{b,A}	0.83^{a,A}
	NFCY	4.39^{a,A}	6674^{a,A}	699^{a,AB}	8.70^{a,A}	0.78^{a,A}	0.76^{b,A}
	JAY	2.38^{b,A}	3623^{c,A}	272^{c,B}	3.45^{c,A}	0.84^{a,A}	0.64^{c,A}
	OFY	2.30^{b,A}	3744^{c,A}	280^{c,B}	4.03^{c,A}	0.83^{a,B}	0.63^{c,A}
	MCIY	2.51^{b,A}	4019^{c,A}	374^{bc,A}	3.83^{cA}	0.97^{a,A}	0.61^{c,A}
	LCIY	2.51^{b,A}	4961^{b,A}	559^{ab,A}	4.48^{c,A}	0.62^{a,B}	0.68^{c,B}
28	FFCY	2.28^{b,A}	3454^{c,A}	426^{ab,A}	4.38^{b,B}	0.61^{b,A}	0.68^{a,A}
	NFCY	4.15^{a,A}	5969^{a,A}	573^{a,B}	7.70^{a,A}	1.09^{ab,A}	0.66^{a,B}
	JAY	1.98^{b,A}	4291^{bc,A}	288^{c,B}	3.89^{b,A}	0.91^{ab,A}	0.65^{a,A}
	OFY	2.48^{b,A}	3499^{c,A}	243^{c,B}	3.28^{b,A}	1.34^{a,A}	0.52^{b,B}
	MCIY	2.64^{b,A}	3822^{bc,A}	227^{c,A}	4.28^{b,A}	1.25^{a,A}	0.57^{ab,A}
	LCIY	2.88^{b,A}	5183^{ab,A}	527^{a,A}	4.38^{b,A}	1.11^{a,A}	0.64^{a,C}

[1] See Table 7.1 for details.

[2] Apparent viscosity (Pa.s) at $\dot{\gamma} = 2$ s^{-1}.

[3] Area under the upward curve when plotting shear stress versus shear rate.

[4] Differences in area under the upward part of the upward and downward curve.

[5] Determined by fitting to the Herschel-Bulkley model.

[a, b, c] Means in the same column with different small letter superscripts indicate significant difference at $P \leq 0.05$ between yoghurt batches at a particular storage time.

[A, B, C] Means in the same column with different capital letter superscripts indicate significant difference at $P \leq 0.05$ within yoghurt batches affected by storage time.

No significant changes were found in η_{app} and A_{up} in all samples during the shelf life. Yield stress values of all yoghurts were also maintained, except a marked decrease for FFCY at day 28 of storage. However, storage time was a significant factor for consistency index (K) and flow index (*n*) in various samples. For example, a decrease in flow index (*n*) and increase in K were noticed in OFY and LCIY at the end of storage.

- **Viscoelastic properties**

This dynamic testing provides useful information on the viscoelastic properties of yoghurt, i.e. the storage modulus (G′) and loss modulus (G″) which denote the degree of solid-like (elastic) and liquid-like (viscous) behaviour, respectively, as well as complex viscosity (η∗) and loss tangle (tan δ). Figure 7.3 shows changes in G′ profiles recorded during stress sweep of day-1 yoghurt samples.
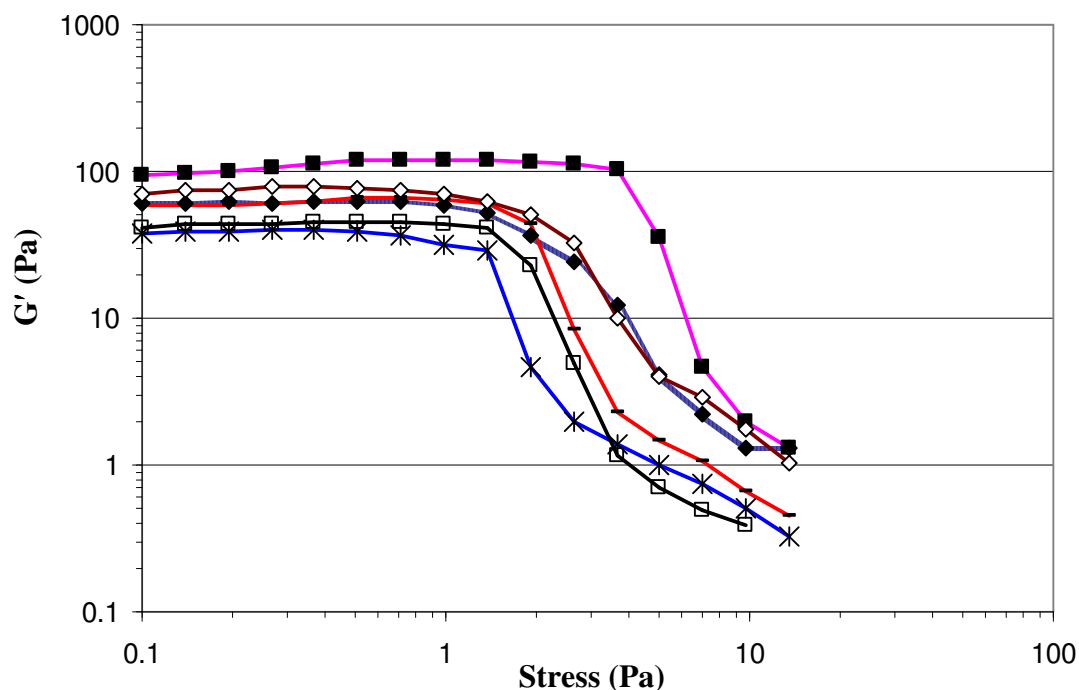
Figure 7.3 **The storage modulus (G′) of inulin-containing and control yoghurts on day 1 as a function of stress**

FFCY ◆ NFCY ■ OFY MCIY ✳ JAY ▫ LCIY ◇

The length of the linear viscoelastic region (LVR) indicating that G′ value is independent of the oscillation stress (Lapasin and Pricl 1995) was different for each treatment. The G′ values of the NFCY remained unchanged up to a shear stress of 3.7 Pa before gel structure started breaking down whereas inulin-containing samples and FFCY showed structural damage at a weaker force within the range of 1.4-1.9 Pa. Since the LVR for all samples was similar within the stress range of 0.1-1.4 Pa, the stress level of 1 Pa was taken as the starting value for frequency sweep.

The viscoelastic properties of inulin-containing and control yoghurts as a function of frequency (ω) are shown in Table 7.5. For comparison purposes, G′, G″, tan δ and η∗ values recorded at 1 Hz were considered. The results indicated that towards the end of storage period the NFCY gel was firmer (G′ values of 88-94 Pa) than inulin-containing yoghurts (34-49 Pa) and FFCY (40-54 Pa). Moreover, the inulin-containing yoghurts showed a weaker gel than NFCY as demonstrated by their low η∗ (32-56 Pa.s vs. 100-107 Pa.s) and slightly higher tan δ (0.32-0.39 vs. 0.32-0.33) values. Among yoghurts made with inulin, only LCIY exhibited comparable viscoelastic characteristics to FFCY for all attributes. This demonstrates the functional suitability of long-chain inulins as fat replacers in non-fat yoghurts.

A plot of log (G′) or log (G″) versus log angular frequency (ω) gave reasonable straight lines for all yoghurts as shown in Figure 7.4. The G′ values of all yoghurts were greater than G″ over the tested frequency range which is expected for weak viscoelastic systems (Rohm and Kovac 1994). The slope values of the linearised plots of log (G′) or log (G″) vs. log (ω) indicate the frequency dependence of tested samples (Hassan *et al*. 2003). In the present study, G′ and G″ showed similar

frequency dependency but those of FFCY and LCIY were significantly lower than the

rest of the yoghurts (data not shown).

Table 7.5 Viscoelastic properties for inulin-containing and control yoghurts during storage at 4°C

Day	Batch codes[1]	Viscoelastic properties			
		G′ at 1 Hz (Pa)	G″ at 1 Hz (Pa)	η∗ at 1 Hz (Pa.s)	Tan δ at 1 Hz
1	FFCY	$53.9 \pm 6.7^{b,A}$	$15.6 \pm 1.2^{b,A}$	$60.8 \pm 7.3^{b,A}$	$0.29 \pm 0.02^{c,A}$
	NFCY	$94.3 \pm 9.8^{a,A}$	$26.8 \pm 3.7^{a,A}$	$107.0 \pm 10.6^{a,A}$	$0.32 \pm 0.03^{bc,A}$
	JAY	$30.7 \pm 2.1^{c,A}$	$11.2 \pm 0.9^{c,A}$	$38.5 \pm 4.21^{c,A}$	$0.36 \pm 0.03^{ab,A}$
	OFY	$35.9 \pm 8.4^{c,A}$	$13.2 \pm 2.0^{bc,A}$	$41.5 \pm 9.3^{c,A}$	$0.38 \pm 0.03^{a,A}$
	MCIY	$39.6 \pm 6.1^{bc,A}$	$14.0 \pm 0.8^{bc,A}$	$45.5 \pm 6.5^{bc,A}$	$0.36 \pm 0.04^{ab,A}$
	LCIY	$44.7 \pm 15.43^{bc,A}$	$14.7 \pm 3.1^{bc,A}$	$51.1 \pm 16.9^{bc,A}$	$0.34 \pm 0.05^{abc,A}$
7	FFCY	$40.5 \pm 4.0^{bc,A}$	$13.3 \pm 0.8^{bc,A}$	$46.3 \pm 4.3^{bc,B}$	$0.33 \pm 0.01^{c,A}$
	NFCY	$87.8 \pm 16.0^{a,A}$	$27.5 \pm 3.5^{a,A}$	$99.5 \pm 17.7^{a,A}$	$0.31 \pm 0.01^{c,A}$
	JAY	$31.2 \pm 3.3^{c,A}$	$10.8 \pm 1.9^{c,A}$	$37.4 \pm 4.6^{c,A}$	$0.34 \pm 0.03^{bc,A}$
	OFY	$34.2 \pm 2.1^{c,A}$	$12.0 \pm 1.5^{c,A}$	$31.8 \pm 6.2^{c,A}$	$0.35 \pm 0.03^{bc,A}$
	MCIY	$48.7 \pm 1.9^{b,A}$	$16.2 \pm 1.2^{b,A}$	$55.6 \pm 2.3^{b,A}$	$0.33 \pm 0.02^{c,A}$
	LCIY	$37.7 \pm 9.2^{bc,A}$	$14.0 \pm 2.5^{bc,A}$	$43.6 \pm 10.3^{bc,A}$	$0.38 \pm 0.03^{a,A}$
28	FFCY	$39.9 \pm 8.0b^{b,A}$	$12.4 \pm 1.7^{b,A}$	$45.5 \pm 8.6^{b,B}$	$0.32 \pm 0.04^{b,A}$
	NFCY	$90.0 \pm 16.1^{a,A}$	$27.0 \pm 3.7^{a,A}$	$102.0 \pm 17.8^{a,A}$	$0.31 \pm 0.02^{b,A}$
	JAY	$31.3 \pm 3.6^{b,A}$	$10.8 \pm 1.41^{b,A}$	$43.4 \pm 5.9^{b,A}$	$0.35 \pm 0.02^{ab,A}$
	OFY	$35.9 \pm 3.3^{b,A}$	$11.5 \pm 1.1^{b,A}$	$40.9 \pm 3.7^{b,A}$	$0.33 \pm 0.01^{b,A}$
	MCIY	$43.2 \pm 5.7^{b,A}$	$13.7 \pm 1.7^{b,A}$	$49.2 \pm 6.4^{b,A}$	$0.32 \pm 0.02^{b,A}$
	LCIY	$35.5 \pm 14.7^{b,A}$	$13.3 \pm 4.2^{b,A}$	$41.1 \pm 16.5^{b,A}$	$0.39 \pm 0.04^{a,A}$

[1] See Table 7.1 for definitions.
[a, b, c] Means in the same column with different small letter superscripts indicate significant difference at $P \leq 0.05$ between yoghurt batches at a particular storage time.
[A, B] Means in the same column with different capital letter superscripts indicate significant difference at $P \leq 0.05$ within yoghurt batches affected by storage time.
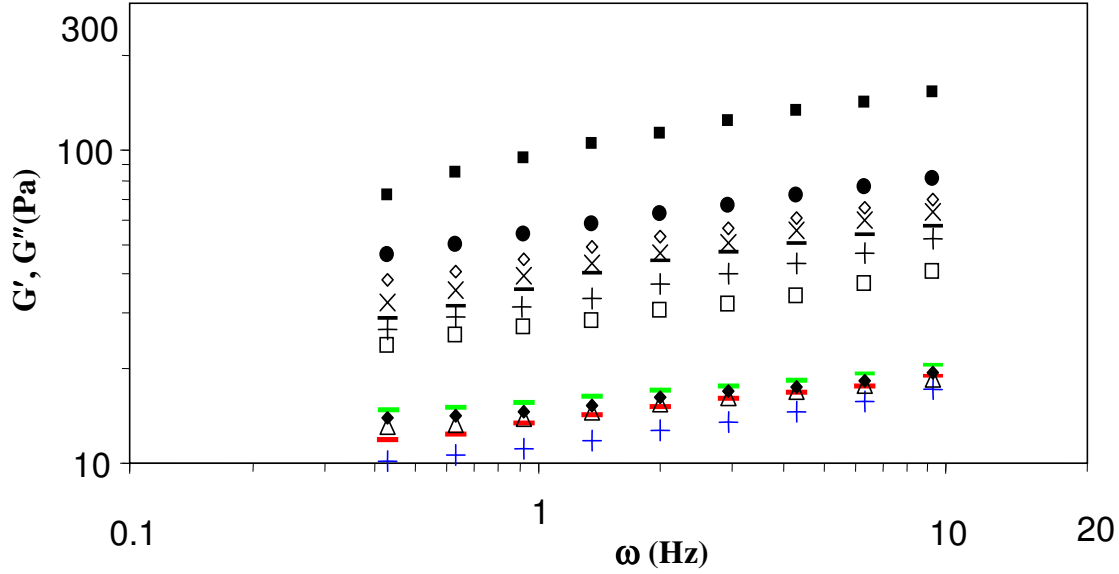
Figure 7.4 **Changes of G′ and G″ of inulin-containing and control yoghurts on day 1 as a function of angular frequency (ω)**
FFCY: G′ (●) & G″(▬); NFCY: G′ (■) & G″ (□); JAY: G′ (✛) & G″ (✛); OFY: G′(▬) & G″(▬); MCIY: G′ (✘) & G″ (△); LCIY: G′ (◇) & G″ (♦)

Prolonged cold storage did not significantly affect viscoelastic properties (G′, G″ and tan δ) of yoghurts. Only η∗ of FFCY declined significantly from 61 ± 7 Pa.s on day 1 to 46 ± 4 Pa.s on day 7.

- **Large deformation properties**

Table 7.6 shows the firmness (in N) of all yoghurts subjected to large deformation test at a constant velocity. On day 1, the gel firmness of the four inulin-containing yoghurts was significantly lower (0.58-0.83 N) than that of NFCY (1.04 N), but closer to that of the control FFCY (0.70 N). The firmness of yoghurts was significantly affected by type of inulin added as long-chain inulin resulted in less firm yoghurts than those added with OF and medium-chain inulin ($P \leq 0.05$). After 7 days of storage at 4°C, compression force (i.e. firmness) of various yoghurts increased slightly which could be attributed to the phenomenon of gel structure reinforcement at low temperature (Oliveira *et al*. 2001). Storage time had a small but statistically

insignificant effect on maximum compression force of each yoghurt sample ($P >$ 0.05).

Table 7.6   Large deformation properties (in N) of yoghurts with and without inulin supplements during storage at 4°C

Batch codes[1]	Storage time		
	1 day	7 days	28 days
FFCY	0.70 ± 0.14^{cd}	0.76 ± 0.22^{bc}	0.64 ± 0.04^{c}
NFCY	$1.04 \pm 0.09^{a,}$	$1.10 \pm 0.12^{a,}$	1.05 ± 0.09^{a}
JAY	0.58 ± 0.04^{d}	0.59 ± 0.03^{c}	0.56 ± 0.03^{d}
OFY	0.79 ± 0.75^{bc}	0.80 ± 0.11^{b}	0.74 ± 0.08^{b}
MCIY	0.83 ± 0.06^{b}	0.84 ± 0.10^{b}	0.79 ± 0.08^{b}
LCIY	0.64 ± 0.04^{d}	0.61 ± 0.04^{c}	0.62 ± 0.03^{cd}

[1] See Table 7.1 for details
[a, b, c] Means in the same column with different small letter superscripts indicate significant difference at $P \leq 0.01$

As expected, the long-chain inulin (Raftiline® HP) shown to be an efficient fat replacer. According to Franck (2002) long-chain inulin has the capacity to form microcrystals which interact with each other and entrap large amount of water, producing a fine texture that mimics milk fat properties. However, medium-chain inulin did not show noticeable differences to OF on yoghurt rheology, although MCIY exhibited slightly firmer texture than OFY and showed rheological functions closer to FFCY. According to manufacturer's specifications, medium-chain inulin (Raftiline® GR) with an average DP of 12 is also recommended for fat replacement due to the presence of high-molecular-weight fractions, while OF (Raftilose® P95) is more suitable for sugar replacement due to the predominance of mono- and disaccharides, and short-chain OFs (Niness 1999).

7.4.2 Influence of inulin supplementation levels

Table 7.7 shows the effects of supplementation levels of long-chain inulin on the rheological properties of non-fat yoghurts. In the work presented here, the rheological testes were performed only on day 1 since the storage time was an insignificant factor for rheological behaviours.

Using only large deformation test (Texture Analyser) may not be sufficiently sensitive to measure significant differences between the experimental samples and FFCY but dynamic oscillatory test found to be useful. There were significant changes in gel characteristics as inulin supplementation levels increased, except for apparent viscosity (η_{app}), yield stress (σ_o) and flow index (n). A 1% supplementation level resulted in yoghurts with significant higher values of G′ (82.6 Pa), A_{up} (724 Pa.s^{-1}), $\eta^\star$ (94 Pa.s) and gel firmness (0.86 N) than those of FFCY at $P \leq 0.05$. The use of inulin above 2% caused an increasingly softer texture and no difference was found in the above-mentioned four parameters compared to FFCY. When inulin supplementation was raised to 3%, a significant difference was observed only in hysteresis loop area, and with 4% inulin all rheological parameters were comparable to FFCY ($P > 0.05$). These results suggested the optimal supplementation levels of inulin in non-fat yoghurt at 3-4%, higher than the usage levels recommended by the manufacturer of 1-3%.

Table 7.7   Effects of inulin supplementation level on rheological parameters of day-1 non-fat yoghurts

Parameters	Supplementation levels (%)							
	1%	P-value	2%	P-value	3%	P-value	4%[1]	P-value
η_{app} (Pa.s)	3.43 ± 0.55	ns	3.76 ± 0.97	ns	2.97 ± 0.54	ns	2.56 ± 0.27	ns
A_{up} (Pa.s^{-1})	724 ± 140	0.014	696 ± 99	ns	632 ± 72	ns	571 ± 59	ns
ΔA (Pa.s^{-1})	6541 ± 840	0.014	6051 ± 806	0.017	5192 ± 319	0.023	4882 ± 202	ns
σ_o (Pa)	7.08 ± 1.49	ns	6.59 ± 2.00	ns	5.16 ± 0.63	ns	5.11 ± 0.20	ns
K (Pa.s^n)	0.93 ± 0.16	0.031	0.80 ± 0.13	0.028	0.73 ± 0.10	ns	0.66 ± 0.09	ns
n (−)	0.69 ± 0.05	ns	0.70 ± 0.06	ns	0.71 ± 0.07	ns	0.73 ± 0.03	ns
G' at 1 Hz (Pa)	82.6 ± 12.5	0.006	64.7 ± 8.2	ns	56.6 ± 8.1	ns	44.7 ± 15.4	ns
G'' at 1 Hz (Pa)	26.1 ± 2.9	0.000	21.3 ± 2.0	0.002	18.6 ± 3.1	ns	14.7 ± 3.1	ns
η^* at 1 Hz (Pa.s)	94.0 ± 13.8	0.005	73.8 ± 9.1	ns	64.6 ± 9.4	ns	51.1 ± 16.9	ns
Firmness (N)	0.86 ± 0.04	0.005	0.79 ± 0.05	ns	0.71 ± 0.08	ns	0.64 ± 0.04	ns

[1] Taken from Table 7.4-7.6.
Results are means ± SD of two determinations from triplicate experiments.
ns = not significant ($P > 0.05$).

7.5 General discussion

The semi-solid texture of set yoghurt gel is a result of the development of a three dimensional network of milk proteins. The main factor responsible for milk gelation is a reduction in the high net negative charge on the casein micelles due to the liberation of acids from microbial activity. Aggregation of casein micelles starts at a pH of *ca*. 5.3 which also causes the solubilisation of colloidal calcium phosphate. Further pH reduction to below 5.0 causes a more complex and extensive interconnection of casein micelles and the gel attains its maximum firmness at pH 4.6, the isoelectric point of casein (Tamime 2006; Amatayakul *et al*. 2006b).

The addition of inulin to non-fat yoghurt milk altered the rheological and textural properties of the product. Inulin-containing yoghurts showed a low magnitude of yield stress value and firmness than did yoghurts without inulin. The firmness of yoghurt is directly dependent on its total solids and specifically, protein content and the type of proteins. Higher protein content would cause a higher degree of cross linking of the gel network resulting in a much denser and more rigid gel structure (Tamime 2006). In the current study, non-fat yoghurt milks were prepared by reconstituting the LHSMP in distilled water (Table 7.1, section 7.3), where NFCY milk was standardised to 16% total solids (*ca*. 5.4% protein) using LHSMP, while the inulin-containing yoghurts were prepared from RSM (12% LHSMP) plus 4% inulin powder, which resulted in lower net protein content (*ca*. 4.1%) compared to NFCY. Besides, the molecules of inulin are dispersed amongst the casein micelles, thus interfering with protein matrix formation. These facts are believed to be responsible for a softer yoghurt gel formation. This is supported by the fact that FFCY prepared from 16% reconstituted WMP (without inulin supplement) also had a weak gel structure compared to NFCY due to proportionally low protein content (*ca*. 4.2%) and interference of fat globules with protein matrix formation

(Becker and Puhan 1989; Salvador and Fiszman 2004). From dynamic oscillation measurements, the gel firmness was lower in inulin-supplemented yoghurts as shown by their low G′ values, which could be explained in terms of the weak network resulting from reduced number of casein-casein bonds.

There were no statistically significant differences ($P > 0.05$) in rheological behaviour between full-fat control and long-chain inulin-containing samples, confirming the suitability of Raftiline® HP as a fat replacer. Kip *et al*. (2006) hypothesised that the positive effect of long-chain inulin on imparting creamy mouthfeel to stirred yoghurt was due to its water-binding ability that helps it act as a thickener by combining with the protein aggregates. According to Sensus Operation CV (2000), inulin gel is composed of a tri-dimensional network of insoluble sub-micron crystalline inulin particles that enclose large amounts of water. The aggregation rates and gelation process depend on the numbers (proportion or concentration) and length of inulin chains and only the longer inulin molecules (DP > 10) participate in gel formation while the smaller molecules are dissolved in the aqueous phase.

The formation of entangled networks of inulin, as an additional structure to the protein network, could cause an increase in hysteresis loop size. When long-chain inulins were added to yoghurt milks, high structural breakdown and reforming (as indicated by high ΔA and A_{up} values) comparable to that of FFCY was observed, indicating the presence of entangled network of inulin. The rest of inulin powders studied produced inferior ΔA and A_{up} values, reflecting the presence of lower numbers of inter- and intra-strand bonds within the gel systems, thus needing less energy to breakdown during shearing and reform into a coherent network structure. It is also likely that JAIP, OF and medium-chain inulin could act as inert fillers or structure breakers to hinder the formation of

a cohesive network of protein strands and thereby decreasing the cross-linking density and G′ of yoghurt gels. This phenomenon is also evident in the studies using exopolysaccharide (EPS) producing starter cultures for yoghurt production where the incompatibility between EPS and proteins decreased the gel firmness (Rohm and Kovac 1994; Hassan *et al*. 2003; de Kruif and Tuinier 2001; Amatayakul *et al*. 2006a, 2006b). Likewise, when whey proteins were added to milk a reduction in yoghurt gel rigidity was observed because of their inability to form cohesive network (Guggisberg *et al*. 2007).

The fat replacement effects of Raftilose® P95, Raftiline® GR and JAIP were less pronounced compared to Raftiline® HP. The Raftilose® P95 contains 95% oligosaccharides, and 5% di- and monosaccharides, therefore it would have a textural effect similar to that of sucrose for example, which produced non-gelled, liquid-like systems. This view was supported by Chiavaro *et al*. (2007) who reported that medium- and long-chain inulins develop gel structure at concentrations exceeding 30 and 20%, respectively, whereas short-chain inulins produce non-gelled system. For JAIP, the powder was produced from JA tubers which are naturally a source of medium DP inulin, rendering the powder difficult to form a gel. The literature data on the chain length distribution of inulin of JA tubers shows that inulins with DP < 10 prevail in the tuber (52% of total inulins), whereas inulins with DP 10-20, 21-40 and > 40 are represented only at 22, 20 and 6%, respectively (Saengthongpinit and Sajjaanantakul 2005; Van Loo *et al*. 1995). In contrast to chicory roots, *ca*. 31 % of total inulins have a DP < 10, whereas the fractions with DP 10-20, 21-40 and > 40 are made up of 24, 28 and 17%, respectively (Van Loo *et al*. 1995). During heat treatment and at low pH conditions, Kim *et al*. (2001) reported that longer-chains of inulin could be hydrolysed into shorter-chain inulins thus lose their gel-forming capacity. Assuming that the

hydrolysis occurred to the same level in all samples during the course of fermentation and heating, the system of JAIP would contain smaller amounts of the remaining long inulin chains to form gel network compared to that of Raftiline® HP. In a similar situation, Raftiline® GR showed lower capacity for replacing fat than Raftiline® HP because of having less long-chain inulin molecules in the product and less water-holding capacity. The Raftiline® GR could possibly contribute better texture at increasing concentration or in the combination with other additives e.g. hydrocolloid (Bishay 1998) where its incorporating level could be lowered.

In the work presented here, the inulin chain length marginally influenced the viscosity of the yoghurts (as indicated by η_{app}) although medium- and long-chain inulins gave slightly higher values than OF and JAIP. The findings of earlier studies by Aryana *et al*. (2007), stating insignificant effect of inulin chain lengths at 1.5% level on the viscosity of fat-free yoghurt seems to find support in this study.

7.6   Conclusions

The results of this study show that yoghurts supplemented with inulins were characterised by lower values of consistency coefficient and yield stress compared to the NFCY. All inulin powders produced a softening effect on yoghurt gel, progressively reducing storage and loss moduli, and complex viscosity values and showing similar characteristics to FFCY. However, only long-chain inulin could achieve rheological characteristics comparable to that obtained from fat when added at 3-4% levels. The JAIP-containing yoghurts exhibited similar rheological characteristics to those containing OF and medium-chain inulin, but the effect of JAIP as fat replacer was not as efficient as that of long-chain inulin.

Chapter 8

General discussion, conclusions and further research recommendations

In this project, attempts were made to develop a process for preparing prebiotic compounds from natural sources and dairy industry waste streams available in Australia, with the ultimate objective of developing replacements for the imported prebiotic ingredients. Our emphasis was on inulin from JA tubers and lactulose from MCP for incorporation in synbiotic products that would combine these prebiotic ingredients with commercially-available probiotic strains that could efficiently utilise these prebiotics. The outcome of this project falls into four major areas as follows:

1. Method optimisation for preparation of inulin from JA tubers

2. Process development for the preparation of lactulose from MCP

3. Studies on the bifidogenic powers of extracted JAI (concentrate and powder), and lactulose-enriched MCP syrup in media broth and fermented milk models

4. Studies on the fat-replacing ability of JAIP in non-fat yoghurts

The findings for each of these are reviewed as a basis for presenting the primary conclusions of this project and a discussion of areas recommended for further research.

The preliminary study of this project revealed that JA grown in Victoria, Australia would be useful for the production of inulin as its tubers contain *ca.* 13 % total fructans on fresh basis (85% of total CHO). Therefore, a protocol was designed for the production of light-coloured JAIS. The optimised process was based on hydrothermal extraction of whole JA tubers, followed by lime-carbonation and activated carbon

treatments and concentration. The development of undesired colour during extraction due to the activity of PPO enzyme was effectively prevented by peeling the tubers and adding small amounts of sodium metabisulphite. The hydrothermal extraction process developed did not achieve a high inulin yield (only *ca.* 30% of the fructans in tubers could be extracted) but was rapid for turning a large amount of fresh tubers into crude inulin extract that then could be stored frozen for subsequent treatments. Thus the risk of degradation of fresh tubers during cold storage could be avoided.

Two different processes (i.e. ethanol and cold fractionation) were tested for their suitability for laboratory-scale fractionation of inulin from concentrated inulin syrup (up to 40°B). Ethanol fractionation of JA concentrate yielded an inulin-rich pasty substance. The response surface methodology allowed establishment of the optimum fractionation conditions at 32°B syrup concentration with 13:1 (v/v) ethanol-to-syrup ratio and a temperature of 42°C. The results also suggested that syrup concentration had a positive effect on inulin yield, but beyond 32°B a reverse effect on average chain length and purity values was observed. In cold fractionation, the insoluble heavier inulin fractions precipitated that could subsequently be separated from the supernatant by centrifugation. The precipitation yield of inulin was affected by initial syrup concentration and temperature. In the range of condition tested, the highest yield of 36% was achieved as the storage temperature dropped from +4 to −24°C and syrup concentration rose from 6 to 12°B. In comparison, ethanol fractionation was more efficient for separating the high- and low-MW components in JA extracts, but less economical and environmentally friendly. In addition, inulin precipitate obtained from cold fractionation could easily be spray-dried to obtain an off-white powder.

Chapter 8

Application of the UF for fresh milk (and whey) concentration leads to high volumes of waste streams (e.g. permeate or MCP) that contain mainly lactose and some salts. Some of the limited options available are to use it for feeding farm animals or as a raw material for processing into lactose powder. In the work reported here, the MCP collected from a local milk processor was used as lactose source for the production of a more valuable ingredient, the lactulose. The shell powders used as catalysts were waste products from restaurants and egg-processing plants. Results of HPLC determination of lactulose content and colour measurement were primarily used to optimise process conditions *viz.* the catalyst loading, and isomerisation temperature and time.

The results obtained showed that ESP loading above 12 mg per mL enhanced the lactose conversion after 60 min of heating. However, in most cases similar final yields of lactulose were achieved after isomerisation for 150 min. With 30 mg per mL ESP maximum lactulose yield of 0.86 g per 100 mL MCP was achieved, whereas catalyst loadings between 12 and 20 mg per mL gave 0.77 and 0.79 g lactulose per 100 mL, respectively. Isomerisation temperatures of 96 and 100°C gave the optimum reaction rate, below which the conversion rate dropped significantly (to only 0.24 g per 100 mL at 90°C even with extended time of 180 min). By considering the minimum change of reaction mixture colour, a catalyst loading of 12 mg per mL and isomerisation temperature of 96°C were considered optimal. The reaction time depended on the conversion level required. A longer isomerisation time caused an increased lactose conversion but also led to a darker coloured solution.

In further investigation, the catalytic powers of OSP and limestone (pure calcium carbonate) in lieu of ESP were compared. Results indicated that both catalysts were equally effective for lactulose production as ESP by raising the pH of reaction mixture

Chapter 8

above 9.0 and yielding maximum conversion of 18-20% after 120 min of heating. When de-proteinated MCP was used, there was little difference in maximum lactulose yield (*ca*. 0.15 g per 100 mL) from original MCP although the buffering action of protein contaminants in original MCP resulted in slow isomerisation rate at the earlier stage. Similar to inulin extract, the treatments with activated carbon or hydrogen peroxide successfully removed brown colour from isomerised MCP without significant loss of lactulose.

Prebiotic ingredients are recognised for their ability to increasing the numbers of intestinal microflora. A recent interest is to incorporate them in combination with probiotic bacteria for enhanced functional properties (Ziemer and Gibson 1998; Roberfroid 2001). Therefore, a comparison of the growth and acid production by two strains of lactobacilli (*L. acidophilus* LA-5 and *L. casei* LC-01) and one strain of bifidobacteria (*B. bifidum* BB12) in the presence of prebiotic compounds developed was undertaken in RSM and modified MRS broth models. These three probiotic strains were chosen mainly on the basis of their common use in yoghurt manufacturers in Australia. As the development of pure crystalline lactulose powder was not the primary objective of this study, the isomerised MCP containing residual lactose and other by-products was further concentrated under reduced pressure at 70°C to obtain lactulose-enriched MCP syrup of 40°B. Similarly, no additional step was taken for the removal of ash and free sugars from JAI.

The results indicated that the effects of tested prebiotic compounds on the growth and acid production by probiotics were strain-specific. Lactulose-enriched MCP syrup (3-4%) was used preferentially by LA-5 rather than LC-01 and BB-12 both in modified MRS broth and in RSM. Of the three strains, only LC-01 showed improved viability in RSM

and media broth supplemented with 3-4% JAIS and JAIP. Higher counts of LA-5 were observed in RSM containing JAIS but not in basal media containing JAIP, and *vise versa* for BB-12. Studies in broth media also showed the relationship between the DP of inulin powders and their fermentability. In agreement with findings by Roberfroid *et al.* (1998), Kaplan and Hutkins (2000), and Aryana *et al.* (2007), probiotic bacteria preferred to grow on short-chain inulin (oligofructose) and most of them failed to grow on longer inulin chains. The ability of JAIP in improving the growth of probiotic bacteria was comparable to Raftiline® GR, but not as efficient as Raftilose® P95.

A question that needed further investigation was whether the incorporation of JAIP into cultured dairy product could improve the survival of probiotic bacteria during prolonged cold storage. Consequently, experimental yoghurt was made with 12% RSM and supplemented with 4% JAIP and inoculated with mixed cultures (1:1, w/w) of LC-01 and traditional yoghurt starters (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) overnight at 37°C. The use of yoghurt cultures was necessary to achieve fast development of acidity and yoghurt with desirable rheological characteristics (Tamime *et al.* 2005). Two yoghurts supplemented with Raftiline® GR and Raftilose® P95 as reference and non-supplemented yoghurt as control were also prepared. The survival and acidifying activity of micro-organisms of all yoghurts were compared during the fermentation and weekly during shelf life of 28 days at 4°C. All inulin powders tested led to a significant improvement in the initial growth ability of LC-01 and provided a longer-lasting viability of LC-01 than non-supplemented yoghurt, maintaining high numbers throughout storage time above the recommended therapeutic levels of 10^6 CFU g^{-1} (Kurmann and Rasic 1991). The most powerful inulin powder for retaining the viability of LC-01 was Raftiline® GR whereas the JAIP was equally effective as Raftilose® P95. None of the inulin-supplemented yoghurts retained higher numbers of ST and LB

during storage period. The post-acidification was marginal in all yoghurts, dropping from 4.2 to 4.5 by *ca*. 0.3 pH units at the end of storage time.

Besides inulin's uses in different foods as dietary fibre (3-6 g per serving) and as a prebiotic ingredient (3-8 g per serving), it also has an important use as fat replacer (2-6 g per serving) (Coussement 1999; Devereux *et al*. 2003). Considering that probiotic growth-sustaining ability of JAIP was close to Raftiline® GR in modified broth, it may also possess similar fat-replacing properties in food systems. In an effort to test the ability of JAIP to mimic fat in non-fat yoghurts, the powder was incorporated at 4% level into 12% RSM. Three batches of yoghurt supplemented with 4% of three commercial chicory inulins to final solids content of 16% were used as reference. Two non-inulin-supplemented yoghurts (16% total solids) prepared from reconstituted WMP and LHSMP were used as controls. The reconstituted milk was employed for yoghurt production rather than fresh milk in order to minimise the seasonal variations in milk composition in particular its protein content during the study. Rheological properties of experimental and control yoghurts were investigated during shelf life of 28 days at 4°C using large deformation test which gives a good evaluation of the firmness of yoghurt, and small deformation tests which relate to flow and viscoelastic behaviours of the product.

The JAIP-supplemented yoghurt behaved as a non-Newtonian fluid with pseudoplastic characteristic and yield stress appearance, and exhibited weak viscoelastic property. The supplementation of JAIP reduced the magnitude of firmness, apparent viscosity, hysteresis loop area and viscoelastic moduli in comparison with non-inulin-supplemented non-fat yoghurt, possibly due to proportionally lower protein content as the supplementation level increased, and interference of inulin molecules with protein

matrix formation. In comparison with full-fat control yoghurt containing similar total solids and protein contents, JAIP reduced the firmness of non-fat yoghurt to similar extent as that of full-fat yoghurt. However, on the basis of the results for small deformation test JAIP addition caused significantly lower yield stress and storage and loss moduli, indicating a weaker network and/or lower numbers of inter- and intra-strand bonds within gel systems. In comparison with commercial chicory inulins, JAIP was not as efficient as Raftiline® HP that produced optimum rheological characteristics close to that of full-fat yoghurt. The rheological effects of JAIP addition were found to be comparable to those of Raftilose® P95 and Raftiline® GR which contained smaller amounts of long-chain inulin molecules and lower water-holding capacity.

There are a number of criteria that should receive more attention in future study as listed here:

1. To improve the potential of JAI and lactulose-enriched MCP for commercial production and successful application in foods, the removal of by-products (e.g. glucose, fructose and sucrose contents in JAI extract, and galactose and acids in lactulose-enriched MCP) and impurities (e.g. ash in JAIP) using chromatographic ion-exchangers is recommended.

2. Both types of shell need to be treated appropriately before using in food applications since oyster shells may contain high levels of heavy metals i.e. lead, mercury and cadmium, while egg shells may be contaminated with pathogens e.g. Salmonella. For heavy metals, it is possible to remove from the reaction mixture at the completion of isomerisation by ion-exchange method as this procedure is commonly used for water and waste water treatment (Dabrowski *et al*. 2004).

3. The information on the MW distribution of inulins in JA tubers and its extract should be examined using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Van Loo *et al*. 1995; Chiavaro *et al*. 2007). By applying this technique, more information on (i) the effects of extraction and clarification processes on the quality of inulin (ii) the effects of long-term cold storage and late harvest of tuber on the degradation of long-chain inulins, and (iii) the fermentation behaviour of JAI can be obtained.

4. A novel technology e.g. ultrafiltration should be combined for clarification of inulin extract instead of treatments with lime-carbonation and activated carbon that are laborious and time-consuming. To improve inulin extraction yield, hydrothermal extraction should be repeated several times to extract maximum CHO from the pulp. The use of enzymes and ultrasound could also be pursued as a method for facilitating the releasing of inulin.

5. Palatability is one of the most important attributes that consumers look for in food selection. Future research should examine (i) whether the supplementation with JAIP has any adverse influence on sensory characteristics and acceptability of yoghurt (ii) the interrelationship between rheological characteristics and sensory attributes of yoghurt. In addition, because hydrocolloids have been widely used to modify the rheological properties of fermented dairy products (Silva 1996), it would be worthwhile to investigate the (synergistic or antagonistic) effect of other hydrocolloids addition on viscosity and mouthfeel of inulin-supplemented yoghurt.

6. The microstructure of inulin-supplemented yoghurts should be studied through scanning electron microscopy (Hassan 2003) or confocal laser scanning microscopy techniques (Lucey *et al*. 1998) to elucidate the distribution of inulin particles in gel

network and the interactions between inulin and whey proteins and/or casein micelles. The rheological behaviours (e.g. gelling point) of inulin-supplemented yoghurts during incubation should also be monitored to gain better understanding of the role of inulin on yoghurt gel formation.

7. The responses of probiotic bacteria to prebiotic substrates can be viewed not only in terms of their growth rate and acid production but also in terms of their ability to produce extracellular enzymes that are able to hydrolyse prebiotics (Rossi *et al.* 2005). Therefore, evaluating the enzymic potential of the selected probiotic bacteria for fermenting JAI or lactulose-enriched MCP syrup is recommended.

In closing, with increasing health consciousness consumers and awareness about functional foods, the future market for products containing prebiotics seems to be promising. The incorporation of prebiotic compounds into probiotic fermented milk is an opportunity to improve the quality of food by delivering extra nutritional and physical values and could possibly enable the development of synbiotic combinations with improved survivability in the GIT. The work reported in this thesis provides perspectives on more extensive use of JA tubers and MCP for prebiotic production which may turn interest the local agro-industry into developing value-added products from these materials.

References

Note In this thesis the referencing style adopted has followed the recommendations found in the Journal of Food Science (see IFT 2008 below).

Aider M, de Halleux D. 2007. Isomerization of lactose and lactulose production: review. Trends Food Sci Tech 18(7):356-364.

Akalin AS, Fenderya S, Akbulut N. 2004. Viability and activity of bifidobacteria in yoghurt containing fructooligosaccharide during refrigerated storage. Int J Food Sci Tech 39:613-621.

Akalin AS, Gonc S, Unal G, Fenderya S. 2007. Effects of fructooligosaccharide and whey protein concentrate on the viability of starter culture in reduced-fat probiotic yoghurt during storage. J Food Sci 72:M222-M226.

Akin MB, Akin MS, Kirmaci Z. 2007. Effects of inulin and sugar levels on viability of yoghurt and probiotic bacteria and the physical and sensory characteristics in probiotic ice-cream. Food Chem 104:93-99.

Amatayakul T, Halmos AL, Sherkat F, Shah NP. 2006a. Physical characteristics of yoghurts made using exopolysaccharide-producing starter cultures and varying casein to whey protein ratios. Int Dairy J 16:40-51.

Amatayakul T, Sherkat F, Shah NP. 2006b. Physical characteristics in set yoghurt made at 9% (w/w) and 14% (w/w) total solids with varying casein to whey protein ratios and types of EPS-producing starter cultures. Food Hydrocol 20:314-324.

Andrews G. 1986. Formation and occurrence of lactulose in heated milk. J Dairy Res 53:665-680.

Andrews G, Prasad K. 1987. Effect of the protein, citrate and phosphate content of milk on formation of lactulose during heat treatment. J Dairy Res 54:207-218.

Angus F, Smart S, Shortt C. 2005. Prebiotic ingredients with emphasis on galacto-oligosaccharides and fructo-oligosaccharides. In: Tamime AY, editor. Probiotic dairy products. Oxford: Blackwell Publishing. p 120-137.

Anon. 2003. Probiotics or prebiotics? potential in the Australian market. Available from: www.nutraingredients.com/news/ng.asp?id=37521. Accessed May 1, 2008.

Anon. 2007. Australian yoghurt market growing at 12%, driven by shift toward healthier food. Available from: www. goliath.ecnext.com/coms2/gi_0199-6775053/ Australian-yoghurt-market-growing-at.html. Accessed July 4, 2008.

References

ANZFA. 2003. Fermented Milk Products. Standard 2.5.3. Available from: www.foodstandards.gov.au. Accessed July 4, 2008.

AOAC. 2000a. Official methods of analysis. Method 990.20. Determination of solids by direct forced air oven drying method. 17th ed. Washington DC:AOAC.

AOAC. 2000b. Official methods of analysis. Method 945.46. Determination of ash by gravimetric method. 17th ed. Washington DC:AOAC.

AOAC. 2000c. Official methods of analysis. Method 947.05. Determination of acidity by titrimetric method. 17th ed. Washington DC:AOAC.

Aryana KJ, McGrew P. 2007. Quality attributes of yoghurt with *Lactobacillus casei* and various prebiotics. LWT 40:1808-1814.

Aryana KJ, Plauche S, Rao RM, McGrew P, Shah NP. 2007. Fat-free plain yoghurt manufactured with inulins of various chain lengths and *Lactobacillus acidophilus*. J Food Sci 72:M79-M84.

Baldini M, Danuso F, Turi M, Vannozzi GP. 2004. Evaluation of new clones of Jerusalem artichoke (*Helianthus tuberosus* L.) for inulin and sugar yield from stalks and tubers. Indust Crop Prod 19:25-40.

Ballongue J. 1998. Bifidobacteria and probiotic action. In: Salminen S, Wright AV, editors. Lactic acid bacteria: Microbiology and functional aspects. 2nd ed. New York: Marcel Dekker. p 519-587.

Becker T, Puhan Z. 1989. Effect of different processes to increase the milk solids non-fat content on the rheological properties of yoghurt. Milchwissenschaft 44:626-629.

Benezech T, Maingonnat JF. 1994. Characterization of the rheological properties of yoghurt. J Food Eng 21:447-472.

Berg H, van Boekel M. 1994. Degradation of lactose during heating of milk. I. Reaction pathways. Neth Milk Dairy J 48:157-175.

Berghofer E, Cramer A, Schmidt U, Veigl M. 1993. Pilot-scale production of inulin from chicory roots and its use in foodstuff. In: Fuchs A, editor. Inulin and inulin-containing crops. Amsterdam: Elsevier Science. p 77-84.

Biedrzycka E, Bielecka M. 2004. Prebiotic effectiveness of fructans of different degrees of polymerization. Trends Food Sci Tech 15:170-175.

Bielecka M, Biedrzycka E, Majowsa A. 2002. Selection of probiotics and prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. Food Res Int 35:125-131.

Bishay IE. 1998. Rheological characterization of inulin. In: Williams PA, Phillips GO, editors. Gums and stabilisers for the food industry. Cambridge, UK: Royal Society of Chemistry. p 201-210.

Bouhnik Y, Flourie B, Riottot M, Bisetti N, Gailing M, Guibert A, Bornet F, Rambaud J. 1996. Effects of fructooligosaccharides ingestion on faecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. Nutr Cancer 26:21-9.

Bourne MC. 2002. Food texture and viscosity: Concept and measurement. 2nd ed. New York: Academic Press. 427 p.

Bozanic R, Rogelj I, Tratnik L. 2002. Fermentation and storage of probiotic yoghurt from goat milk. Mijekarstvo 52(2):93-111.

Braun DB, Rosen MR. 1999. Rheology modifiers handbook: Practical use and application. New York: William Andrew Publishing. 505 p.

Brian B. 1992. Practical HPLC methodology and applications. New York: John Wiley & Son. 452 p.

Bridson EY. 1995. The Oxoid Manual. 7th ed. Basingstoke: Unipath Ltd.

Bruno FA, Lankaputhra WEV, Shah NP. 2002. Growth, viability and activity of *Bifidobacterium* spp. in milk containing prebiotics. J Food Sci 67:2740-2744.

Capela P, Hay TKC, Shah NP. 2006. Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. Food Res Int 39:203-211.

Carobbi R, Bimbi G, Cipolletti G, inventors; Inalco Spa, assignee. 2001 Oct 04. Process for the preparation of a lactulose syrup by lactose isomerisation. U.S. patent 6,214,124.

Carobbi R, Innocenti F, inventors; Sirac Srl, assignee. 1990 Sep 18. Process for preparing lactulose from lactose by epimerization with sodium aluminate. U.S. patent 4,957,564.

Carobbi R, Miletti S, Franci Vittorio, inventors; Sirac Spa, assignee. 1985 Aug 20. Process for preparing lactulose from lactose, in the form of a syrup or a crystalline product. U.S. patent 4,536,221.

Carubelli R. 1966. Transformation of disaccharides during borate ion exchange chromatography: isomerisation of lactose into lactulose. Carbohyd Res 2:480-485.

Cashman K. 2002. Prebiotics and calcium bioavailability. In: Tannock GW, editor. Probiotics and prebiotics: where are we going?. Wymondham: Caister Academic Press. p 149-174.

References

Champagne CP, Roy D, Garner N. 2005. Challenges in the addition of probiotic cultures to foods. Crit Rev Food Sci Nutr 45(1):61-84.

Chandan RC, O'Rell KR. 2006. Principle of yoghurt processing. In: Chandan RC, White CH, Kilara A, Hui YH, editors. Manufacturing yoghurt and fermented milks. Oxford: Blackwell Publishing Ltd. p 195-209.

Chiavaro E, Vittadini E, Corradini C. 2007. Physicochemical characterization and stability of inulin gels. Eur Food Res Tech 225:85-94.

Chr. Hansen. 2004. Product information: FD-DVS YC-380-Yoflex®. Available from: http://www.chr-hansen.com. Accessed June 27, 2008.

Christman S. 2003. *Helianthus tuberosus*. Available from: http://www.floridata.com/ ref/h/heli_tub.cfm. Accessed March 14, 2005.

Claeys WL, van Loey AM, Hendrickx ME. 2002. Intrinsic time temperature integrators for heat treatment of milk. Trends Food Sci Tech 13:293-311.

Coussement PAA. 1999. Inulin and oligofructose: safe intakes and legal status. J Nutr 129:1412S-1417S.

Crittenden RG. 1999. Prebiotics. In: Tannock GW, editor. Probiotics: a critical review. Wymondham: Horizon Scientific Press. p 141-156.

Crittenden RG, Morris LF, Harvey ML, Tran LT, Mitchell HL, Playne MJ. 2001. Selection of a *Bifidobacterium* strain to complement resistant starch in a synbiotic yoghurt. J Applied Microb 90:268-278.

Crittenden RG, Playne MJ. 1996. Production, properties and applications of food-grade oligosaccharides. Trends Food Sci Tech 7:353-361.

Cummings JH, Macfarlane GT, Englyst HN. 2001. Prebiotic digestion and fermentation. Am J Clin Nutr 73:415S-420S.

Dabrowski A, Hubicki Z, Podkoscielny P, Robens E. 2004. Selective removal of the heavy metal ions from waters and industrial wastewaters by ion-exchange method. Chemosphere 56:91-106.

Dairy Australia. 2007. Australia dairy industry in focus 2007. Available from: www.dairyaustralia.com.au. Accessed May 1, 2008.

Dave RI, Shah NP. 1997a. Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. Int Dairy J 7:31-41.

Dave RI, Shah NP. 1997b. Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures. Int Dairy J 7:435-443.

Dave RI, Shah NP. 1998. Ingredients supplementation effects on viability of probiotic bacteria in yogurt. J Dairy Sci 81:2804-2816.

De Faveri D, Torre P, Perego P, Converti A. 2004. Statistical investigation on the effects of starting xylose concentration and oxygen mass flow rate on xylitol production from rice straw hydrolyzate by response surface methodology. J Food Eng 65(3):383-389.

de Haar WT, Pluim H, inventors; Duphar International Research, assignee. 1991 Jun 25. Method of preparing lactulose. U.S. patent 5,026,430.

de Kruif CG, Tuinier R. 2001. Polysaccharide protein interactions. Food Hydrocol 15:555-563.

de la Fuente M, Juárez D, de Rafael, Villamiel M, Olano A. 1999. Isomerization of lactose catalysed by alkaline-substituted sepiolite. Food Chem 64:1-6.

De Leenheer L, Hoebergs H. 1994. Progress in the elucidation of the composition of chicory inulin. Starch 46:193-196.

Dello Staffolo M, Bertola N, Martino M, Bevilacqua A. 2004. Influence of dietary fibre addition on sensory and rheological properties of yoghurt. Int Dairy J 14:263-268.

Delzenne NM. 2003. Oligosaccharides: state of the art. P Nutr Soc 62:177-182.

Delzenne NM, Williams CM. 2002. Prebiotics and lipid metabolism. Curr Opin Lipidol 13(1):61-67.

Dendene K, Guihard L, Nicolas S, Bariou B. 1994. Kinetics of lactose isomerisation to lactulose in alkaline-medium. J Chem Tech Biotech 61:37-42.

Desai AR, Powell IB, Shah NP. 2004. Survival and activity of probiotic lactobacilli in skim milk containing prebiotics. J Food Sci 69(3):FMS57-60.

Devereux HM, Jones GP, McCormack L, Hunter WC. 2003. Consumer acceptability of low fat foods containing inulin and oligofructose. J Food Sci 68:1850-1854.

De Vries W, Stouthamer AH. 1969. Factors determining the degree of anaerobiosis of *Bifidobacterium* strains. Archiv Microb 65:275-278.

Doleyres Y, Lacroix C. 2005. Technological with free and immobilised cells for probiotic bifidobacteria production and protection. Int Dairy J 15:973-988.

Donkor ON, Nilmini SLI, Stolic P, Vasiljevic T, Shah NP. 2007. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. Int Dairy J 17:657-665.

References

Dreher M. 1999. Food sources and uses of dietary fibre. In: Cho S, editor. Complex carbohydrates in food. New York: Marcel Dekker. p 385-394.

Dykalo NJ, Kim VV, Semenov EA, Evdokimov IA, Serov AV, Kisele NA, inventors. 1999 Aug 23. Method of the production of the concentrate of lactulose. Russian patent 2,133,778.

Elli M, Zink R, Reniero R, Morelli, L. 1999. Growth requirements of *Lactobacillus johnsonii* in skim and UHT Milk. Int Dairy J 9:507-513.

El-Nagar G, Clowes G, Tudorica CM, Kuri V, Brennan CS. 2002. Rheological quality and stability of yog-ice cream with added inulin. Int J Dairy Tech 55:89-93.

Flamm G, Glinsmann W, Kritchevsky D, Prosky L, Roberfroid M. 2001. Inulin and oligofructose as dietary fiber: A review of the evidence. Crit Rev Food Sci Nutr 41:353-362.

Fleming SE, GrootWassink JWD. 1979. Preparation of high-fructose syrup from the tubers of the Jerusalem artichoke. CRC Crit Rev Food Sci Nutr 12:1-28.

Fonden R, Saarela M, Matti J, Mattila-Sandholm T. 2003. Lactic acid bacteria in functional dairy products. In: Mattila-Sandholm T, Saarela M, editors. Functional dairy products. Cambridge: Woodhead Publishing Ltd. p 244-262.

Franck AME. 2000. Inulin and oligofructose. In: Gibson G, Angus F, editors. LFRA ingredient handbook: Prebiotics and probiotics. Surrey: Leatherhead Publishing. p 1-18.

Franck AME, De Leenheer L. 2005. Inulin. In: Steinbuchel A, Rhee SK, editors. Polysaccharides and polyamides in the food industry: Properties, production, and patents. Weinheim: Wiley-VCH. p 281-322.

Frese L. 1993. Production and utilization of inulin part I - Cultivation and breeding of fructan-producing crops. In Suzuki M, Chatterton NJ, editors. Science and technology of fructans. Florida: CRC Press, Inc. p 303-317.

Fuller R. 1989. Probiotics in man and animals. J Appl Bacteriol 66:365-378.

Fumihiko M, inventor; Japan Organo Co. Ltd., assignee. 1990 May 14. Separation of lactulose. Japanese patent 02124895.

Gasparotti FA, inventor; Molteni & C, assignee. 1981 Apr 28. Process for producing lactulose. U.S. patent 4,264,763.

Gibson GR. 2004. Fibre and effects on probiotics (the prebiotic concept). Clin Nutr Suppl 1(2):25-31.

Gibson GR, Beatty ER, Wang X, Cummings JH. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroentero 108:975-982.

Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401-1412.

Gibson GR, Wang X. 1994. Bifidogenic properties of different types of fructo-oligosaccharides. Food Microb 11:491-498.

Greig BD, Payne GA. 1985. Epimerization of lactose to free lactulose in heated model milk solutions. J Dairy Res 52:409-417.

Guggisberg D, Eberhard P, Albrecht B. 2007. Rheological characterization of set yoghurt produced with additives of native whey proteins. Int Dairy J 17:1353-1355.

Guven M, Yasar K, Karaca O, Hayaloglu AA. 2005. The effect of inulin as a fat replacer on the quality of set-type low-fat yoghurt manufacture. Int J Dairy Tech 58:180-184.

Hamilton-Miller JMT. 2005. Probiotics and prebiotics in the elderly. Postgrad Med J 80:447-451.

Hammes WP, Vogel RF. 1995. The genus *Lactobacillus*. In: Wood BJB, Holzapfel WH, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional. p 19-49.

Hansen OC, Madsen RF, inventors; Danisco A/S, assignee. 1992 July 7. Method for preparing a mixture of saccharides. U.S. patent 5,127,956.

Harju M. 2001. Milk sugars and minerals as ingredients. Int J Dairy Technol 54(2):61-63.

Hassan AN, Ipsen R, Janzen T, Qvist KB. 2003. Microstructure and rheology of yoghurt made with cultures differing only in their ability to produce exopolysaccharides. J Dairy Sci 86:1632-1638.

Havenaar R, Ten Brink BT, Huis In't Veld JHJ. 1992. Selection of strains for probiotic use. In: Fuller R, editor. Probiotics: The scientific basis. London: Chapman & Hall. p 209-224.

Hicks KB, Parrish FW. 1980. A new method for preparation of lactulose from lactose. Carbohyd Res 82:393-397.

Hicks KB, Raupp D, Smith W. 1984. Preparations and purification of lactulose from sweet cheese whey ultrafiltrate. J Agric Food Chem 32:288-292.

Hidaka H, Eida T, Takizawa T, Tokunaga T, Tashiro Y. 1986. Effects of fructooligosaccharides on intestinal flora and human health. Bifdobact Microflora 5(1):37-50.

Hinrichs J. 2001. Incorporation of whey proteins in cheese. Int Dairy J 11:495-503.

References

Holcomb JE, Frank JF. 1991. Viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in soft serve frozen yoghurt. Cult Dairy Prod J 26:4-5.

Holsinger VH. 1997. Physical and chemical properties of lactose. In: Fox PE, editor. Advanced dairy chemistry 3: Lactose, water, salts and vitamins. London: Chapman and Hall. p 1-31.

Holsinger VH. 1999. Lactose. In: Wong NP, Jenness R, Keeney M, Marth EH, editors. Fundamentals of dairy chemistry. 3rd ed. Maryland: Aspen Publishers Inc. p 279-342.

Holzapfel WH, Schillinger U. 2002. Introduction to pre- and probiotics. Food Res Int 35(2-3):109-116.

Hopkins MJ, Cummings JH, Macfarlane GT. 1998. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultures on oligosaccharides and other simple carbohydrate sources. J Applied Microb 85:381-386.

Hornby AS, Ashby M, Wehmeier S, McIntosh C. Turnbull J. 2005. Oxford advanced learner's dictionary of current English. 7th ed. Oxford: Oxford University Press. 945 p.

Hough L, Jones JKN, Richards EL. 1953. The reaction of amino compounds with sugars. II – The action of ammonia on glucose, maltose and lactose. J Chem Soc 2005-2009.

Hughes DB, Hoover DG. 1991. Bifidobacteria: their potential for use in American dairy products. Food Technol 45:74, 76, 78-80, 83.

Hull RR, Roberts AV, Mayes JJ. 1984. Survival of *Lactobacillus acidophilus* in yoghurt. Aust J Dairy Technol 39:164-166.

Hutchings JB. 1999. Food colour and appearance. Great Britain: Blackie Academic and Professional. 610 p.

Institute of Food Technologists. 2008. Complete style guide for IFT scientific journals. Available from: http://members.ift.org/IFT/Pubs/JournalofFoodSci/jfsauthorinfo/ jfsstyle.htm. Accessed May 4, 2008.

Jaros D, Haque A, Kneifel W, Rohm H. 2002. Influence of the starter culture on the relationship between dry matter content and physical properties of set-style yogurt. Milchwissenschaft 57:325-326.

Kailasapathy K, Supriadi D. 1996. Effect of whey protein concentrate on the survival of *Lactobacillus acidophilus* in lactose hydrolysed yoghurt during refrigerated storage. Milchwissenschaft 51:565-569.

Kalantzopoulos G. 1997. Fermented products with probiotic qualities. Anerobe 3:185-190.

Kaplan H, Hutkins RW. 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl Environ Microb 66:2682-2684.

Kaur N, Gupta AK. 2002. Applications of inulin and oligofructose in health and nutrition. J Biosci 27(7):703-714.

Kiebling G, Schneider J, Jahreis G. 2002. Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol. Eur J Clin Nutr 56:843-849.

Kim Y, Faqih MN, Wang SS. 2001. Factors affecting gel formation of inulin. Carbohyd Polymer 46:135-145.

Kip P, Meyer D, Jellema RH. 2006. Inulins improve sensoric and texture properties of low-fat yoghurts. Int Dairy J 16:1098-1103.

Klaver FAM, Kingma F, Weerkamp AH. 1993. Growth and survival of bifidobacteria in milk. Neth Milk Dairy J 47:151-164.

Kneifel W, Rajal A, Kulbe KD. 2000. *In vitro* growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic importance. Microb Ecol Health Dis 12:27-34.

Koca N, Metin M. 2004. Textural, melting and sensory properties of low-fat fresh kashar cheeses produced by using fat replacers. Int Dairy J 13:631-641.

Kolida S, Tuohy K, Gibson GR. 2002. Prebiotic effects of inulin and oligofructose. Brit J Nutr 87(Suppl.2):S193-S197.

Kontula P, Suihko ML, Von Wright A, Mattila-Sandholm T. 1998. The effect of lactose derivatives on intestinal lactic acid bacteria. J Dairy Sci 82(2):249-256.

Kozempel M, Kurantz M. 1994. The isomerisation kinetics of lactose to lactulose in the presence of borate. J Chem Tech Biotech 54:25-29.

Krumbholz RE, Dorscheid MG, inventors; Duphar International Research, assignee. 1991 Oct 12. Method of manufacturing lactulose. U.S. patent 5,071,530.

Ku Y, Jansen O, Oles CJ, Laza EZ, Rader JI. 2003. Precipitation of inulins and oligofructose by ethanol and other solvents. Food Chem 81:125-132.

Kunz M, Munir M, Vogel M, inventors; Sudzucker AG, assignee. 1995 Dec 26. Process for the preparation of long-chain inulin with inulinase. U.S. patent 5,478,732.

Kurman JA, Rasic JL. 1991. The health potential of products containing bifidobacteria. In: Robinson RK, editor. Therapeutic properties of fermented milks. London: Elsevier Applied Food Sciences. p 117-158.

Lankaputhra WEV, Shah NP. 1997. Improving viability of *Lactobacillus acidophilus* and bifidobacteria in yoghurt using two step fermentation and neutralises mix. Food Aust 49:363-366.

References

Lankaputhra WEV, Shah NP, Britz ML. 1996. Survival of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide. Milchwissenschaft 51:65-70.

Lapasin R, Pricl S. 1995. Rheology of industrial polysaccharides: Theory and application. Glasgow: Blackie Academic and Processional. 620 p.

Laroia S, Martin JH. 1991. Effect of pH on survival of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in frozen dairy desserts. Cult Dairy Prod J 26(4):13-21.

Laurenzo KS, Navia JL, Neiditch DS, inventors; McNeil-PPC Inc., assignee. 1999 Oct 19. Preparation of inulin products. U.S. patent 5,968,365.

Lee YK, Salminen S. 1995. The coming of age of probiotics. Trends Food Sci Tech 6:241-245.

Leite Toneli JTC, Murr FEX, Martinelli P, Fabbro IMD, Park KJ. 2007. Optimization of physical concentration process for inulin. J Food Eng 80:832-838.

Lingyun W, Jianhua W, Xiaodong Z, Da T, Yalin Y, Chenggang C, Tianhua F, Fan Z. 2007. Studies on the extracting technical conditions of inulin from Jerusalem artichoke tubers. J Food Eng 79:1087-1093.

Livesey G. 2003. Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties. Nutr Res Rev 16:163-191.

Lopez-Molina D, Navarro-Martinez MD, Rojas-Melgarejo F, Hiner ANP, Chazarra S, Rodriguez-Lopez JN. 2005. Molecular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.). Photochem 66(12):1476-1484.

Lourens-Hattingh A, Viljoen BC. 2001. Yoghurt as probiotic carrier food. Int Dairy J 11:1-17.

Lucey JA, Tamehana M, Singh H, Munro PA. 1998. Effect of interactions between denatured whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. J Dairy Res 65:55-567.

Macfarlane GT, Cummings JH. 1999. Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health?. Brit Med J 318:999-1003.

Mahran GA, Haggag H, Mahfouz MB, Zaghloul AH, Abd El-Salam MH. 1995. Effect of isomerization conditions on the formation of lactulose from UF milk and whey permeates. Egypt J Dairy Sci 23:197-204.

Makras L, Van Acker G, De Vuyst L. 2005. *Lactobacillus casei* subsp. *casei* 8700:2 degrades inulin-type fructans exhibiting different degrees of polymerisation. Appl Environ Microb 71:6531-6537.

Manfred V, inventor; Sudzucker AG Mannheim, assignee. 1992 Aug 03. Production of inulooligosaccharide product low in glucose, fructose and sucrose contents. Japanese patent 04211388.

Manning TS, Gibson GR. 2004. Prebiotics. Best Pract Res Cl Ga 18(2):287-298.

Marchand JF, inventor; Marchand JF, Assignee. 1951 Jun 05. Method for the preparation of inulin. U.S. patent 2,555,356.

Marconi E, Messiaa MC, Amineb A, Mosconec D, Vernazzad F, Stocchid F, Palleschic G. 2004. Heat-treated milk differentiation by a sensitive lactulose assay. Food Chem 84(3):447-450.

Martinez-Castro I, Olano A, Corzo N. 1986. Modifications and interactions of lactose with mineral components of milk during heating processes. Food Chem 21:211-221.

McCleary BV, Blakeney AB. 1999. Measurement of inulin and oligofructan. Cereal Foods World 44(6):398-406.

Medina L, Jordano R. 1994. Survival of constitutive microflora in commercially fermented milk containing bifidobacteria during refrigerated storage. J Food Prot 56:731-733.

Menne E, Guggenbuh N, Roberfroid M. 2000. Fn-type chicory inulin hydrolysate has a prebiotic effect in humans. J Nutr 130:1197-1199.

Mitchell C, Mitchell P, inventors; California Natural Products, assignee. 1995 Jun 6. Instant dried dahlia inulin juice and its method of production and usage. U.S. patent 5,422,346.

Mizota T, Tamura Y, Tomita M, Okongi S. 1987. Lactulose as a sugar with physiological significance. Bulletin IDF 212:69-76.

Moerman FT, Van Leeuwen MB, Delcour JA. 2004. Enrichment of higher molecular weight fractions in inulin. J Agric Food Chem 52:3780-3783.

Montgomery DC. 1996. Design and analysis of experiments. New York: John Wiley & Sons. 418 p.

Montgomery EM, Hudson CS. 1930. Relation between rotary power and structure in the sugar group. XXVII. Synthesis of a new disaccharide ketose (lactulose) from lactose. J Am Chem Soc 52:2101-2106.

Montilla A, del Castillo MD, Sanz ML, Olano A. 2005. Egg shell as catalyst of lactose isomerisation to lactulose. Food Chem 90(4):883-890.

Moreno FJ, Villamiel M, Olano A. 2003. Effect of high pressure on isomerisation and degradation of lactose in alkaline media. J Agric Food Chem 51:1894-1896.

References

Mullin WJ, Modler HW, Farnworth ER, Payne A. 1994. The macronutrient content of fractions from Jerusalem artichoke tubers (*Helianthus tuberosus*). Food Chem 51:263-269.

Murphy O. 2001. Non-polyol low-digestible carbohydrates: food applications and functional benefits. Brit J Nutr 85(Suppl.1):47-53.

Myers R. 1976. Response surface methodology. Boston: Allyn and Bacon. 246 p.

Nagasawa T, Tomita M, Tamura Y, Obayashi T, Mizota T, inventors; Morinaga milk industry Co. Ltd., assignee. 1974 Jun 11. Process for preparing a lactulose syrup. U.S. patent 3,816,174.

Nakakuki T. 2002. Present status and future of functional oligosaccharide development in Japan. Pure Appl Chem 74(7):1245-1251.

Naughton PJ, Mikkelsen LL, Jensen BB. 2001. Effects of non-digestible oligosaccharides on *Salmonella enterica* serovar typhimurium and non-pathogenic *Escherichia coli* in the pig small intestine *in vitro*. Appl Environ Microb 37(8):3391-3395.

Niness KR. 1999. Nutritional and health benefits of inulin and oligofructose. J Nutr 129:1402S-1406S.

O'Brien J. 1997. Reaction chemistry of lactose: non-enzymatic degradation pathways and their significance in dairy products. In Fox P, editor. Advanced dairy chemistry, Vol. 3: Lactose, water, salts and vitamins. London: Chapman & Hall. p 155-231.

Oku T, Nakamura S. 2002. Digestion, absorption, fermentation, and metabolism of functional sugar substitutes and their available energy. Pure Appl Chem 74(7): 1253-1261.

Olano A, Calvo M, Corzo N. 1989. Changes in the carbohydrate fraction of milk during heating processes. Food Chem 31:259-265.

Olano A, Corzo N, Paez MI, Martinez-Castro I. 1987. Isomerization of lactose during heat treatment of liquid and freeze-dried simulated milk ultrafiltrates. Effect of pH and calcium. Milchwissenschaft 42:628-630.

Oliveira MN, Sodini I, Remeuf F, Corrieu G. 2001. Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. Int Dairy J 11:935-942.

O'May GA, Macfarlance GT. 2005. Health claims associated with probiotics. In: Tamime AY, editor. Probiotic dairy products. Oxford: Blackwell Publishing. p 138-166.

O'Neill J. 2008. The lifelong benefits of inulin and oligofructose. Cereal Foods World 53(2):65-68.

O'Sullivan DJ. 2001. Screening of intestinal microflora for effective probiotic bacteria. J Agri Food Chem 49:1755-1760.

Ozer D, Akin S, Ozar B. 2005. Effect of inulin and lactulose on survival of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB-02 in acidophilus yoghurt. Food Sci Tech Int 11:19-24.

Parameswaran M. 1994. Jerusalem artichoke: Turning an unloved vegetable into an industrial crop. Food Aust 46(10):473-475.

Parameswaran M. 1996. Urban waste water use in plant biomass production. Resources Conserv Recyc 27:39-56.

Parrish FW, inventor; Army US, assignee. 1970 May 26. Isomerization of glucose, maltose and lactose with amino compounds. U.S. patent 3,514,327.

Parrish FW, Tayler FB, Ross KD, Clark J, Phillips JG. 1979. Sweetness of lactulose relative to sucrose. J Food Sci 44:813-815.

Pearson D. 1976. The chemical analysis of foods. London: Churchill Livingstone. 575 p.

Pollock CJ, Cairns AJ. 1991. Fructan metabolism in grasses and cereals. Annu Rev Plant Physio Plant Mol Biol 42:77-101.

Praznik W, Beck RHF. 1987. Inulin composition during growth of tubers of *Helianthus tuberosus*. Agric Biol Chem 51(6):1593-1599.

Prosky L. 1999. Inulin and oligofructose are part of the dietary fiber complex. J AOAC 82:223-226.

Purwandari U, Shah NP, Vasiljevic T. 2007. Effects of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological and rheological properties of set-type yoghurt. Int Dairy J 17:1344-1352.

Radke-Mitchell L, Sandine E. 1984. Associative growth and differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*: A review. J Food Prot 47:245-248.

Rakhimov DA, Arifkhodzhaev AO, Mezhlumyan LG, Yuldashev OM, Rozikova UA, Aikhodzhaeva N, Vakil MM. 2003. Carbohydrates and proteins from *Helianthus tuberosus*. Chem Nat Com 39(3):312-313.

Rao VA. 2001. The prebiotic properties of oligofructose at low intake levels. Nutr Res 21(6): 843-848.

Rastall RA. 2003. Enhancing the functionality of prebiotics and probiotics. In: Mattila-Sandholm T, Saarela M, editors. Functional dairy products. Cambridge: Woodhead Publishing Ltd. p 301-315.

Rastall RA, Gibson GR. 2004. Functional foods. Bioscience explained 2(1):1-7.

References

Rastall RA, Maitin V. 2002. Prebiotics and synbiotics: towards the next generation. Curr Opin Biotechnol 13(5):490-496.

Ravula RR, Shah NP. 1998. Selective enumeration of *Lactobacillus casei* from yogurts and fermented milk drinks. Biotechnol Tech 12(11): 819-822.

Roberfroid MB. 1993. Dietary fiber, inulin, and oligofructose: A review comparing their physiological effects. Crit Rev Food Sci Nutr 33:103-148.

Roberfroid MB. 1998. Prebiotics and synbiotics: concepts and nutritional properties. Brit J Nutr 80(Suppl. 2):S197-S202.

Roberfroid MB. 2001. Prebiotics: preferential substrates for specific germs?. Am J Clin Nutr 73(2):406S-409S.

Roberfroid MB, Delzenne NM. 1998. Dietary fructans. Ann Rev Nutr 18:117-143.

Roberfroid MB, Van Loo JAE, Gibson GR. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. J Nutr 128:11-19.

Rohm H. 1993. Influence of dry matter fortification on flow properties of yoghurt. 2. Time dependent behaviour. Milchwissenschaft 48(11):614-617.

Rohm H, Kovac A. 1994. Effects of starter cultures on linear viscoelastic and physical properties of yoghurt gels. J Texture Studies 25:311-329.

Rossi M, Corradini C, Amaretti A, Nicolini M, Pompei A, Zanoni S, Matteuzzi D. 2005. Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl Environ Microbiol 71:6150-6158.

Ruiz-Matute AI, Sanz ML, Corzo N, Martin-Alvarez PJ, Ibanez E, Martinez-Castro I, Olano A. 2007. Purification of lactulose from mixtures with lactose using pressurised liquid extraction with ethanol-water at different temperatures. J Agric Food Chem 55:3346-3350.

Rumney C, Rowland IR. 1995. Non-digestible oligosaccharides-potential anti-cancer agents?. BNF Nutr Bull 20:194-203.

Rycroft CE, Jones MR, Gibson GR, Rastall RA. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J App Microbiol 91:878-887.

Saarela M, Hallamaa K, Mattila-Sandoholm T, Matto J. 2003. The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic *Lactobacillus* strains. Int Dairy J 13:291-302.

Saarela M, Lahteenmaki L, Crittenden R, Salminen S, Mattila-Sandholm T. 2002. Gut bacteria and health foods: The European perspective. Int J Food Microb 78:99-117.

Saavedra J, Tschernia A, Moore N, Abi-Hanna A, Coletta F, Emenhiser C. 1999. Gastro-intestinal function in infants consuming a weaning food supplemented with oligofructose, a prebiotic. J Pediatr Gastr Nutr 29(4):A95.

Saengthongpinit W, Sajjaanantakul T. 2005. Influence of harvest time and storage temperature on characteristics of inulin from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers. Posth Biol Tech 37:93-100.

Salminen S. 1996. Uniqueness of probiotic strains. IDF Nutr News Lett 5:16-18.

Salminen S, Roberfroid M, Ramos P, Fonden R. 1998. Prebiotic substrates and lactic acid bacteria. In: Salminen S, Wright AV, editors. Lactic acid bacteria: microbiology and functional aspects. 2 nd ed. New York: Marcel Dekker. p 343-358.

Salvador A, Fiszman SM. 2004. Textural and sensory characteristics of whole and skimmed flavored set-type yoghurt during long storage. J Dairy Sci 87:4033-4041.

Samona A, Robinson RK. 1994. Effect of yoghurt cultures on the survival of bifidobacteria in fermented milks. J Soc Dairy Technol 47:58-60.

Saxelin M, Grenov B, Svensson U, Fonden R, Reniero R, Mattila-Sandholm T. 1999. The technology of probiotics. Trends Food Sci Tech 10:387-392.

Saxelin M, Korpela R, Mayra-Makinen A. 2003. Functional dairy products. In: Smit G, editor. Dairy processing: improving quality. Abington: Woodhead Publishing Ltd. p 229-245.

Schaafsma G. 1996. State of art concerning probiotic strains in milk products. IDF Nutr News Lett 5:23-24.

Schaafsma G, Meuling WJA, van Dokkum W, Bouley C. 1998. Effects of a milk product, fermented by *Lactobacillus acidophilus* and with fructo-oligosaccharides added, on blood lipids in male volunteers. Eur J Clin Nutr 52:436-440.

Schaller-Povolny LA, Smith DE. 2001. Viscosity and freezing point of a reduced fat ice cream mix as related to inulin content. Milchwissenschaft 56:25-29.

Schillinger U. 1999. Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. Int J Food Microbiol 47:79-87.

References

Scholz-Ahrens KE, Schaafsma G, van den Heuvel EGHM, Schrezenmeir J. 2001. Effects of prebiotics on mineral metabolism. Am J Clin Nutr 73(2):459S-464S.

Schrezenmeir J, de verse M. 2001. Probiotics, prebiotics and synbiotics: Approaching a definition. Am J Clin Nutr 73(2):361S-364S.

Schumann C. 2000. Lactulose. In: Gibson G, Angus F, editors. LFRA ingredient handbook: Prebiotics and probiotics. Surrey: Leatherhead Publishing. p 47-67.

Schumann C. 2002. Medical, nutritional and technological properties of lactulose: an update. Eur J Clin Nutr 41(Suppl.1):1/17-11/25.

Sensus Operation CV. 2000. Frutafit® inulin. In Phillips GO, Williams PA, editors. Handbook of hydrocolloids. Cambridge: Woodhead Publishing. p 397-399.

Shah NP. 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. Int Dairy J 5:515-521.

Shah NP. 1997. Bifidobacteria: Characteristics and potential for application in fermented milk products. Milchwissenschaft 52(1):16-21

Shah NP. 2000. Probiotic bacteria: selective enumeration and survival in dairy products. J Dairy Sci 83:894-907.

Shah NP. 2006. Probiotics and fermented milks. In: Chandan RC, White CH, Kilara A, Hui YH, editors. Manufacturing yogurt and fermented milks. Oxford: Blackwell Publishing Ltd. p 341-354.

Shah NP, Jelen P. 1990. Survival of lactic acid bacteria and their lactases under acidic conditions. J Food Sci 55: 506-509.

Shin HS, Lee JH, Pestka JJ, Ustunol Z. 2000. Growth and viability of commercial *Bifidobacterium* spp. in skim milk containing oligosaccharides and inulin. J Food Sci 65(5):884-887.

Silva RF. 1996. Use of inulin as a natural texture modifier. Cereal Foods World 41(10):792-795.

Silver BS, inventor. 2003 May 27. Processes for making novel inulin products. U.S. patent 6,569,488.

Smart JB, Pillidge CJ, Garman JH. 1993. Growth of lactic acid bacteria and bifidobacteria on lactose and lactose-related mono-, di- and trisaccharides and correlation with distribution of β-galactosidase and phospho-β-galactosidase. J Diary Res 60:557-568.

Smits G, Daenekindt L, Booten K, inventors; Tiense Suikerrafinaderij NV, assignee. 2001 Oct 16. Fractionated polydisperse compositions. U.S. patent 6,303,778.

Sodini I, Remeuf F, Haddad S. 2004. The relative effect of milk base, starter and process on effects of milk fortification and heating on microstructure and physical properties of stirred yoghurts. Int Dairy J 13:773-782.

Southgate DAT. 1991. Determination of food carbohydrates. 2nd ed. New York: Elsevier Science Publishers Ltd. 232 p.

SPSS Inc. 2006. SPSS for Windows, version 15.0, SPSS Inc., Chicago, IL.

Stable Micro Systems. 1995. Texture Expert for Windows, version 1.0, Stable Micro Systems, Surrey, England.

Stanton C, Gardiner G, Meehan H, Collins K, Fitzgerald G, Lynch PB, Ross RP. 2001. Market potential for probiotics. Am J Clin Nutr 73(2):476S-483S.

StatSoft Inc. 1995. STATISTICA for Windows, Version 5.1. Tulsa, OH, US.

Steffe JF. 1992. Rheological methods in food process engineering. Michigan: Freeman Press. 228 p.

Strohmaier W. 1998. Lactulose: status of health-related applications. IDF, Bulletin no. 9804:262-271.

Suarez E, Lobo A, Alvarez S, Riera FA, Alvarez R. 2006. Partial demineralization of whey and milk ultrafiltration permeate by nanofiltration at pilot-plant scale. Desalination 198:274-281.

Suskovic J, Kos B, Goreta J, Matasic S. 2001. Role of lactic acid bacteria and bifidobacteria in synbiotic effect. Food Technol Biotechnol 39(3): 227-235.

Talwalkar A, Kailasapathy K. 2004. A review of oxygen toxicity in probiotic yoghurts: Influence on the survival of probiotic bacteria and protective techniques. Comp Rev Food Sci Food Saf 3:117-124.

Tamime AY. 2006. Fermented Milks. England: Blackwell Science Ltd. 262 p.

Tamime AY, Marshall VME, Robinson RK. 1995. Microbiological and technological aspects of milks fermented by bifidobacteria. J Dairy Res 62:151-187.

Tamime AY, Saarela M, Sondergaard AK, Mistry VV, Shah NP. 2005. Production and maintenance of viability of probiotic micro-organisms in dairy products. In: Tamime AY, editor. Probiotic dairy products. Oxford: Blackwell Publishing. p 39-72.

Tarrega A, Costell E. 2006. Effect of inulin addition on rheological and sensory properties of fat-free starch-based dairy desserts. Int Dairy J 16:1104-1112.

References

Tashiro Y, Oike H, Aramaki M, Hirayama M, Adachi T. 1997. *In vitro* fermentation of fructooligosaccharides in comparison with other oligo- and polysaccharides. Proceedings of the International Symposium − Non-digestible oligosaccharides: healthy food for the colon?; 1997 Dec 4-5; Wageningen, the Netherlands. p 128.

Thomann RJ, Habel A, Mersiowsky E. 1995. Recent investigation on the fractionation and use of products from Jerusalem artichoke. Proceedings of the Fifth Seminar on Inulin; 1995 Oct. 27; Wageningen, the Netherlands. p 65-66.

Thompson A, Taylor BN. 2008. Guide for the use of the International System of Units (SI). Gaithersburg, MD: National Institute of Standards and Technology. 89 p.

Trowel H, Burkitt D. 1986. Physiological role of dietary fiber: a ten year review. J Dent Child 53:444-447.

Troyano E, de Rafael D, Martinez-Castro I, Olano A. 1996. Isomerisation of lactose over natural sepiolite. J Chem Tech Biotech 65:111-114.

Tungland BC, Meyer D. 2002. Nondigestible oligo- and polysaccharides (dietary fibre): their physiology and role in human health and food. Comp Rev Food Sci Food saf 1:73-92.

Tunick MH. 2000. Rheology of dairy foods that gel, stretch, and fracture. J Dairy Sci 83(8):1892-1898.

van Boekel MAJS. 1998. Effect of heating on Maillard reactions in milk. Food Chem 62(4):403-414.

Van Loo J, Booten K, Smits G, inventors; Raffinerie Tirlemontoise SA, assignee. 1997 Aug 26. Method for separating a polydispersed saccharide composition, resulting products and use thereof in food compositions. U.S. patent 5,660,872.

Van Loo J, Coussement P, De Leenheer L, Hoebregs H, Smits G. 1995. On the presence of inulin and oligofructose as natural ingredients in the Western diet. CRC Crit Rev Food Sci Nutr 35:525-552.

Van Loo J, Cummings J, Delzenne N, Englyst H, Franck A, Hopkins M, Kok N, MacFarlane G, Newton D, Quigley M, Roberfroid M, van Vliet T, van den Heuvel E. 1999. Functional food properties of non-digestible oligosaccharides: A consesus report from the ENDO project (DGXII AIRII-CT94-1095). Brit J Nutr 81:121-132.

Varga L, Szigeti J, Csengeri E. 2003. Effect of oligofructose on the microflora of an ABT-type fermented milk during refrigerated storage. Milchwissenschaft 58:55-58.

Villamiel M, Corzo N, Foda MI, Montes F, Olano A. 2002. Lactulose formation catalysed by alkaline-substituted sepiolites in milk permeate. Food Chem 76(1):7-11.

Villegas B, Costell E. 2007. Flow behaviour of inulin-milk beverages. Influence of inulin average chain length and of milk fat content. Int Dairy J 17:776-781.

Vinderola CG, Bailo N, Reinheimer, JA. 2000. Survival of probiotic microflora in Argentinean yoghurts during refrigerated storage. Food Res Int 33:97-102.

Vinderola CG, Mocchiutti P, Reinheimer JA. 2002. Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. J Dairy Sci 85:721-729.

Voragen AGJ. 1998. Technological aspects of functional food-related carbohydrates. Trends Food Sci Tech 9:328-335.

Weidmann M. Jager M. 1997. Synergistic sweeteners. Food Ingredients Int Nov-Dec:51-56.

Yamazaki H, Modler HW, Jones JD, Elliot JI, inventors; Canadian Patents and Development Ltd., assignee. 1989 Oct 3. Process for preparing flour from Jerusalem artichoke tubers. U.S. patent 4,871,574.

Zarate G, Chala AP, Gonzalez S, Oliver G. 2000. Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. J Food Prot 63:1214-1221.

Zhu J. 2004. Changes of pH value, acidity and lactic acid bacteria in yoghurt with fructooligosaccharide during storage. Shipin Gongye Keji 25(2):70-1.

Ziemer CJ, Gibson GR. 1998. An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. Int Dairy J 8:473-479.

Zokaee F. Kaghazchi T. Soleimani M, Zare A. 2002a. Isomerisation of lactose to lactulose using sweet cheese whey ultrafiltrate. J Chin Inst Chem Eng 33:307-313.

Zokaee F, Kaghazchi T, Zare A, Soleimani M. 2002b. Isomerisation of lactose to lactulose-study and comparison of three catalytic systems. Process Biochem 37:629-635.