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Development of a Probiotics Rich Yogurt Dry Mix

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Food Technology

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ABSTRACT

Background.

The probiotic *Lactobacillus acidophilus* NCFM has been scientifically researched to promote health beneficial effects in humans when consumed in sufficient numbers ($\approx 10^7$ cfu.mL^{-1}). The incorporation of the NCFM strain in foods has been widely applied world-wide, mainly in liquid fermented milks. Probiotics and other microorganisms can remain viable in high concentration when present in liquid or high water activity products. Products with high water activity have relatively low shelf-life, particularly at ambient temperature. This has generated international interest to investigate the survival of probiotics in low water products. This challenge forms the basis of the current study on the survival of probiotics in dehydrated yogurt mixes. The development of dehydrated food bases, such as yogurt dry mixes has created opportunities for the delivery of probiotics. Such products bring convenience to the consumer as they give flexibility to preparation and the quantities prepared. However, probiotics are sensitive to environmental factors such as water activity, oxygen, and storage temperature; and little is known about their survival mechanisms in dehydrated food systems. Therefore, the aim of this study was to develop probiotic-rich dehydrated yoghurt bases (DYB) with shelf-life of up to 18 months in modified-atmosphere packaging when stored at ambient temperature. The stability of the ready-to-eat (RTE) yogurt during refrigerated storage was also investigated.

Materials and Methods

Milk powder characterization

The degree of whey protein (α-lactalbumin & β-lactoglobulin) denaturation was analysed using the dye-binding method at 615 nm and by HPLC (GF-250 column equipped with UV detector at 280 nm) set at 30 °C. Standard and the NIR methods (800-2500 nm) were used to analyse the levels of fat (gravimetric), moisture (oven drying), and protein (Kjeldahl).

Selection lactic starter cultures and probiotic strain

To determine the suitability of the lactic acid bacteria (*Streptococcus thermophilus* (ST); *Lactobacillus bulgaricus* (LB); probiotic *Lactobacillus acidophilus* (LA) NCFM) used for the development of the DYB, growth kinetics of the cultures were conducted using a 96-well plate reader at 595 nm. Of the freshly prepared (18-24 h) stock cultures, 15 µl (10^{-1} to 10^{-7} dilution) and 135 µl of respective broths were dispensed into the 96 wells and allowed to grow anaerobically at 37°C for 24 h. The lactic acid bacteria (LAB) growth kinetic profiles at various initial inoculation rates (1, 2, 3%) of cultures used in 10% milk medium for 8 h at 43°C were conducted using viable counts. The M17 and MRS+clindamycin agar/broth were used to enumerate ST, NCFM, and the difference between Man de Rogosa (MRS) & MRS+clindamycin medium was used to estimate the levels of LB.

Characterization of DYB

Full factorial 2^3 experimental design was applied to develop twelve DYB formulations containing fat (1.4 & 3.5%), total sugar (15.4 & 14.4%), flavour (natural and strawberry). The DYB formulations were blended using the ribbon-type blender and then packaged in PE/foil/PET & PE/foil/nylon/PET packages. The DYBs were blended thoroughly and packaged under 100% N_2. Viable cell counts the LAB (NCFM, ST and LB), [O_2] and a_w at 20°C were analysed at intervals of three weeks for 9 weeks. Of the 12 formulations initially developed, 3 of them (formulations) with high LAB counts (>10^6 cfu/g), low a_w (<0.15) and [O_2] (<16%) were selected for further characterization and fermentation of yogurt. The first order kinetics was used to monitor the changes in the cell counts of LAB

in the DYB at various storage temperatures (22, 35, 45, 55°C). The results were then used to predict its survival at 4 and 22 °C using Arrhenius law.

Characterisation of liquid yogurts

The selected formulations were high fat high sugar (HFHS), high fat natural (HFN), and low fat low sugar (LFLS). The three formulations were fermented at 43°C for 8 h to produce yogurt and stored at 4°C for 2 weeks , during which analyses of viable cell counts, titratable acidity (% lactic acid), texture (N), viscosity (mPa.s), and syneresis (%) were conducted. pH measurement was conducted in the products and consumer acceptance using the 9-point hedonic scale was also conducted.

Results and Discussion

The protein, fat, and moisture contents of skim milk powder (SMP) were ±36%, <1%, and <4%; while for whole milk powder (WMP) were ±26%, ±28, and 2.9%. The undenatured whey protein was <2% using HPLC and <3 mg/g using dye-binding method for both powders.

The three strains of bacteria grew appreciably in milk and broth media, which followed sigmoidal growth in the latter medium. The growth profile of NCFM during fermentation in the absence and presence of ST and LB was comparable in broth and in reconstituted milk media indicating that the bacteria could be used together.

The a_w and $[O_2]$ in selected DYB formulations were <0.15 and <16% respectively, which may play a crucial role in maintaining the NCFM, ST, and LB at $>10^8$, $>10^6$, and $>10^7$ cfu/g. No significant difference ($p>0.05$) between packages was observed during storage as shown by comparable $[O_2]$ throughout storage. In its liquid form, concomitant increase of sigmoidal LAB growth (up to 4 logs) and acidity (pH 6.5 to 4.4) was observed during fermentation. The texture, viscosity and syneresis index were comparable during 2 weeks storage at 4°C; where low fat yogurt performed better than yogurt containing higher fat contents. Meanwhile, the loss of LAB counts as a result of acid accumulation (pH 4.55-4.2; lactic acid 0.7-1.5%) throughout refrigeration storage was observed. The LAB cell counts however were still maintained at $>10^7$ cfu/mL after 2 weeks.

Flavour, sweetness, and sourness were the main descriptors that drive consumer acceptance using Principal Component Analysis (PCA). Based on the cluster analysis, 62% (n=77) of consumer panellists showed clear differences in sample acceptability with 57% of the panellists indicating their likeness for samples HFHS, LFLS, and HFN. The shelf life of selected DYBs was >18 months at 4°C. The LAB survival, particularly ST, was markedly reduced at elevated temperature showing survival rates of $\geq 10^5$ cfu/g after 6, 10, and 14 months for LFLS, HFN, and HFHS, respectively at 22°C.

Conclusion

The cultures used in the current study were stable in the DYBs and liquid yogurts for the formulations used. The products were liked by consumer panellists with predicted storage life of up to 14 months at 22°C.

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LIST OF ABBREVIATION

ANOVA	Analysis of variance
ATP	adenosine triphosphate
A_w	Water activity
B	Bifidobacterium
cfu	Colony forming units
CO_2	Carbon dioxide
DE	Dextrose equivalent
DNA	Deoxyribonucleic acid
DYB	Dehydrated yogurt base
EC	European Committee
EFSA	European Food Safety Authority
ETC	Electron Transport Chain
EU	European Union
FDA	Food and Drug Association
FOSHU	Food for Specialized Health Use
FSANZ	Food Standard Australia New Zealand
GI	Gastrointestinal
GLM	General linear model
GMP	Good manufacturing practice
h	Hour
H_2O_2	Hydrogen peroxide
HACCP	Hazard analysis critical control point
k	Rate constant
IBS	Irritable Bowel Syndrome
LA	*Lactobacillus acidophilus*
LAB	Lactic acid bacteria
LB	*Lactobacillus bulgaricus*
MRS	Man de Rogosa
MSNF	Milk solid non-fat
NCFM	North Carolina Food Microbiology
NZFSA	New Zealand Food Safety Authority

N_2	Nitrogen
NADH	Nicotinamide Adenine Dinucleotide
O_2	Oxygen
Pa	Pascal
PCA	Principal Component Analysis
RSM	Reconstituted skim milk
RTE	Ready-to-eat
s	Seconds
S.D	Standard deviation
S.E	Standard error
SMP	Skim milk powder
ST	*Streptococcus thermophilus*
WMP	Whole milk powder
WPNI	Whey protein nitrogen index

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1. INTRODUCTION

Yogurt has long been known in human history as a way of preserving milk. Despite the distinctive acidity of natural yogurt, the consumption of yogurt or other cultured milk products is believed to have additional health promoting benefits to the host (Lee & Salminen, 2009). Since then, production of cultured milk products have become commercially important worldwide. Today, the technology of yogurt-making has become more advanced, which delivers more functional health benefits (e.g. probiotics) as well as application of strict hygiene control (e.g. HACCP and GMP) along with a variety of yogurt types to suit individual tastes (e.g. low fat, reduced sugar). With many yogurts of various types becoming available in the market, few contain less yogurt bacteria than the level recommended by professionals (Saavedra and Degnan, 2009).

According to the Food Standard Australia New Zealand (FSANZ) (2002) Standard 2.5.3, yogurt is defined as "fermented milk where the fermentation has been carried out with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with or without lactic acid producing micro-organisms where they have to be viable during shelf-life at pH 4.5 with minimum protein content (measured as crude protein) of 30 g/kg". For the health benefits, addition of probiotics is a common practice in yogurt manufacture. According to the European Commission (2003), probiotics are live-microorganisms which can establish themselves in the microbial population of the lower gut and can beneficially affect the host by improving host intestinal microbiological balance. The amount of added probiotics have not been harmonized by the European Union but viable bacteria of more than 10^6 cfu/g throughout shelf-life of the product are recommended as the health benefits commence at consumption level of 10^8 to 10^9 cfu/g. Although international standards have recommended for minimum viable bacteria in yogurts, the regulations however have not been harmonized and therefore vary in each country.

Since health-promoting effects of probiotics can only be delivered when adequate number of live bacteria is ingested, maintaining the viability of these bacteria becomes a challenge. During yogurt manufacture, exposure to heat and air are not uncommon. Such factors, compounded with the acid environment in yogurt may impact on their survival. The safety and efficacy of yogurt starter and other LAB microorganisms in liquid yogurt, as a model system, has been extensively

investigated (Behrens et al., 2004; Choi et al., 2011; Kailasapathy & Rybka, 1997). However, little is known about how these bacteria react in a dehydrated food system. The challenge of keeping the LAB alive in a dehydrated food system and throughout the shelf-life of the final product in its ready-to-eat (liquid) form is a topical issue. Like other microorganisms, yogurt bacteria require water and essential nutrients for growth. The reduction of moisture, however, can prolong the shelf-life of the product which can be conveniently stored at ambient temperature.

L. acidophilus NCFM is a probiotic strain of human origin available commercially in conventional foods (milk, yogurts, infant formula, and juice) and dietary supplements. The safety and the properties of the strain have been characterised *in-vitro*, *in-vivo*, and in human. It is presumed that the strain is functionally important to human due to their health beneficial effects which have been demonstrated to alleviate, reduce, and/or prevent various diseases such as colon cancer, diabetes, hay-fever, and lactose intolerance (Sanders and Klaenhammer, 2001).

No studies have been reported on the evaluation of the behaviour of LAB in the dehydrated yogurt base (DYB) systems where other food additives may be present in the dehydrated system. Further, limited studies have been reported on the viability of LAB in liquid yogurts made from dehydrated yogurt mixes (Wang et al., 2004; Wirjantoro & Phianmongkhol, 2009). Therefore, the characterization of dry yogurt bases and the respective ready-to-eat (liquid) forms is the main interest of this study.

1.1. Aim and Objectives

Aim:

To develop dehydrated yogurt bases (DYB) containing *L. acidophilus* NCFM with shelf-life of up to 18 months in modified-atmosphere packaging and up to two weeks as liquid yogurt.

Objectives:

1. Characterise the milk powder intended for yogurt production and developed 12 unique DYB formulations
2. Conduct a systematic review of literature on probiotic bacteria and their application in yogurt making
3. Characterise growth profiles of *S. thermophilus, L. bulgaricus*, and *L. acidophilus* NCFM in broth media using plate reader and evaluated the effects of various initial inoculation rates of yogurt cultures in milk medium
4. Characterise the DYB (dry) and the respective liquid forms during which analyses of [O_2], viable cell counts, water activity and shelf-life were conducted in its dehydrated form while the acidity, rheology, syneresis, and sensory evaluation were carried out in the liquid form. Experimental data were analysed statistically and the findings were presented in thesis report and oral presentations.

2. PROBIOTICS

2.1. Why Probiotics?

Probiotics have been studied worldwide due to their relationship between the gut and general well-being of human health (Ohashi & Ushida, 2009). The definition of probiotics has been modified over decades as more and more scientific knowledge about the microorganisms has been gained over time. In 1965, probiotics were defined as growth-promoting factors produced by microorganisms; the definition has now been modified to "live microorganisms which confer health benefits to the host when administered in adequate amounts" (FAO/WHO, 2001). The massive sales market of probiotics was worth about US$ 12 million in Europe, US$ 5 billion in Japan, and over US$ 25 billion worldwide in 2005, and still growing with an annual rate of 15%, probiotics therefore play an important role in food undustries (Chang, 2009).

Rapid expansion of probiotics market is mainly associated with emerging clinical evidence of its health benefits (Ohashi & Ushida, 2009). Various strains of *Lactobacillus* and *Bifidobacteria* have been commercially available for food applications. Extensive studies on this particular issue have been conducted on general population. The studies concluded that the benefits are strain specific (Ohashi & Ushida, 2009). Hence, strain characterization and its associated health advantages are paramount for use in commercial food products. Thus, the section reviews the health claims of probiotics, particularly the *Lactobacillus acidophilus* NCFM strain, which is of human origin and has been used commercially in food applications such as yogurt, infant foods, and supplements for more than 25 years (Sanders and Klaenhammer, 2001). The strain NCFM meets the pre-requisites properties of probiotics, which are resistant to bile acid, low pH, and digestive enzymes, and adhere to human epithelial cell lines and intestinal mucus (Ouwehand and Lahtinen, 2009).

2.2. Health Claims of Probiotic *Lactobacilus acidophilus* NCFM on Human Studies

It is no doubt that the health benefits are realised well when the probiotics are viable (Sanders and Klaenhammer, 2001). The advantages however are not limited to non-viable bacteria.

Previous studies have shown that although adhesion properties of non-viable probiotics were less than when they are in viable state, the bacteria are not without effects (Ouwehand & Salminen, 1998; Ouwehand et al., 2000b). During fermentation, more available bioactive compounds are produced as by products such as isoflavone which could reduce risk of hormonal related diseases (Ewe et al., 2011). The study showed that production of isoflavones which were less available in unfermented soymilk was enhanced in soymilk fermented with UV-treated *Lactobacillus acidophilus*. Since studies on the effects of non-viable bacteria that promote health benefits are scarce, it will not be discussed further; the focus will be on viable probiotics.

2.2.1. Maintaining Insulin Sensitivity in Diabetes Patients

Insulin is important in maintaining glucose homeostatis in the bloodstream (Andreasen et al., 2010). It is produced in the liver as a response to glucose level in the body. In patients with type-2 diabetes, their liver is not sensitive to glucose thus insufficient amount of insulin is produced (Andreasen et al., 2010). Recent studies conducted by Andreasen et al. (2010) revealed that the means insulin sensitivity of 24 patients (healthy and diabetic) which were treated with oral implementation of NCFM for a period of 4 weeks was improved while reduction in means insulin sensitivity was found in the placebo group (healthy and diabetic).

2.2.2. Increased Immunity

Probiotic bacteria may increase immunity in individuals through their ability to influence the immune response (Sanders and Klaenhammer, 2001). Of the immunoglobulin cells produced, 70% are produced in the intestine (Ibrahim et al., 2010). The decrease in immunity is one of the major problems in elderly people. These changes include the reduction of "natural killer" (NK) cells, which are responsible for killing unwanted substances such as tumour cells. The NCFM has been reported to increase NK cells in healthy elderly male volunteers (aged 72 to 103 years), although more studies remain open with respect to the beneficial effects of the probiotics for general well-being of the elderly (Ibrahim et al., 2010). An increase ($p<0.05$) of immunoglobulin responses were also observed in healthy volunteers aged 18-62 years old when single strain of

selected probiotics, including NCFM, were orally administered for three weeks (Paineau et al., 2008).

2.2.3. Lactose Intolerance

Lactose maldigestion is the inability of the body to metabolize lactose due to reduction of endogenous lactase, the enzyme is present abundantly during infancy and its activity decreases with age (Montes et al., 1995). In the absence of lactase, colonic bacteria ferment the undigested lactose and produce H_2, CO_2, CH_4, and other organic acids as fermentation by-products, which may result in abdominal pain, diarrhoea, and bloating (Montes et al., 1995). NCFM has been shown to reduce the symptoms when unfermented milk was inoculated with 10^{10} colonies per 250 mL milk although H_2 excretion was not always reduced (Montes et al., 1995). The proposed mechanism was attributed to the release of microbial enzyme, β-galactosidase, into the gastrointestinal (GI) tract to substitute the poor activity of endogenous lactase (Montes et al., 1995).

2.2.4. Alleviation of Hay Fever

Pollen allergy or so called hay fever is not unusual in western countries, with approximately 15% of teenagers in Western Europe suffer from the birch pollen allergy (Ouwehand et al., 2009). The symptom is seasonal and the severity varies between individuals from sneezing, runny nose, eye-lid swelling to itchy and skin rash. It occurs when an allergen from the surrounding air is inhaled which triggers the antibody production. It is hypothesized that probiotics could be used to reduce the symptoms of allergic rhinitis by altering the gut microbiota to confer immune effects (Ouwehand et al., 2009). The authors reported that probiotics (a combination of NCFM and *Bifidobacterium lactis* BI-04) interventions alleviated the nasal symptoms in pollen allergic children as opposed to the placebo.

2.2.5. Prevention of Cold and Influenza-like Symptoms Incidence

It is very common that children, immune compromised people, even healthy individuals could suffer from cold and influenza-like symptoms such as cough, fever, runny nose, and sore throat during cold winters. Although this may be insignificant, however, such symptoms could affect children's performance at school. *Lactobacillus acidophilus* NCFM alone or in combination with *Bifidobacterium animalis* subsp *lactis* Bi-07 have shown to prevent the cold and influenza-like symptoms in healthy children aged 3 to 5 years old. Children receiving either one strain or two strains of the probiotics (daily dose of 10^{10} cfu/g) had less number of missed school days attributable to influenza-like illness compared to placebo (Leyer et al., 2009).

2.2.6. Improvement of End-stage Kidney Failure

Failure of kidney to remove toxins metabolites such as urea, a compound found abundantly in urine, results in toxins accumulation in the body. When the kidney function falls by 20%, dialysis is one type of treatment given to sustain a person's life, a condition known as end-stage kidney disease. The most common cause associated with this condition is toxin-accumulation produced by particular bacterial metabolites in the small intestine. In healthy individuals, bacterial population in upper intestine is relatively low, ranging from 100 to 1000 cfu.mL^{-1}. In kidney failure patients, the bacteria microflora may reach 10^7 cfu.mL^{-1} in the small intestine. Oral administration of *Lactobacillus acidophilus* NCFM and BG2F04 (twice daily oral dose of 10^9 cfu per capsule) has been shown to reduce carcinogens levels in the blood and body fluids; and improve body weight and caloric intake in patients with end-stage kidney failure (Dunn et al., 1998).

2.2.7. Anti-carcinogenic Agent

Today, colon cancer has become a critical issue among people whose diet is high in all nutrients (e.g. fat, sugar) except fibre. A human study carried out by Goldin and Gorbach (1984) showed potential use of the NCFM in reducing three carcinogenic precursor enzymes (β-glucuronidase, nitroreductase, and azoreductase). Consumption of unfermented milk inoculated with NCFM

(10^9 cfu/day) for a period of 4 weeks reduced the activity of these enzymes by 2-4 fold (Goldin & Gorbach, 1984).

2.2.8. Pathogen Exclusion in Urogenital Tract

Similarly to antiseptic, yogurt can be used as an alternative solution for vaginal douches. The NCFM has been reported to inhibit the adhesion of uro-pathogens by producing bio-surfactants against them, although the oral route delivery showed fewer efficacies and would not be the optimal choice as functional foods at this case (Reid, 2000).

2.3. Regulatory Status of Probiotics

Apart from strict consideration of selecting microorganisms to be used as effective probiotics, the incorporation of probiotics into human food, either as liquid or powder type, should comply with the regulations of the country of origin and/or destination, where "Generally Regarded as Safe" (GRAS) status is compulsory. This section reviews the current regulatory policies and status of probiotics in NZ, Australia, Europe, Japan, and the USA.

2.3.1. Codex Alimentarius

In 2003, FAO/WHO joint organization declared that starter cultures of fermented milks should be present at level of more than 10^7 cfu/g; and where claims are made on the product for specific microorganisms other than starter cultures, the product shall have a minimum amount of the culture at 10^6 cfu/g (FAO/WHO, 2003).

2.3.2. European Union

At this stage, no legal definition and specific legislation have been made for the term probiotic by the European Union (EU). Harmonization of regulation for functional foods in Europe has been adopted recently in 2007. Under the new regulation, manufacturers intend to claim health benefits of the product should submit their list of claims under Article 13.1, which is based on

generally accepted scientific evidence; Article 13.5 for newly developed scientific evidence; or Article 14 for claims regarding reduction of disease risk or children's development and health. Claims submitted under Article 13.1 were reviewed by the European Committee (EC) and a list of permitted and accepted claims is expected to be announced in 2010 by European Food Safety Authority (EFSA) (Ouwehand and Lahtinen, 2009b). Of the probiotic claims that have been submitted, some of them have been accepted and published by the EFSA, although it is not guaranteed that such claims will be approved by the EC (EFSA, 2010).

While the new regulation of EC 1924/2006 Article 13 is still under review (expected completion date is 31 December 2011), health statements can be made (assuming that they have submitted the claims and that it would be approved by EFSA). Health claims, however, shall be based, and supported by good scientific evidence and the following information should be labelled on the package (EU, 2003);

- The quantity of the food and the required consumption pattern to obtain the claimed health effects;
- Where appropriate, a statement addressed to persons who should avoid the product;
- Where appropriate, a statement warning not to exceed quantities of the product that might represent health risk

The regulation also states that probiotic bacteria shall be present in the product at concentration of more than 10^7 cfu/g or sufficient numbers to obtain the claimed health effects and that the species should be viable from the date of production throughout shelf-life of the product.

2.3.3. Australia and New Zealand

The joint regulation of Australia and New Zealand is governed by Australia New Zealand Food Authority (ANZFA). At this stage, the legal standard of probiotics and its health claims has yet to be harmonized. The use of probiotics as feed additives, however, has been approved and regulated separately from human foods (Ouwehand and Lahtinen, 2009b). Although the regulation on probiotics has not been harmonized, according to the Food Standard Australia New Zealand (FSANZ) (2002) Standard 2.5.3, yogurt is defined as "fermented milk where the fermentation has been carried out with *Streptococcus thermophilus* and *Lactobacillus delbrueckii*

subsp. *bulgaricus* with or without lactic acid producing micro-organisms where they have to be viable during shelf-life at pH 4.5 with minimum protein content (measured as crude protein) of 30 g/kg".

2.3.4. Japan

Japan had its own regulation on food containing probiotics before the FAO/WHO of the United Nations guidelines had been harmonised. The regulation was initiated to protect consumers and to promote growth of fermentation industries such as Yakult and Morinaga (Chang, 2009). Food manufacturers can apply for certification from the Ministry of Health, Labour, and Welfare for "food for specialized health use" (FOSHU) system. The system acknowledges probiotics as functional food; however manufacturers who wish to make specific health claims against certain illnesses fall under different categories of the system. The Japanese Fermented Milks and Lactic Acid Bacteria Beverages Association was appointed by the government to set the legal requirement for probiotics food. The organization stipulated that a probiotic food should contain more than 10^7 cfu/g during shelf-life along with the frequency and recommended intake, the strain involved, and the general health of consumers. In 2005, there were 69 products allowed to make health claims and of these, 57% were intended for gastrointestinal disorder (Chang, 2009).

2.3.5. USA

The Food Drug Administration (FDA) regulates the legal status of probiotics according to how the product is going to be used, either as "food", "food additives, "drug", "new drug", or "dietary supplement". At present, no legal or recognized regulations govern probiotics labelling in the product in the USA. Classifications, labelling, and claims of probiotics containing products are regulated by the FDA. As long as the microorganisms have obtained GRAS status of its intended use (e.g. microorganisms obtain GRAS status for a food does not mean that it also qualifies for food supplement), manufactures can launch their products onto the market, provided that Good Manufacture Practice (GMP) is implemented (Saavedra and Degnan, 2009). However, many of the products do not meet the recommended viable amounts of probiotics (Trahan, 2008). It may

be misleading to consumers since probiotic foods are expected to contain sufficient viable cells to deliver health benefits (Saavedra and Degnan, 2009).

The FDA, however, has a special authority to regulate health claims of foods and dietary supplements, with only claims authorised and approved by FDA being lawfully labelled on the product. Thus, market pathways of probiotiocs products in the USA are numerous depending on the intended use of the products (Saavedra and Degnan, 2009).

2.4. Safety of the *Lactobacillus acidophilus* NCFM

An extensive review carried out by Sanders and Klaenhammer (2001) reported the safety of the *Lactobacillus acidophilus* (LA) NCFM colonization in neonatal and adult mice at inocula levels of 10^6-10^7 and 10^8-10^9 cfu/g faeces, respectively. The history of safety of LA NCFM consumption in commercial products was also confirmed by the IDF (IDF, 2002). Although the translocation of LA into the blood may occur, however, it was only observed in immunocompromised individuals. Of noteworthy, the infection as a consequence of the translocation of the strain, did not harm the host and the infection in the system was cleared within three weeks (IDF, 2002). Moreover, the rate of translocation of LA into the blood occurrence varies between strains of *Lactobacillus* due to the adhesion properties of the bacterium as discussed in section 2.5 (Apostolou et al., 2001).

2.5. Mechanism of Action of *L. acidophilus* in the GI Tract

It is important to consider the survival of *Lactobacillus acidophilus* (LA) NCFM through passage in the stomach and its implantation in the colon as microflora of human gut varies between individuals (Lee & Salminen, 2009). To survive passage through the stomach, the NCFM has to tolerate extreme acidity (≈ pH 2) and various bile acid concentrations which are the challenges in the production of food containing frobiotics. These factors therefore highlight the importance of food system as carrier of the probiotics due to the buffering effects of the foods which may increase pH of the stomach (Ross et al., 2005). Cells that survive passage through the stomach can then move towards the intestines. Adhesion of probiotic

microorganisms to intestinal epithelial cells and mucosa serves as a mechanism of survival to prevent them being washed out of the system. The epithelium cells, which line up to form mucosa, shields the cells from certain microorganisms while providing binding sites for the bacteria to proliferate at the same time. The epithelial cells then transfer the nutrients needed towards the capillaries. While transient colonization of probiotics in the human gut may stimulate immune system, it may be disadvantageous in the case of adherence of pathogens (Lee & Salminen, 2009).

The colonization of LA cells in human intestinal cells is host-specific. The precise specificity of the binding abilities of probiotics is due to specific transporter genes (Fukuda et al., 2011), structure, and components of the bacteria (Greene & Klaenhammer, 1994). The former study indicated that the ability of bacteria to utilize available nutrients (e.g carbohydrate) in the gut varies between strains due to various specific transporter genes of the bacterium, which may therefore indicate its efficacy. The cell wall polysacharides and surface protein layers of the LA are useful for cell colonization in the human GI tract (Greene & Klaenhammer, 1994). Apart from its surface recognition properties, the surface protein S-layer protects the bacteria by changing its structural features under stress conditions (e.g. bile acids, acidity) and adapt to the changes accordingly (Frece et al., 2005). An *in vitro* study by Buck et al. (2005) showed a significant reduction in adhesion of NCFM to Caco-2 cells when the surface layer gene, *slpA* is inactivated. When direct adhesion of NCFM to epithelial cells is not possible, it may adhere to specific epithelial extracellular matrix component, such as fibronectin protein, using gene *FbpA* which acts as a bridge between bacteria surface and host cells (Buck et al., 2005). The adhesion of bacteria to mucin, a glycoprotein produced by epithelial tissue of gastrointestinal tract, is another possible way of the adherence of NCFM (Azcarate-Peril et al., 2009; Buck et al., 2005). Further, the mechanism of protein-intestinal adhesion could also be mediated by lipoteichoic acid which acts as a bridge between the bacteria and the intestine. Lipoteichoic acid is a macromolecule present in the cell wall of a wide range of probiotic groups (*L. acidophilus*, *L.reuteri*, and *L. fermentum*) (Sherman & Savage, 1986).

Adhesion of probiotics bacteria LA can be influenced by many chemical factors such as calcium, bile acids, digestive enzymes, or the presence of other microorganisms (Lee & Salminen, 2009).

In the presence of calcium, an *in vitro* study on the adhesion of *acidophilus* to epithelial cell lines showed an increase in its attachment (Larsen et al., 2007) which may probably be due to the "bridging" properties of calcium which links between the negatively charged bacterial surfaces and the host cells (Kleeman & Klaenhammer, 1982). Decreased in adhesion properties of probiotics *lactobacilli* and *bifidobacteria* were more distinct in the presence of bile acids and digestive enzymes (Greene & Klaenhammer, 1994; Khaleghi et al., 2010; Ouwehand et al., 2001). The gradual increase in acidity (pH 6 to 3) improved the adherence ability of the NCFM to caco-2 cells (Greene & Klaenhammer, 1994). In the study of Hood and Zottola (1988), the ability of LA to bind to human intestinal cells was not affected by acid pre-treatment. The author summarised that although the bacterium was previously exposed to low pH (pH 2) and no viable cells were recovered after the acid exposure, adherence was not compromised; hence active metabolism was not a prerequisite for adherence.

In the high diverse microbial population of the intestine, survival is crucial. In this regard, probiotic bacteria may produce bacteriocins or antimicrobial substances for competitive exclusion (Sanders and Klaenhammer, 2001). Previous reports have shown that probiotic NCFM produce a bacteriocin, lactacin B, and is reported to have an antagonism effect to *L. bulgaricus, Enterococcus faecalis, L. fermentum spp.*, and *Lactococcus lactis* (Barefoot & Klaenhammer, 1983; Sanders & Klaenhammer, 2001). While the production and effect of the bacteriocin remains unclear in the intestine, a decrease in adherence of *Lactobacillus rhamnosus* GG to caco-2 cells was reported in the presence of aflatoxins (Kankaanpää et al., 2000). The adhesion properties of probiotic *Lactobacillus* spp. in the presence of other microorganisms, however, can be mutualistic (Ouwehand et al., 2000a) or neutral (Ouwehand et al., 1999; Ouwehand et al., 2004).

The ability of bacteria to aggregate with its species, pathogens or other microorganisms present in the intestinal mucosa is one of the criteria for selection of probiotics. With respect to the NCFM strain, the *apf* gene plays an important role in aggregation, cell shape maintenance, and adherence to epithelial cells. The gene was up-regulated during the stationary phase and responsible for the adherence of the probiotic to caco-2 cell, mucin, and fibronectin (Goh & Klaenhammer, 2010).

While studies on probiotics adhesion to intestinal cells described in preceding section were carried out in healthy cells, particular strains of probiotics (*L.rhamnosus* GG and *L.reuteri*) were found to be able to attach to the mucus model of inflammatory bowel disease. This finding may serve as a tool for potential use of probiotics to treat particular intestinal-associated diseases (Ouwehand et al., 2003).

Dietary habits play significant roles in maintaining the *L. acidophilus* (LA) as well as probiotic micro flora in general. Alcoholic beverages contain various compounds which can affect the maintenance of the NCFM and its effects vary considerably in individuals. Alcohol has adverse negative effects on the survival of LA and consumption should be limited for maintaining healthy digestive system (Sellars, 1991). On the contrary to alcohol intake, a diet rich in fibres such as linseed which contained abundant amount of plant lignans promoted the growth of *Lactobacilli* species (Lahtinen et al., 2002). Wu et al. (2011) reported that the microbiota in the gut varies significantly according to their long-term dietary pattern. Typically, diets of meat and saturated fat have more ratios of *Bacteroides*; consumption of lots of alcohol and unsaturated fat resulted in the abundant numbers of *Ruminicoccus*; and carbohydrate-rich diets favoured *Prevotella*. A pilot study conducted in Asian countries comparing the diet of healthy teenagers in big and rural cities from each Asian country showed the presence of *Butyricicoccus pullicaecorum*. The bacterium is commonly found in chicken (Lee et al., 2011). The shift in the representation of dominant phyla in obese people has been well-reviewed by Ley (2010) who emphasized the importance of gut microbiota in regulating caloric intake which may have an impact on gut inflammation, insulin resistance and fat storage. The importance of gut microbiota in regulating nutrients obtained through a daily diet was also supported by Ley et al. (2006) and Jumpertz et al. (2011).

3. PROPERTIES OF MILK USED FOR YOGURT MANUFACTURE

High quality yogurts are characterised by the rich mouth-feel sensation, which are derived from milk fat and milk-solid-non-fat composition. Undoubtedly, this could only be achieved when good sources of materials are used. This section reviews the importance of milk composition on the quality of milk powder and the end-product, which is the dehydrated yogurt base (DYB). The DYB in this study will be made by blending the milk powder and the freeze dried bacteria as well as other dried food ingredients. The DYB will then have to be fermented to make the "ready-to-eat" yogurt. In this study, the word "milk" refers to bovine milk unless otherwise stated.

3.1. Milk Composition

With milk being the main component of yogurt, the quality of raw milk is of significant importance for sensory, chemical, and microbiological aspects of the final product. Assuming that hygiene practice has been applied; good quality milk should have low acidity (0.16-0.17% lactic acid) and coliform counts, free from antibiotics and have to be milked from healthy cows. Milk is a complex nutritious fluid in which various constituents are held in a multi-dispersed phase of emulsion, colloidal suspension, or solution (Chandan, 2006). The main components of milk composition are illustrated in Figure 1. Seasonal variation of milk composition (e.g. protein and fat contents) is influenced by breed genetics and feeds (e.g. grass). According to the Food and Drug Administration (FDA), an A grade milk should contain no less than 8.25% of milk solids non-fat (MSNF) and no less than 3.25% of milk fat exclusive of colostrum (Chandan, 2006). The term MSNF refers to all milk constituents excluding milk fat and water; while total solids are defined as MSNF plus milk fat. Depending on the season of the year, milk comprises of 3-3.5% of fat, 8.5-9% of solids non-fat with water making up the remaining constituents. Among the MSNF, around 4.5% is lactose, 3.3% protein (2.6% casein and 0.7% whey proteins) and the remaining being minerals (calcium, magnesium, zinc, etc) (Chandan, 2006; Robinson et al., 2006).

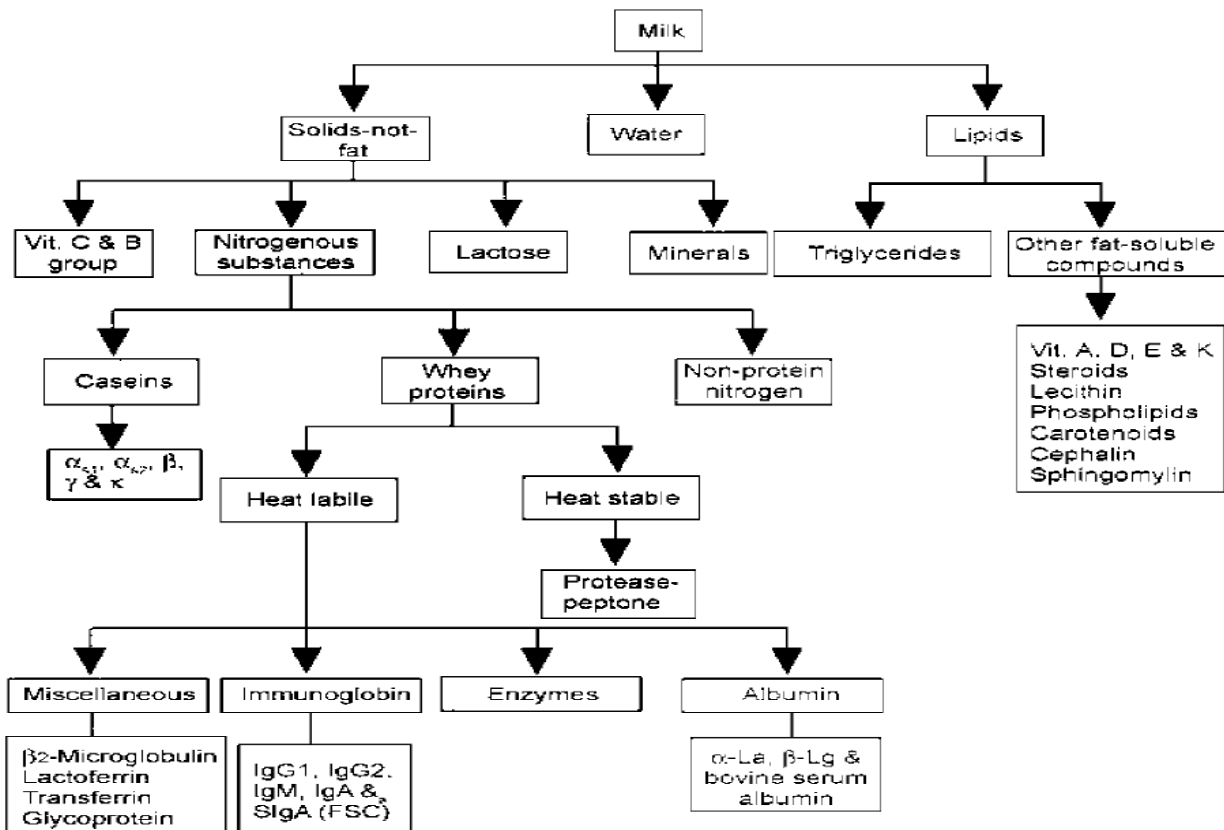


Figure 1. Typical example of the main chemical component of cow's milk. Note that milk contains dissolved gas (O_2, CO_2, N_2), enzymes, cellular matter, and microorganisms (Tamime and Robinson, 2007).

3.1.1. Lipids

Milk is an oil-in-water emulsion where the fat globules are dispersed in aqueous phase. The size of milk fat globules varies (3.4 - 4.5µm) depending on breeding regime and species of cow. Unsaturated and saturated fats are present in milk. The ratio of unsaturated and saturated fats is strongly influenced by feeds and seasonal variation, where more concentration of saturated fatty acids is found during the cold season (Spreer, 1998). Nearly all fats in milk are in the form of fat globules (Walstra et al., 2006). To maintain a stable emulsion, fat globules are stabilised by thin plasma membrane which consists of proteins (e.g. caseins), phospholipids, glycerides, water and small fractions of cholesterols, enzymes, and trace elements (Chandan, 2006; Tamime and Robinson, 2007). Proteins and enzymes (e.g. phosphatase) are incorporated towards the aqueous phase which plays an important role in fat stability. At the surface of the fat globules, there is electrical charge. The electrical charge changes considerably following the milk pH; negative is

observed at higher pH (>4.5) while positive charge is found in lower pH (<4.5). At the layer close to the core area, vitamin A and stearins are located (Spreer, 1998). Milk fat is important to give milk its rich-creamy taste and to act as the transporter of the fat soluble vitamins. However it may also lead to the development of rancidity due to fat oxidation (Chandan, 2006; Tamime and Robinson, 2007). With the health issues related to fat content, the level is often partially or almost completely reduced in particular food products (e.g. non-fat yogurt). Undoubtedly, this may affect the flavour and emulsion stability which would lead on to rheological and sensory changes. In yogurt, higher fat content correlates to lower viscosity as fat globules interrupt the gel network (Walstra et al., 2006). While no significant impact of fat level has been found on the viability of ST, LB, and LA in liquid yogurt (Obi et al., 2010), no studies have been done on the effects of fat level on dry yogurt.

3.1.2. Lactose

Lactose is the major sugar present in milk and it plays a significant role on the solubility of milk powder. The disaccharide comprises of glucose and galactose (Walstra et al., 2006). In solution (milk), lactose crystal exists as α-lactose, β-lactose or a mixture of both forms. The α-lactose crystals are very hard, slightly hygroscopic, and dissolved slowly; while the β-lactose dissolved quickly and have fairly good solubility. The rate and degree of crystallization is influenced by milk processing temperature. In spray drying, lactose exist as anhydrous lactose in its glassy state, which has similar properties (hardness, density, and specific heat) as crystals but the packing of the molecule is not in perfect order (Walstra et al., 2006). Crystallization of anhydrous lactose in milk powder brings negative impacts on the reconstituted milk quality (e.g. lumpiness). For crystallization to occur, moisture is required. The bonding between water and α-lactose crystal is very strong which makes it difficult to be broken. For this reason, manufacturer of milk powder keep the moisture content of anhydrous lactose to about 5% or lower to prevent crystallization (Walstra et al., 2006). Apart from governing the solubility of milk powder, lactose provides the energy required for growth of starter cultures in yogurt production (Chandan, 2006).

3.1.3. Proteins

The total solids content of milk is around 10-13% with 3-4% being milk protein which contains heterogeneous mixture of protein. Caseins are the most abundant proteins which account for about 80% of total protein content followed by whey (15-20%) and proteose/peptones (2-6%) (Chryssanthopoulos and Maridaki, 2010; Tamime and Robinson, 2007). Apart from the significant role of protein to form gel network during yogurt manufacture, the macromolecule (particularly whey protein) also contains high amount of essential amino acids, which cannot be synthesised in the human body. Alpha (α-) lactalbumin, for example, has high content of amino acid tryptophan, a precursor of niacin (vitamin B_3). In yogurt, the amount of free amino acids is even higher due to the proteolytic activity of yogurt cultures, particularly *L. bulgaricus* and remains active throughout the shelf-life of yogurt (Chryssanthopoulos and Maridaki, 2010).

Caseins

Casein is a milk protein comprising of α_{s1}, α_{s2}, β, κ-fractions and contributes to 2.4-2.8 g/100 mL of milk protein content with 50% being α_{s1} casein, 30% being β- casein, and 15% being κ- casein (Tamime and Robinson, 2007). Casein has disordered structures and consists of α_{s1}-, α_{s2}-, β-caseins in the core area and κ-casein in the outer area, which wraps around the surface. The hydrophobic interaction between the caseins holds them together to maintain globular stability of the caseins. Casein is so called sub-micelle and the interaction of various caseins and clusters of calcium phosphate form casein micelles. The aggregation of submicelles influences the adsorption behaviour of the milk protein in the emulsion. Caseins are soluble and stable under heat, pressure (homogenization), and other dairy processes (Chandan, 2006). Caseins, however, are sensitive to pH changes. The isoelectric point of caseins is around pH 4.5-4.6. When pH decreases below its isoelectric point, caseins are destabilised and precipitated (Spreer, 1998). Additionally, caseins are unstable when exposed to enzymes (phosphatises, glucosidases, and proteinase) and coagulation can also be formed. The formation of gel is important in yogurt and cheese making (Spreer, 1998).

β-lactoglobulin Whey Protein

Whey or so called serum proteins is the second most abundant protein (after caseins) in milk. It contributes to 0.5-0.7 g/100 mL of milk proteins, comprises mainly of β-lactoglobulin (58%) and α-lactalbumin (13%) and small fractions of immunoglobulin, albumin and proteose peptone. The latter form is heat stable while the remaining is sensitive to high temperature or other heat treatments (Chandan, 2006; Walstra et al., 1984). β-lactoglobulin contains two disulfide, one free sulfhydryl and no phosphate group. The name β-lactoglobulin is consistent with the tertiary structure of the protein which consists of nine β-sheet and one α-helix which is located at the surface. The molecule is highly hydrophobic at the centre of β-sheet which plays a significant role, but limited to, in retinol binding (Kilara, 2008).

Unlike caseins, whey protein is soluble at a wide range of pH and is useful as water binding agent which is desirable for beverages application. The hygroscopic property of whey protein is often used as an indicator of whey protein denaturation. When the structure is disrupted through heating or other milk treatment, it becomes insoluble and can form a gel with κ-caseins which is crucial in yogurt manufacture (Chandan, 2006; Kilara, 2008; Walstra et al., 1984). The heat-stability of β-lactoglobulin is decreased by the presence of calcium ions. Insoluble proteins absorb a lot of water. The extent of whey denaturation and its water binding properties are therefore important in yogurt production to reduce syneresis (Kilara, 2008).

α-Lactalbumin Whey Protein

Being the second most abundant whey protein, α-lactalbumin contains four disulfide bonds and no phosphorus. Although it is sensitive to heat, the protein shows increased heat resistance ability in the presence of calcium and that the mechanism remains unknown. Additionally, α-lactalbumin plays an important role in lactose synthesis in milk. It has been reported that it modifies the activity of galactosyl transferase and speeds up the synthesis of lactose (Kilara, 2008). Both caseins and whey are highly nutritious in which their applications in foods (e.g. infant formula) as well as pharmaceutical formulae are of significant importance (Spreer, 1998).

3.1.4. Minerals and Vitamins

Without fortification, milk contains 0.7% of ash with calcium and potassium being the major constituents. Both fat-soluble (e.g. A, D, E, and K) and water-soluble (e.g. B and C) vitamins are present in milk and the concentration varies according to the season and feed profile of cows (Chandan, 2006).

3.1.5. Enzymes

Phosphatase enzyme is present in milk, particularly in the membranes of the fat globules. Generally, phosphatase activity is used by milk manufacturers as an indicator of adequate pasteurization practice. Inactivation of the enzyme ensures that non-spore forming pathogenic bacteria that may be present in milk have been killed (Walstra et al., 2006). Lipase is another milk enzyme which is capable of liberating free fatty acids, which may cause rancidity in milk. The enzyme is bound with casein micelles in milk. However, the enzyme is inactivated during heat treatments (e.g. 70°C for 1 minute). Proteinases, which hydrolyse proteins to yield caseins and proteose peptone, are present in milk and play an important role for growth of yogurt bacteria (Walstra et al., 2006).

3.2. Manufacture of Milk Powder for the Development of DYB

Milk powder manufacture involves gentle removal of water at low temperature under reduced pressure to minimize damage to milk properties and retain desirable properties of milk (e.g. nutritional values, colour, and flavour). A wide range of milk powders are manufactured according to the intended end-use (e.g. instant or regular whole milk powder). Whole milk powder (WMP) contains 38% lactose, 26% fat, 25% protein, 7% ash and 3.5% moisture. Skim milk powder (SMP), 52% being lactose, 35% being protein, 8% being ash, 4.3% being moisture and only small fraction (less than 1%) of the remaining being fat. Upon delivery at the manufacturing plant, milk is pasteurised at 72°C for 15 seconds. Depending on the purpose, the manufacture of milk powders (Figure 2) commences after separation of skim milk and cream. Cream may then be added to skim milk if WMP is to be manufactured. The standardised milk is

then held in buffer silos prior to preheating. At this stage, milk is heated to temperatures between 75 and 120°C for few seconds up to a few minutes. The heating temperature may vary depending on the intended end-use, which is closely related to the degree of denatured whey protein. Based on the pre-heating treatment, SMP is categorised as low heat (75°C for 15 s), medium heat (75°C for 1-3 min), and high heat (80°C for 30 min or 120°C for 1 minute). WMP, however is not generally heat-classified, but is typically heated at 85-95°C for several minutes to inactivate the milk enzyme lipase and to expose the sulphydryl groups, which is milk antioxidant (Kelly, O'Connell, & Fox, 2003). The next step is the evaporation of water from milk. Milk is concentrated by removing more than 85% of its moisture content. Evaporation is achieved by boiling milk at temperatures below 72°C under vacuum. If WMP is to be manufactured, concentrated milk is homogenized at around 15 mPa at low temperature (e.g. below 70°C). This is done to reduce fat droplet size to prevent fat-separation in the final product (refer to section 3.6.2). Concentrated milk is then ready for atomization and subsequent drying. The inlet temperature can be as high as 200°C and the outlet temperature typically ranges at 70-90°C, the milk droplets temperature however, may never reach 70°C due to evaporative cooling (Kelly et al., 2003). During atomization, moisture content is greatly reduced, leaving fine milk powder of diameter less than 0.1 with 6% moisture. Secondary drying may then take place in fluidized bed to give milk powder with moisture content of 2-4% (maximum moisture content of 5% according to Standard 2.5.7, NZFSA). The purpose of secondary drying is to improve powder quality (optimum moisture content and solubility), reduce cost (higher inlet temperature and lower outlet temperature, which is 100°C), and for the opportunity to produce agglomerated powder. If WMP is manufactured, lecithin may be spread during the secondary drying to give the milk powder "instant" properties. Pre-treated and spray-dried milk undergoes significant changes in its composition, particularly protein (e.g. casein micelles) and lipids, therefore consideration on processing condition is important (Baldwin and Pearce, 2005; Pearce, 2010; Tamime et al., 2007b).

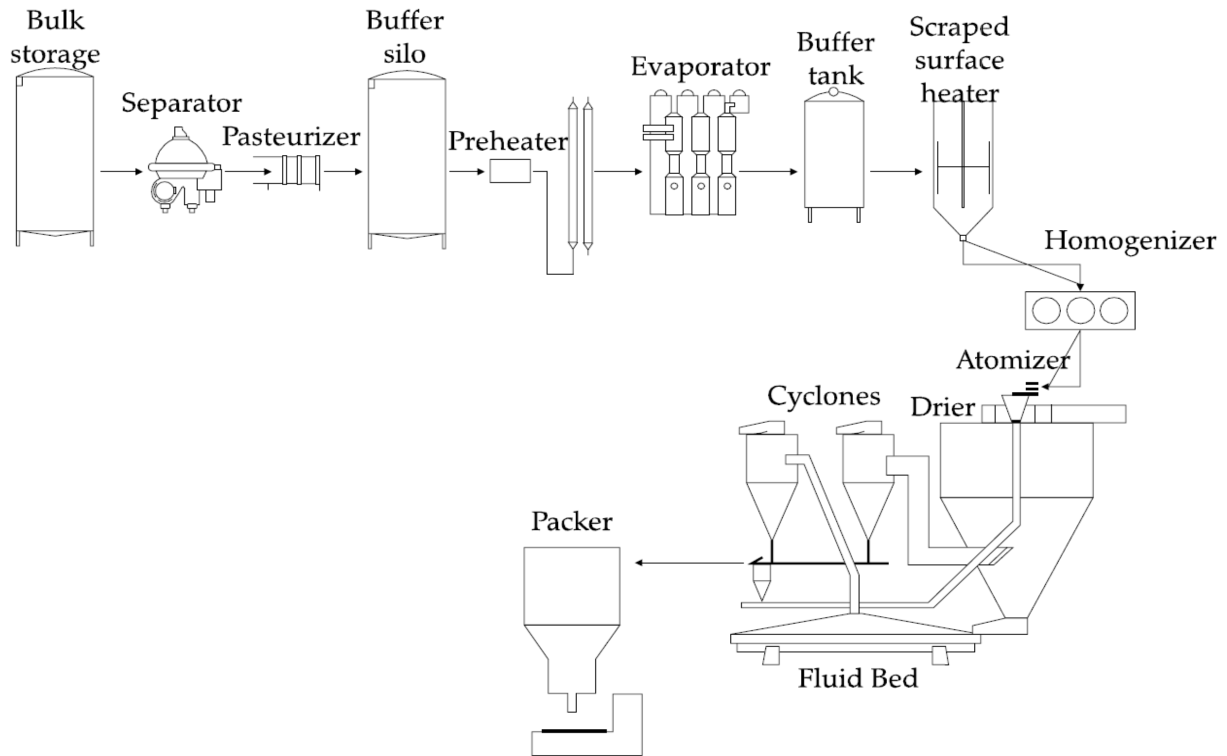


Figure 2. Manufacturing process for milk powder intended for the production of dry yogurt base (Pearce, 2010)

3.3. Changes of Milk Composition during Milk Powder Manufacture

Preheating temperature applied during milk powder manufacture is typically above 75°C at pH 6.8 (normal pH of milk) (Kelly et al., 2003). Significant alterations can occur during preheating, particularly in the structure of whey protein. On preheating, β-lactoglobulin, to a lesser extent, α-lactalbumin is denatured and starts to interact with κ-caseins via the sulphydryl-disulphide bonding. Concomitantly, β-lactoglobulin and α-lactalbumin form complexes with bovine serum albumin via disulphide and hydrophobic bonding respectively. The degree of temperature dictates the protein-protein aggregation. Lower temperature (< 75°C) and slower heating favour whey-to-whey protein interactions, while higher temperatures favour casein-whey interactions. This is owing to the free sulphydryl groups of β-lactoglobulin which can be activated at lower temperature. Caseins do not possess sulphydryl groups which explain the lack of casein-whey interactions at low temperature (Kelly et al., 2003).

Preheating and evaporation causes significant changes on the crystallization of lactose. Crystals are often formed in foods and during food processing (Walstra, 2003). Two lactose isomers, α- and β-lactose are present in milk (liquid); the former is more stable. The ratio between α and β isomers is influenced by the presence of salt. However, the isomers are temperature dependent (Vuataz, 2002). In liquid milk, lactose and other milk compounds (proteins, minerals, and to a lesser extent, lipids) are dissolved in water. During drying, a significant amount of water is removed; and lactose transforms into metastable amorphous form, which is highly hygroscopic. An increase in temperature or relative humidity, or both can easily convert the labile amorphous form into crystal β-lactose, which enhances caking and stickiness properties of milk powders (Roos, 2002; Vuataz, 2002). Moreover, Baechler et al. (2005) reported that heat-treatment applied to WMP (90°C for up to 70 min) in an air sealed environment increases a_w and β-lactose crystal due to the release of water during lactose crystallization resulting in an increase of water content in the matrix and a_w of the powder. The activity is compounded with high initial level of a_w of the powder (e.g. faster rate of crystallization at a_w 0.35 than at a_w 0.28) (Baechler et al., 2005).

In WMP, amorphous lactose forms continuous matrix in which proteins, fat globules, and air vacuoles are dispersed. The proteins and fats are coated with lactose (Vuataz, 2002). The increase in temperature and water content promotes crystallization of β-lactose crystals. Lactose crystallization promotes the expulsion of fat from the interior core causing particle aggregation during which the contents of free fat and particle density are increased while the surface area and pore volume are decreased. The migration of fat from the core to the particle surface causes substantial porosity in the core of particles (Vuataz, 2002). According to Baechler et al. (2005), the porous particle induces powder agglomeration and lactose crystallization on the particle surface which may affect the milk powder attributes (Faldt & Bergenstahl, 1996). More importantly, the speed of fat expulsion was even greater when higher initial level of a_w (e.g. 0.28 *vs* 0.35) was observed in the powder (Baechler et al., 2005). Furthermore, heat treatment (90-95°C for 15-30 s) in milk ensures the inactivation of lipase, the enzyme which is capable of breaking fats causing fat oxidation (Spreer, 1998). Evaporation of milk and subsequent drying decrease the size of fat globules, which can influence the level of free fat in the powder (Mulder & Walstra, 1974). While drying can cause significant changes on the structure of milk particles,

homogenization in milk decreases the size of milk fat globules and increases the surface area of fat globules. The increased surface area enhances the ability of milk fat globules to bind with protein (Robinson, 2002). Homogenization also helps to improve the fat dispersion during WMP manufacture (Spreer, 1998).

During preheating and evaporation, changes in mineral salts occur. Medium-high heat treatments (>75°C for few min or more) increase the precipitation of soluble calcium phosphate into the colloidal form. The increase in solids content (lactose and salts) during evaporation shifts the salt balance and pH of the concentrate; thus increasing the calcium phosphate precipitation (Kelly et al., 2003). The overall changes of milk composition as a result of spray-drying alter the powder microstructure, which is related to milk powder properties as described in section 3.5.

3.4. Changes in Water Sorption during Spray Drying and Subsequent Storage

When a product is in its liquid state, the vapour pressure of the interphase between free water and vapour at equilibrium is saturated. Removal of water changes the vapour pressure at the interphase and subsequent changes in structures follow (Kessler, 1981). The amount of water in foods is categorised into three levels; monolayer water, multilayer water, and free water. Free water fills in the space within foods and can be easily removed by drying. Multilayer water is situated above monolayer water. Monolayer water is bound to the food substance of proteins and sugar groups through hydrogen bonding. Bound water is neither available to microbes nor as a solvent. During drying, both multilayer and free water can be removed except monolayer water. The drying periods therefore are characterised in two steps; the first is the removal of free water. After the complete removal of free water, drying still takes place at a slower rate, during which multilayer water is removed. The final point at which no further water can be removed is called the critical point. Drying beyond the critical point results in significant heat damage to the foods such as browning and loss of vitamins (Early, 1998).

Spray-drying is the atomization of liquid by passing a stream of hot air as quickly as possible, during which a glass state is formed as the final product as shown in Figure 3 (Roos, 2002). The powders exiting the spray dryer at glassy state should have a viscosity of $>10^{12}$ Pa.s to maintain

its solid-like properties. The amorphous powder is metastable; thus it can transform to rubbery state or even sticky liquid easily. For this reason, milk solids should be stored at temperature and water content below its glass temperature (Tg), known as Tg (Roos, 2002). In dehydrated milk, lactose exists in a supersaturated concentration where the Tg of SMP is shown in Figure 4. From the graph (Figure 4), it can be seen that the solid-like properties of amorphous lactose is highly dependent on storage temperature and water content. An increase in temperature causes the formation of lactose crystallization which results in an increase of release water and encapsulated fat to the matrix; thus water activity increases and non-enzymatic browning (Maillard reaction) is triggered (Roos, 2002). Faster colour changes as a consequence of non-enzymatic browning was observed in WMP containing higher initial level of a_w (0.35) (Baechler et al., 2005). The delay in crystallization of lactose can be achieved by decreasing the storage temperature as shown in Figure 5 (Vuataz, 2002).

Apart from temperature and moisture contents of the powder during storage, the presence of other milk components (e.g. proteins and fats) in the powder may delay the amorphous lactose crystallization as shown in the study of Listiohadi et al. (2005). The authors stated that caseins in milk powders may absorb moisture first prior to amorphous lactose and the moisture absorbed by caseins was less available to amorphous lactose; thus the competition in moisture adsorption between them may delay crystallization (Listiohadi et al., 2005).

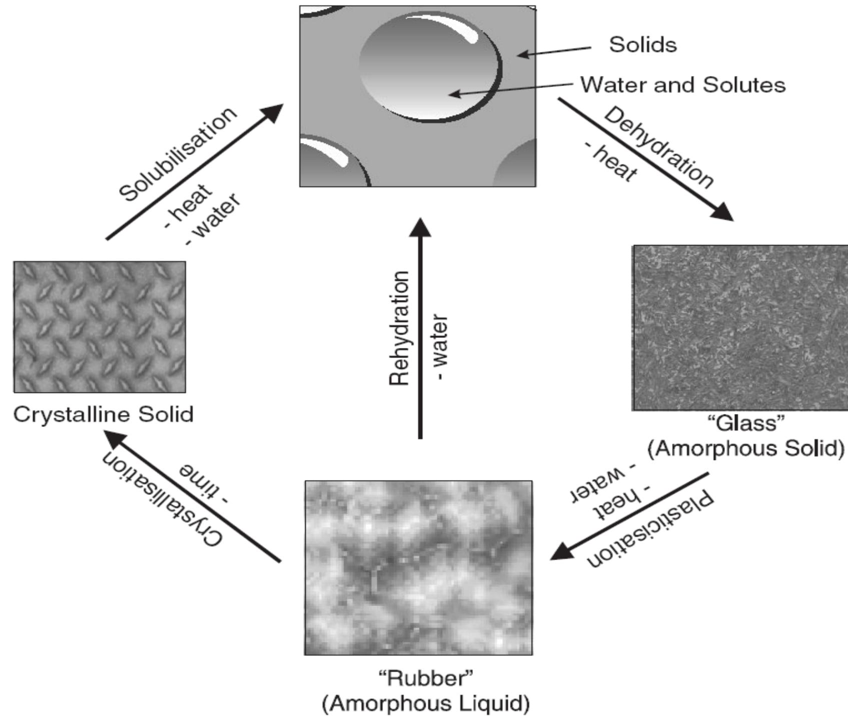


Figure 3. Formation of amorphous structures in dehydration and the relationships between equilibrium (solution, crystalline solid) and non-equilibrium (amorphous solid and liquid) states (Roos, 2002).

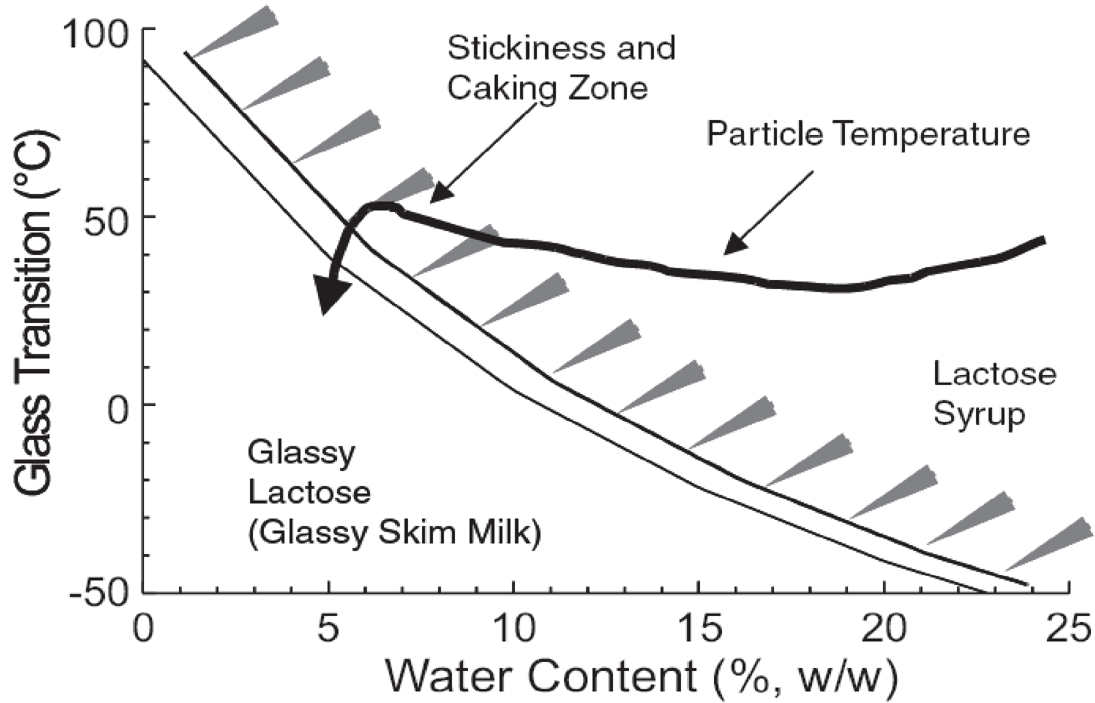


Figure 4. Glass transition of skim milk solids with a hypothetical particle temperature during water removal in spray drying, and formation of the glassy solid particles at the end of drying (Roos, 2002).

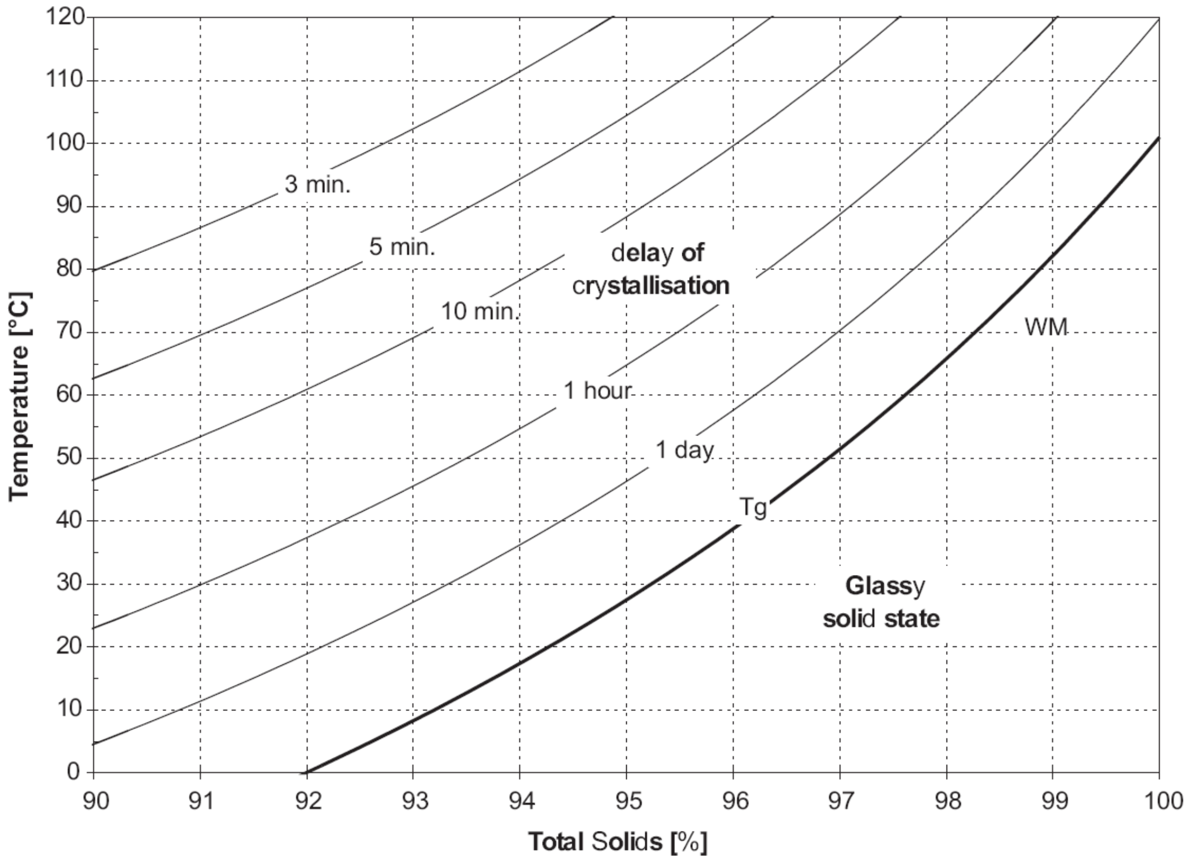


Figure 5. Dry region of the WMP state diagram (Vuataz, 2002).

3.5. Properties of Milk Powder

3.5.1. Bulk Density

The amount of weight in a unit volume of powder (g/mL) is defined as bulk density (Kelly et al., 2003). It measures the density of the air trapped between and within (called vacuoles) powder particles. The preheating and evaporation treatment applied to milk influences its bulk density properties; where milk powder having low lactose contents has more porous structures. If similar drying conditions were applied to SMP and WMP, the bulk density of WMP would be lower than SMP due to the higher density of protein and lactose as compared to fat. Furthermore, the degree of whey protein denaturation enhances the foaming properties of milk powder, presumably due to the unfolding properties of its tertiary form. Bulk density is also influenced by the atomization process, which governs the particle size distribution of the powder (Kelly et al., 2003).

3.5.2. Wettability

The ability of water to overcome the surface tension between the powder and water, so called "solubility" is defined as wettability. Pumping, homogenization, and the formation of lactose crystal affect the degree of wettability of milk powders to the extent of releasing fat, known as free fat (Kelly et al., 2003).

3.5.3. Flow-ability

The flow-ability of milk powder is defined as the ability of the powder to resist flowing. The flow-ability of SMP is higher than WMP due to the presence of fat content which resist the powder to flow. The decrease in particle size increases flow-ability of powders while an increase in moisture decreases its loose-ability properties (Kelly et al., 2003).

3.5.4. Whey Protein Nitrogen Index (WPNI)

The WPNI is a measure of whey protein denaturation in milk powders. The principle is based on the measurement of undenatured protein present in milk after heating. Basically, the precipitated denatured protein is retained through filtration and the filtrate is analysed for nitrogen content which is expressed as WPNI, the quantity of undenatured protein per gram of powder (Singh, 2007). WPNI is greatly influenced by preheating treatment applied before evaporation during milk powder manufacture (Kelly et al., 2003). The higher the heat-treatment, the higher the degree of whey-denaturation and the lower the WPNI values are. Depending on the extent of whey denaturation, WPNI values are classified into three classes; high-heat (WPNI $\leq$1.5), medium-heat ($1.5<\text{WPNI}<6$), and high-heat (WPNI $\geq$6) (Figure 6).

Economically, WPNI is a good indicator of whey protein levels in raw milk, especially during lactation period where protein level varies. It is also one of the important tools that give the analyst information about powder solubility and shelf-life. A higher degree of denaturation results in more protein aggregation and higher casein micelle sizes, but gives poorer powder solubility (Singh, 2007).

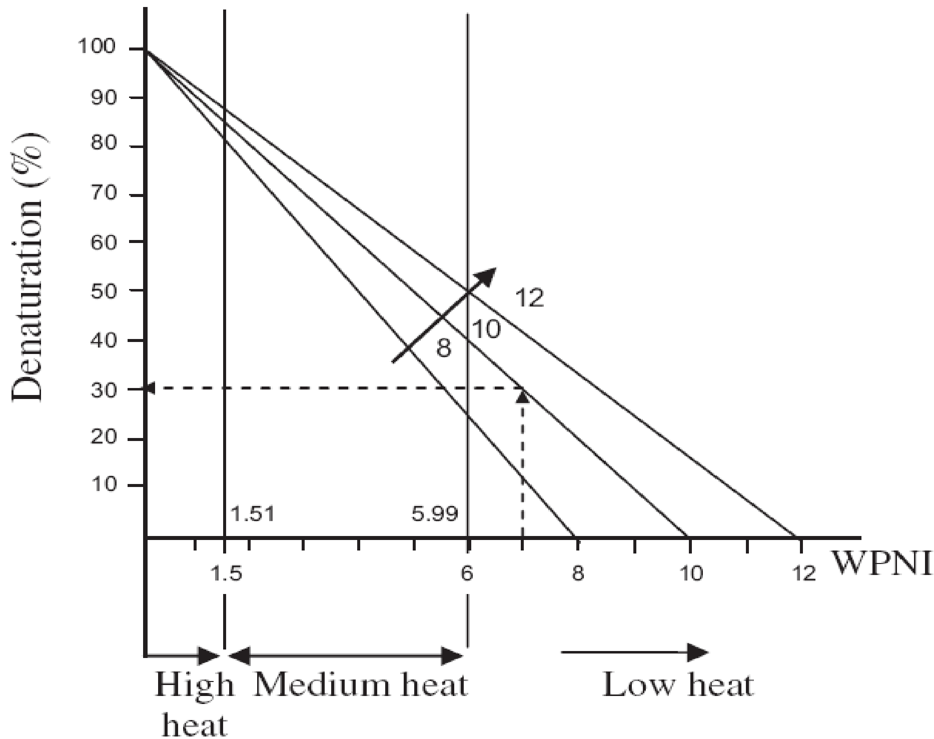


Figure 6. Denaturation and WPNI at varying levels of whey proteins in raw milk (Singh, 2007)

3.6. The Importance of Milk Powder Processing on Yogurt Quality

3.6.1. Heat-Treatment

The main purpose of heat-treatment on milk intended for yogurt manufacture is to eliminate pathogens and other competitive microorganisms to create a favourable environment for growth of yogurt cultures. It also reduce the oxygen content as well as provides more readily available amino acids to the cultures (Ozer, 2010). Depending on heat treatment, the type of milk is classified into UHT and pasteurised milk. The temperature may range from as low as 65°C to 150°C for few seconds. For yogurt manufacture, temperature of more than 70°C is recommended, not only for eliminating pathogens but to cause desirable changes (e.g. denaturation of whey protein, more available free amino acids) on whey protein (Özer, 2010; Tamime et al., 2007a). Chandan and O'Rell (2006) suggested using 97°C for 10 min for plain set yogurt without added stabilizers. For yogurt without added stabilizers, milk proteins are the only components responsible for affecting the viscosity of the final product. The rate of whey protein denaturation varies depending on heating temperature and lactose concentration. Heat-treatment

above 70°C results in irreversible changes on whey protein denaturation. The attachment of glucosyl residue to α-lactalbumin stabilises the protein against heat-treatment. Therefore, temperatures above 90°C for 10 min are usually used in yogurt manufacture to break the bond between lactose and whey protein (Chandan, 2006). As a consequence of heating, the heat-sensitive whey protein becomes unstable and interacts with other milk proteins to form gel network, which normally occurs at pH 4.5 (Tamime, 2007). Insoluble whey proteins bind a lot of water and can coagulate milk to form a gel which has improved water retention capability. The firmer gel structure and a reduction of whey syneresis are important factors for quality of yogurt (Spreer, 1998). It was reported that heat-treated milk has shorter gelation time compared to unheated milk (Özer, 2010; Tamime et al., 2007a). The fact that a larger volume of casein-fat globules is formed after homogenization, heating may further modify the structure of casein-fat globules by displacing the caseins (fat globules are surrounded by caseins) with β-lactoglobulin to form β-lactoglobulin-fat interaction (Tamime, 2007). The interaction between β-lactoglobulin and α-lactalbumin increases the hydrophilicity of yogurt milk which contributes to faster gel formation (Tamime, 2007).

Although casein is more heat-tolerant than whey protein, changes in casein micelles are inevitable. In heat-treated milk, an increase in casein particle size and formation of chains leads to strong gel networks, which impair mobilization of aqueous phase resulting in the reduction of syneresis and improve viscosity. The denatured whey protein (β-lactoglobulin) may react with the casein micelles (κ-caseins) and forms casein aggregates through -SH and -SS bonds (Özer, 2010; Tamime, 2007; Tamime et al., 2007a). The interaction of these proteins results in larger and more complex caseins aggregates which traps more water during the gelation of yogurt due to enhanced hydrophilic capacity which is desirable for improving gelling properties of yogurt and reducing syneresis. Milk heated at 82°C for 30 min gives optimum denaturation of proteins and results in better yogurt quality compared to very high temperature (e.g. 149°C for 3 s) treatment (Tamime, 2007). In unheated milk, casein micelles aggregate to form clusters. This creates more spaces for water and aqueous spaces to mobilise within the matrix and as a consequence, the product is more susceptible to syneresis (Özer, 2010; Tamime et al., 2007a).

Heat-treatment may also produce stimulatory/inhibitory effects (e.g. cysteine) to yogurt cultures; depending on the type of strains and the metabolic activity of those strains. The proteolytic activity of starter bacteria decreases with increasing temperature (e.g. heat treatment applied to milk) (Tamime, 2007; Tamime and Robinson, 2007). Furthermore, heating (85°C for 5-10 min) can decompose lactose to formic acid. Formic acid is an essential nutrient for growth of LB during fermentation in yogurt manufacture (Walstra et al., 2006).

Apart from protein denaturation, heating may also cause some changes on other milk components such as Maillard reaction and destruction of heat-labile vitamins (e.g. B_1, B_{12}, and C) and folic acid (Walstra et al., 2006). Maillard reaction is a reaction between amino acids (lysine) and sugar (lactose) under heat and can cause browning as well as changes in taste and smell (Spreer, 1998). More importantly, heating may affect balance of milk salts, particularly calcium phosphate. In native state, calcium may exist as soluble ions or in colloidal phase of caseins. As temperature increases and pH decreases, the amount of soluble salts declines and more salts are observed in casein submicelles (Tamime, 2007). A series of chemical and physical changes as a consequence of heating are shown in Table 1.

Table 1. Chemical and physical effects of heat on yogurt milk (Tamime and Robinson, 2007).

Milk constituent	Heat-induced changes	Relevance in yoghurt manufacture	Consequences for yoghurt
Nitrogenous			
Whey proteins	Denaturation and aggregation, inactivation of immunoglobulins	Almost complete	Destruction of lactenins, reduction in creaming ability
	Active SH group production	Maximum at 90 ºC/10 min	Cooked flavour, lowering of Eh formation of antioxidant properties
	α-La and β-Lg interaction	Occurs before and/or interaction with κ-casein	Contributes to gel stability
	β-Lg and κ-casein interaction	Very significant	Minimises syneresis, increases micelle size, stabilises gel
Casein	Partial hydrolysis, release of glycopeptide from κ-casein	Of limited significance	Slight increase in free amino acids and peptides
	Dephosphorylation	Very little	Slight redistribution of phosphorus
	Aggregation, disaggregation, interchain cross-linking, e.g. by isopeptide bonding	Occurs especially with smaller micelles	Increase in micelle size and formation of protein network
Enzymes	Inactivation	Destruction of lipases and proteases from milk and bacteria	Minimises rancid and bitter off-flavours
Other	Decomposition of amino acids to flavour compounds	Significant effect	Contributes to flavour
	Amino acid lasctose interaction, Maillard reaction, Schiff's base formation, reduction in available lysine	Occurs to only small degree, e.g. lysine loss *c.* 0.3%	Slight decrease in nutritive value, significant where yoghurt fortified with high-heat powders and concentrates
	Amino acid–amino acid interaction, e.g. formation of lysine–alanine	Occurs to a limited degree	Minimal
Carbohydrates			
Lactose	Decomposition to form organic acids, furfural and hydroxymethylfurfural	Occurs to small extent	Reduces pH and Eh, produces formic acid and affects growth of starter cultures, contributes to yoghurt flavour
	Reaction with amino acids (see above)		
Other	Decrease in sialic acid and hexosamines, increase in hexoses	Occurs at 85 ºC for 10 min	Unknown
Miscellaneous			
Fat	Formation of lactones, methyl ketones and other volatile ketones	Occurs to small degree	Contributes to flavour
	Hydrolysis	Insignificant	Insignificant
Vitamins	Destruction of some water-soluble vitamins	C, B_1, B_6, B_{12}, folic acid, reduced	Reduction in nutritive value
Minerals	Redistribution of Ca, P, Mg between soluble and colloidal forms	Significant effect, modifies surface structure of casein micelle	Reduces pH, affects curd particles, decreases coagulation time
Microorganisms	Destruction	Elimination of pathogens and other organisms	Ensures public safety and minimises quality defects
Gases	Reduction in level of dissolved oxygen, nitrogen and carbon dioxide	Produces micro-aerophilic environment for starter culture	Ensures public safety and minimises quality defects

3.6.2. Homogenization

The density of milk fat globules is lower than the aqueous phase (water) and for that reason, fat would form clusters which rise to the surface and form cream when milk is left to stand (e.g. during yogurt fermentation). To avoid such problems, milk is subjected to high speed homogenizer under high pressure (100-250 bar) at low temperature (55-80°C), then forcing it

through small orifice (Özer, 2010; Tamime and Robinson, 2007). Lower homogenization temperature (<40°C) may cause partial crystallization of milk fat and therefore should be avoided at all means (Walstra et al., 2006). Homogenization reduces fat globule sizes and increases the surface area, which results in an increase of adsorption efficiency with milk proteins, particularly casein micelles, therefore altering the milk colour to become whiter and enhance the amount of suspended matter which leads to viscosity improvement (Özer, 2010; Tamime and Robinson, 2007). The amount of adsorbed caseins decreases with increasing homogenizing temperature (Mulder & Walstra, 1974).

The newly formed protein-fat globules (coalescence) contain caseins, to the greater part, at the surface and some serum protein. Fat globules with smaller diameter size adsorb more casein compared to the globules having larger radii. Further, increasing homogenization temperature results in faster spreading rate of casein micelles to the fat globule surface (Walstra et al., 2006).

3.7. The Effect of Blending and Post-blending on Powder Properties

Raw materials required in producing the dehydrated yogurt bases (DYB) were blended at manufacturing plant for the current study. All the ingredients were placed on top of a convection blender of the ribbon type-blender (100 kg capacity) which utilises gravity to move the particles into a bottle-neck storage bin. The blending cycles lasted for about 30 min in which agitator design and speed of the propeller play important roles in mixture homogeneity (Maynard, 2008). During blending, particles are moved rapidly from one location to another thus particle collision is unavoidable. When this occurs, particles with larger size may break and agglomeration or coating may take place (Maynard, 2008). A blend of smaller particles of identical size to form larger particles will not segregate after discharge from the blender. However, if the size is not homogenous, disaggregation may occur and can cause problems in bulk density or reactivity later on (Maynard, 2008). Further, a blend may consist of similar particles which have the tendency to not attach to dissimilar particles. In this case, the blend can reach saturation and may disintegrate (Maynard, 2008).

When the particle mixtures are not similar in size, powder with larger density may sink to the bottom of the blender thus giving rise to variation between sample sachets of the same batch production. It is crucial that dry mixtures are used timely (e.g. samples should not be stored for too long post-blending) (Maynard, 2008). Another important factor worth considering is the charge between powders and surface of the materials, e.g. stainless steel. Friction and collision that occur during blending may alter charge of each material. Coarse particles have positive charge and fine particles are negatively charged but all particles become negatively charged when relative humidity increases (Bailey, 1993). An increase in temperature is inevitable during blending. Particle attachment to the surface of materials may therefore contribute to the homogeneity of the powder mixes. A good practice of sub-sampling from three different locations of the hopper to determine particle distribution may be useful as if particle distribution is not optimal as it can facilitate dry-blending before packaging (Bailey, 1993; Maynard, 2008). Further, consideration on the time, rate of mixing, and the energy input during mixing is advisable (Earle, 1983).

4. THE SCIENCE AND TECHNOLOGY OF YOGURT-MAKING

4.1. Yogurt Manufacture

According to the physical nature of the product, commercial yogurts are classified into three main categories; set, stirred, and drinking, the latter is often referred as stirred yogurt of low viscosity. The main difference between the products is the type of incubation. Set-yogurt is incubated in the packaging container while stirred-type yogurt is incubated in the large manufacturing vat prior to packaging as illustrated in Figure 7. Depending on the manufacturers' preferences, milk used for yogurt manufacture could be either from fresh milk, powder, or combination of both (Robinson et al., 2006; Tamime and Robinson, 2007).

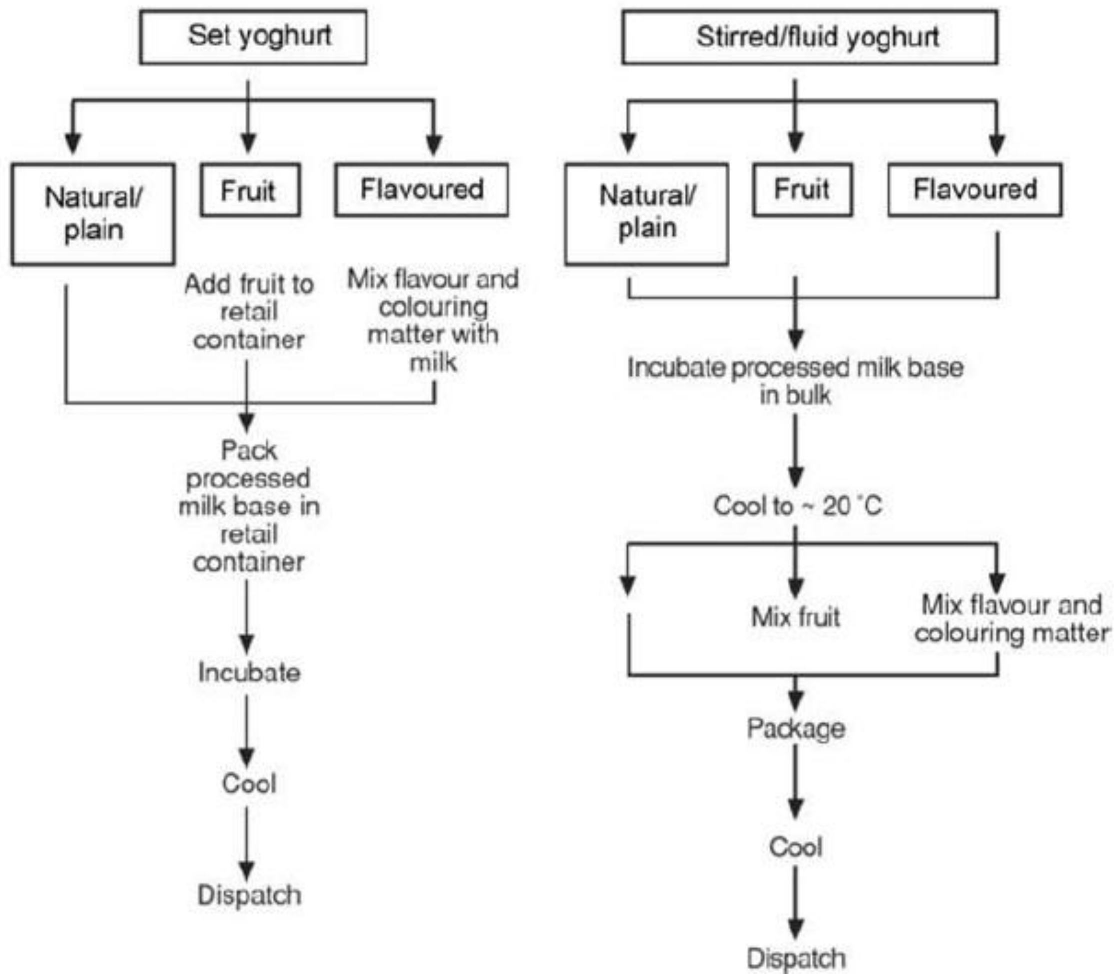


Figure 7. Generalised manufacturing stages of ready-to-eat set and stirred yogurt based on Tamime and Robinson (2007).

Many yogurt products are available in the market as liquid system, and only few are in its dehydrated form. This may explain why only few studies on DYB are available. Although DYB does not gain popularity as ready-to-eat yogurts, yogurt powder may be equally important in the developing countries where many do not have a cooler as food storage. In the current study, consumers at home ferment yogurt mix. In another study (Wang et al., 2004), manufacturer has already fermented the yogurt mix powder and consumers just need to reconstitute them with water.

4.1.1. Standardization of Yogurt Base (Milk)

As milk composition varies according to the season of the year, standardization of yogurt base is of critical importance for yogurt manufacture to maintain consistency of product. As mentioned earlier (chapter 3), milk solids non-fat (MSNF) and lipids are important attributes of yogurt profile. Fat gives the luxury mouth-feel taste while MSNF is important for texture. Typically, fat content and MSNF in yogurt range between 1-4.5 g/100 mL and 12-18 g/100 mL respectively, but they may be adjusted in order to meet existing or proposed standards or target consumers (Robinson *et al*., 2006). According to Ozer (2010), total milk solids of high quality yogurt ranges between 18 and 22% to support the growth of *S. thermophilus* and *L. bulgaricus*, which is optimum at SNF level of 14% and 12% respectively. An increase of milk solids in yogurt is also believed to improve viscosity, mouth-feel, texture and taste (Ozer, 2010).

Addition of milk powder to the product is a common practice applied to increase the milk solids content (e.g. to enrich protein level). Whole milk or skim milk powder can be added but skim milk powder (SMP) is preferred over whole milk powder (WMP) due to potentially lower fat oxidation issues. Typically, the fortification of MSNF with SMP is applied at concentration levels of 3-4% as excessive addition of SMP may result in lumpiness and powdery taste. Although it is not a common practice at industrial scale, addition or reconstitution of skim milk powder with buttermilk powder is possible (Ozer, 2010). Standardization of fat in yogurt can be done easily using the Pearson's square to determine the desired fat content (Tamime and Robinson, 2007).

Another way of milk fortification in yogurt manufacture is the use of whey protein powders. The incorporation of whey protein (WP) to fortify milk is limited in yogurt manufacture to the extent of texture and overall sensory appearance. High level fortification of WP (50% of SMP and 50% of WP) results in a yellowish colour and reduced viscosity, due to lower protein content (6%) of WP compared to SMP (34%) (Sodini and Tong, 2006). Therefore, to minimize the risk of undesirable physical properties in yogurt, 2-3% WP and 20-25% whey protein concentrate (WPC) of total solids fortification level has been recommended by Ozer (2010).

The use of sodium or calcium caseinates, singly or in combination with whey protein concentrate to maintain the milk serum and casseinates ratio has also been applied in yogurt manufacture to increase milk solids. The ratio of serum protein to caseinates of cow's milk is about 20:80. The addition of caseinates inclusive of WPC reduces fermentation time and graininess, forms a more open and less branched structure and improves the viscosity (Ozer, 2010; Sodini and Tong, 2006).

4.1.2. Addition of Stabilizer (Optional)

The main purpose of adding stabilizers in yogurt is to improve rheological and textural properties. The mode of action of stabilizers in yogurt is water binding to retard the movement of water within the protein gel network leaving less free water for syneresis. As a result, protein network is stabilized and viscosity is improved. In some cases, hydrocolloids may lead to gel formation. Stabilizers may improve mouth-feel, act as fat substitutes, and thus maintaining low levels of calories of the product (Ozer, 2010). Stabilizers may be reconstituted for low quality yogurt. Small scale yogurt manufacturer relies on stabilizers to achieve the desired texture and sensory appearance for their products instead of milk solids, which can be costly in some countries. The concentration of stabilizers added to yogurt varies depending on the purpose (Table 2). Generally, the use of stabilisers is about 2% because the desired functionality is achieved at these level (Fennema, 1996).

Further, stabilizers may form complexes with caseins and decrease the casein micelles size to become denser and shorter, which eventually leads to viscosity improvement (Ozer, 2010).

Irrespective of the purpose, stabilizers in yogurt should be effective in high acid environment (e.g. high ester pectin), easily soluble at normal incubation temperature, do not impart any negative effects on the food system. More importantly, stabilizers should not affect the metabolic activity of the yogurt cultures (Chandan, 2006; Fennema, 1996; Tamime and Robinson, 2007). As stabilizers will not be used in the current study, it will not be further discussed.

Table 2. Concentration of stabilizers used in yogurt (Özer, 2010).

Stabilizers	Concentration (%)
Pectin or modified starch	0.02-0.7
Pectin	0.05
Agar-agar, guar gum, alginate, gelatine	0.05-0.6
Carrageenan or carboxymethylcellulose	0.05-0.6
Starch preparations	1.00-2.00
Guar gum	0.1-0.5
Sugar beet fibre	0.5-2.00
Gelodan	0.35
Na-alginate	0.30
Na-alginate + β-cyclodextrin	0.2 + 0.1

4.1.3. Addition of Sweetener (Optional)

In terms of nutrition, sucrose is one of the three major sugars (lactose and starch) that can be hydrolysed to glucose and fructose by the enzyme sucrase in the human intestinal tract as source of energy (Fennema, 1996).

Sweetener is often added in yogurt to mask the acid flavour as an option for people who cannot bear the strong acid taste of unsweetened yogurt (Chandan, 2006). The amount of sweetening agent used varies according to yogurt ingredients and cultures. Sucrose, in either liquid or granulated form, is the most common sweetener used in yogurt manufacture at concentration levels between 8 and 13% (Chandan, 2006). As sugar is highly hygroscopic, excess amount of sweetening agents used can lower the water activity required for growth of cultures. Thus, it is necessary to maintain the recommended total solids ratio (Chandan, 2006; Tamime and Robinson, 2007). Ozer (2010) recommended that the addition of sucrose should not exceed 10% as it may suppress the growth of yogurt cultures (Table 4). However, Akin et al. (2007)

contradicted the report of Ozer (2010) by indicating that better survival of *L. acidophilus* at a sugar level of 18%. For set yogurt, sweetener is commonly added before fermentation proceeds, while in stirred yogurt, it is added just before packaging. For the weight-conscious consumer, artificial sweeteners can be used to replace the use of nutritive sweetener (Özer, 2010).

There are many food grade sweetening agents (Table 3) available nowadays, and their degree of sweetness varies accordingly. For the sake of simplicity and clarity, the term "dextrose equivalent" (DE) is applied to indicate the percentage of reducing sugar calculated as dextrose (glucose). Lower DE value indicates higher molecular weight of product and lower sweetness intensity. If the sweetness intensity of sucrose is defined as 1, then the sweetness intensity of dextrose is 0.8. This system is used as hydrolysis of sucrose yields different products (e.g. corn syrups, maltose syrups, high fructose corn syrups, crystalline fructose, etc) at various sweetness intensity levels (Chandan, 2006; Tamime and Robinson, 2007). The sugar alcohol sweetener (dulcitol, sorbitol, and mannitol) listed in Table 3 are hygroscopic. Such sweetener is not completely non-nutritive, however it contributes to less calories compared to nutritive sweetener such as sucrose. For this reason, it can be used in diets for individuals with special dietary requirements (e.g. diabetics). Moreover, such sweeteners can be used in dehydrated foods to improve rehydration properties of the dried products (Fennema, 1996).

Table 3. Sweetening agents and their intensity of sweetness relative to sucrose (Fennema, 1996; Tamime and Robinson, 2007).

Sweetening compound	Relative sweetness: sucrose = 1
Lactose	0.4
Dulcitol	0.4
Maltose	0.4
Sorbitol	0.5
Mannose	0.6
Galactose	0.6
Glucose	0.7
Xylose	0.7
Mannitol	0.7
Glycine	0.7
Invert sugar	0.7-0.9
Glycerol	0.8
Sucrose	1.0
Fructose	1.1-1.5
Cyclamate	30-80
Acesulfame K	150-200
Aspartame	200
Saccharine	240-350
Sucralose	600
Neohesperidin DC	1500-2000
Alitame	2000
Thaumatin	3000

Notes: sweetness intensity of sucrose is 1

Table 4. Sweeteners and their inhibitory effects on yogurt starter microorganisms (Özer, (2010).

Sweeteners	Inhibitory Effect (%)
Sucrose	>4.00
Fructose	>2.70
Aspartame	>0.02
Fructo-oligosaccharides	>7.30
Isomalto-oligosaccharides	>7.70

4.1.4. Addition of Colorant, Flavouring, and Preservatives (Optional)

The addition of colorant, flavouring, and preservatives is discretionary in NZ (FSANZ, 2011). Yogurt is categorised as a low risk products in terms of microbial contamination due to its acidic environment that suppresses other microbial growth. However, yeast and moulds can grow in

such conditions and are the spoilage microorganisms in yogurt-making. Sorbic acid, sulphur dioxide, and benzoic acid are common preservatives used in yogurt to suppress growth of yeast and moulds. The levels and type of preservatives used differ according to preference of the manufacturer, but the maximum limit of such preservatives should not exceed 50 mg/kg (singly or combination) according to the legal limit set by FAO/WHO (Tamime and Robinson, 2007). The FSANZ (2011) permit the use of sulphur dioxide, sorbic and benzoic acid up to 350 mg/kg and 1000 mg/kg respectively, depending on the type of the products. The FSANZ standard 13.1 (2011) lists the permitted additives that can be added to fermented milks. According to the FSANZ (2011), the colorant should not be more than 290 mg/kg in foods while 70 mg/L is the maximum limit for beverage products.

4.1.5. Homogenization

The purpose of milk homogenization is to decrease the size of fat globules. In yogurt making, homogenization using pressures of 15-20 MPa at 65-70°C is critical to stabilise the oil-in-water emulsion. Before homogenization, milk fat globules in their native state (raw milk) are encapsulated within the protein and phospholipid membrane. Homogenization breaks the lipid membrane thus reducing its size. The newly formed small fat globules interact with casein micelles and other milk components to form a new membrane which differs in composition from its native state. The interaction increases water-holding capacity, yogurt viscosity and enhances light reflection, which makes milk appear whiter (Tamime and Robinson, 2007). The effects of homogenization on milk were discussed earlier in section 3.6.2.

4.1.6. Heating

It is still arguable to heat yogurt mix before or after homogenization due to contamination issue, however homogenization prior to heating is widely practiced to avoid contamination (Tamime and Robinson, 2007). The importance of heating of milk in yogurt-making was discussed earlier in section 3.6.1.

4.1.7. Fermentation

Following homogenization and heating, yogurt base is cooled to 40-45°C, which is the optimum temperature for growth of the cultures. Although the growth of bacteria may vary between products, inoculation of starter cultures (bulk or freeze-dried) usually consists of a well-balanced ratio (1:1) of *S. thermophilus* and *L. bulgaricus* (Tamime and Robinson, 2007). Fermentation usually takes place at 42-43°C and is stopped when the pH reaches 4.5-4.6. In the case where probiotics are added, incubation temperature may be reduced to 37°C depending on the optimum temperature of the probiotic cultures to facilitate the growth of the probiotic bacteria (Ozer, 2010).

During fermentation, microbiological, chemical, and physical changes occur simultaneously. In terms of microbiological changes, the relationship between *S. thermophilus* (ST) and *L. bulgaricus* (LB) is symbiotic. The ratio of ST and LB during fermentation changes constantly. The proteinase activity of ST is less vigorous than LB; therefore at early stages of fermentation, ST utilizes the available amino acids present in milk to produce formic acid and CO_2, which are the prime sources for LB growth. Concurrently, LB produces small peptides and amino acids (e.g. valine) for the growth of ST (Kessler, 1981). LB exhibits strong activity of proteases and cleaves milk β-casein (preferred nitrogen source) to support the growth of ST. At early fermentation, ST grows quickly. The synergistic relationship between the two cultures ensures rapid conversion of lactose and other available sugars to lactic acid within 3.5-4 hours. Concomitantly, the slower activity of ST as a consequence of the increase in lactic acid creates an optimum acid condition for growth of LB. The ratio between traditional yogurt bacteria after approximately 3 h of fermentation should therefore be equal (1:1 ratio) (Lourens-Hattingh & Viljoen, 2001; Ozer, 2010). When the pH has reached 4.5 - 4.6, the product has to be cooled immediately to prevent over-acidification defects. Similarly, if the fermentation period is too short, the unique acidity of yogurt will not be produced due to imbalanced ratio between the cocci and the rods. Generally, the composition of the acids in yogurt is as follows; 58.9% lactic acid, 28.1% citric acid, 5.3% acetic acid, 2.4% formic acid, and 2.3% succinic acid (Kessler, 1981). Apart from acid production, a small amount of CO_2 and ethanol may also be produced during hetero-fermentation of yogurt (Walstra et al., 2006).

In terms of physical changes, fermentation also determines viscosity and gel strength of the final product. Lower incubation temperature prolongs gelation time resulting in firmer, more viscous, and less syneresis in products. However, the formation of aroma compounds such as acetaldehyde may be weakened at lower temperature (Özer, 2010; Tamime and Robinson, 2007). Acetaldehyde, although present in a small proportion (10 mg/kg), is crucial for yogurt aroma. The acetaldehyde imparts the characteristics yogurt flavour in the product. The precursor for acetaldehyde is threonine, which is produced by LB through proteolysis (Rysstad et al., 1990). Diacetyl (0.8-1.5 mg/kg) is another aroma compound produced by ST from pyruvate as a result of glucose fermentation (Walstra et al., 2006). Another important aroma precursor for acetaldehyde and diacetyl is citrate, which is completely degraded (>99%) during yogurt fermentation (Hugenholtz, 1993; Kneifel et al., 1992). Prior to incubation, surface charge of casein is highly negative at pH 6.7. As fermentation proceeds and pH decreases, the calcium phosphate bonds which link the casein sub-micelles break and casein starts to aggregate and form a gel through hydrophobic bonding as a consequence of surface charge reduction to no net charge. Gel formation occurs at around pH 5.3 in heated milk and at pH 5 in unheated milk. This is owing to the high β-lactoglobulin isoelectric point (pH ~5.3) and its interaction with κ-casein as complexes of β-lactoglobulin/κ-casein initiate gelation. Concomitantly, destabilization of casein micelles as a consequence of calcium phosphate solubilisation at higher pH in heat treated milk explains this difference (Tamime and Robinson, 2007). The interaction between β-lactoglobulin and α-lactalbumin with κ-casein prevents the formation of coarse gel aggregation which produces smooth gel network, that entraps water and other milk compounds (Ozer, 2010).

With respect to chemical changes, the total free amino acid content increases due to the proteolytic activity of the lactic bacteria although the total amino acids in yogurt do not differ substantially from milk. The concentration of lactic acid, galactose, and fatty acids increase; in contrast, the level of lactose decreases due to its metabolism by yogurt bacteria. The level of vitamins may also adversely change. While some bacteria require vitamin B for growth, concurrently several other vitamins are synthesized. Fermentation has little impact on the total mineral contents (Lourens-Hattingh & Viljoen, 2001). Further, polysaccharides chain may also be produced during fermentation by the traditional yogurt cultures (ST and LB). The polysaccharides can then be partially secreted into liquid, called exopolysaccharides, which may

influence the yogurt consistency. However, the production of exopolysaccharides are strain dependent and the substance does not always affect yogurt consistency (Walstra et al., 2006).

4.1.8. Cooling

Chilling is aimed to slow down the growth and metabolic activity of cultures so that excess acid production can be prevented. Another important role of cooling in set-yogurt is to improve the texture. Tamime and Robinson (2007) recommended large installations to cool the yogurt in two stages to avoid temperature shock, which may increase syneresis during storage. The first cooling commences from incubation temperature to 24°C followed by packaging, then further cooling to 10°C in the first 6 hours and continues to 1-2°C for the remaining cooling period.

4.1.9. Packaging and Transportation

Packaging is another important step in yogurt manufacture, not only because it gives protection from contamination but also minimizes the gaseous exchange between inside and outside air. This is crucial in yogurt containing probiotics, as oxygen exposure may greatly influence the survival of microorganisms. More importantly, the packaging has to be acid-resistant and prevent loss of volatile flavours. Suitable primary (inner) packaging materials for yogurt include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC) and polyvinylidene chloride (PVDC) with or without combination of other materials such as aluminium foil (Tamime and Robinson, 2007). The other important factors that can be considered in choosing the packaging materials include strength, flexibility, sealing-ability, and resistance to heat and freezing (Walstra et al., 1999). Aseptic packing may also be applied online to reduce contamination (Walstra et al., 1999). Secondary (outer) packaging is also required to ease handling and transportation. The most widely used secondary packaging is semi-rigid plastic crates and cardboard trays, which are stacked in wooden pallets to be transported using fork-lifts. Refrigeration storage and transport are compulsory for transporting the product before it reaches consumers. This ensures minimum biological and chemical reactions which can cause quality defects (Tamime and Robinson, 2007).

4.2. Selection of Starter Cultures

Strain selection is a main aspect that has to be considered for yogurt making. It dictates the sensory, physical and microbiological quality of the final product. Culture characterisation determines the viscosity, flavour, aroma, texture, and acidity perception while synergistic effects of mixed cultures may influence the cultures' viability in food system. Therefore it is important to use compatible blends of probiotics and lactic starter cultures. Maintaining the viability of probiotics during fermentation and storage is challenging as the bacteria may die due to the environmental changes (e.g. acid accumulation, antimicrobial production) occur during the process. For this reason, it is not unusual to use high initial inocula rate (5-10 mL/100 mL) of probiotics, compared to starter cultures (1 mL/100 mL) (Tamime et al., 2005).

Generally, *Lactobacillus* species (*L. bulgaricus* and *L. acidophilus*) and *Streptococcus thermophilus* employed for yogurt manufacture are categorised as homo-fermenters (Sellars, 1991). Homofermenters utilise (only) hexoses to produce more than 85% lactic acid via the Embden-Meyerhof (glycolysis) pathway; whereas heterofermenters utilise hexoses and pentoses to produce a mixture of lactic acid (50%) and equimolar amount of CO_2, ethanol, acetic acid, and formic acid via the phosphoketolase pathway (Sellars, 1991; Trahan, 2008). During homo-fermentation, lactose is mediated inside bacterial cells and cleaved by β-galactosidase into glucose and galactose. Glucose and galactose are metabolized simultaneously through glycolytic and tagatose 6-pathways respectively (Lourens-Hattingh & Viljoen, 2001). The glucose enters the glycolytic pathway to produce pyruvate, which is further metabolized into lactic acid. The galactose and lactic acid are transported outside the cells and accumulate in the medium. Galactose is then further metabolized by *S. thermophilus* (strain specific) into lactic acid (Robinson et al., 2002). The galactose can then be metabolized by LB. However, the utilization of galactose by LB is slow due to the low production of the enzymes required for galactose hydrolysis. For this reason, galactose may present abundantly in yogurt (Lourens-Hattingh & Viljoen, 2001). In yogurt, LB is useful not only as starter cultures but also as an acetaldehyde producer, which is responsible for the unique distinctive yogurt taste (Sellars, 1991). The characteristics of yogurt cultures used in the current study are shown in Table 5.

Table 5. Characteristics of yogurt cultures (Sellars, 1991, Tamime and Robinson, 2007)

Lactic species	Growth temperatures		Metabolism of sugars			
	10°C	45°C	Sucrose	Lactose	Galactose	Fructose
L. bulgaricus	-	+	-	+	-	+
L. acidophilus	-	+	+	+	+	+
S. thermophilus	-	+	+	+	-	+

Notes: (+) indicates normal growth; (-) indicates no growth

4.2.1. *Streptococcus thermophilus* (ST)

S. thermophilus is a facultative anaerobe (can use oxygen) and shows a spherical or ovoid shape in pairs or chains under the microscope. ST is a Gram-positive homofermenter, mainly produces lactic acid but also produce acetaldehyde and diacetyl from lactose (Tamime and Robinson, 2007). The importance of ST in fermented milk is not only limited to its acid production, but also produces relevant aroma and flavour as well as the lactase activity (IDF, 2002; Sellars, 1991).

4.2.2. *Lactobacillus bulgaricus* (LB)

The full nomenclature for this bacterium is *Lactobacillus delbrueckii* subsp. *bulgaricus* (Tamime and Robinson, 2007). This obligates homofermentative microorganism forms rods with rounded end and occurs in singly or in short chains under the microscope (Tamime and Robinson, 2007). LB was reported to be more acidic tolerant than ST (Robinson et al., 2006).

4.2.3. Probiotics activity

Probiotics are widely accepted by consumers nowadays due to their health beneficial effects. As mentioned in Chapter 2, the benefits exerted by consumption of probiotics are strain specific due to their unique properties. Of the commercially available probiotic products in the market, many of them contain *Bifidobacteria* and *Lactobacillus* species (Lee & Salminen, 2009) because they are typically human intestinal origin and have been approved as Generally Recognize as Safe (GRAS) (Crittenden et al., 2005). The commercially available probiotic microorganisms to date

include *L. acidophilus* LA-5, L. *acidophilus* NCDO, *L. acidophilus* NCFM, *L. casei* Shirota, *L. gasseri* OLL2716 (LG 21), *L. paracasei* ssp. *Paracasei* F19, *L. casei* 431, *L. rhamnosus* GG, *L. rhamnosus* GR-1 + *L. reuteri* RC-14, *B. animalis* ssp. *lactis* BB-12, *B. breve* Yakult, *B. longum* strain BB536 and strain BL46 (Lee & Salminen, 2009). Traditional fermented foods (e.g. cheese) and drinks (e.g. yogurts) are vehicles for delivering probiotics (Ibrahim et al., 2010). When probiotics are not incorporated into foods, they can be consumed as freeze dried powder (e.g. milk infant formula) or capsules. In New Zealand, Ethical Nutrients© is one of the leading companies that sells probiotic powder and capsules (www.ethicalnutrients.com.au). The product range varies from reducing symptoms of travel diarrhoea, eczema in children, or for well balanced of intestinal microflora. The use and potential market of probiotics on New Zealand consumers is summarised in the survey of Schultz et al. (2011). In Asia and the northern hemisphere, leading probiotics companies such as Yakult™ and Danone™ have sold large quantities of their products. Irrespective of the type of products, selection of bacteria which have "true probiotics" attributes (Chapter 2) is of great importance. Nevertheless, it is equivocally important that the bacteria promote such benefits upon consumption. Recent well-established human studies on the benefits of probiotics are shown in Table 6. While few studies conducted on the effect of probiotics were inconclusive on their beneficial advantages (Moran et al., 2011; Sari et al., 2011), the importance of strain selection is still important.

Table 6. Human studies on the beneficial effects of probiotics

Beneficial effects	Probiotics Strain	Reference
HIV treatments	*L. rhamnosus* GR-1	(Dols et al., 2011)
Increase antioxidant activity in endurance athletes	L. *rhamnosus* IMC 501® & L. *paracasei* IMC 502®	(Martarelli et al., 2011)
Reduction in frequency and pain severity of irritable bowel syndrome (IBS) in children	*L. rhamnosus* strain GG	(Brown, 2011)
Reduction in constipation-IBS related	*S. thermophilus, L. acidophilus* & *Bifidobacterium* (B) *infantis*	(Choi et al., 2011)
Improvement in atomic dermatitis	heat-killed *L. paracasei* K71	(Moroi et al., 2011)
	Lactobacillus GG	(Majamaa & Isolauri, 1996)
Stimulate growth of indigenous LAB	*L. acidophilus* NCFM®	(Sui et al., 2002)
	L. rhamnosus DR20	(Tannock et al., 2000)
Enhance immunity	*B. lactis* Bb-12	(Fukushima et al., 1998)
	Lactobacillus GG	(Kaila et al., 1992)
	L. acidophilus La1	(Link-Amster et al.,1994)
Folate production (*in vitro*)	*B. breve, B. infantis*, & *B. longum*	(Rossi et al., 2011)

***Lactobacillus acidophilus* (LA) NCFM**

To date, there are few *Lactobacillus* species which can potentially be used as probiotics in yogurts. However the use of such probiotics must suit the food system of the intended product and more importantly, they must survive the harsh condition of the human gut to impact beneficial effects (Lee & Salminen, 2009). The *Lactobacillus acidophilus* NCFM is the only probiotic strain of *Lactobacillus* species for which the genome sequence has been fully sequenced and annotated (Sanders & Klaenhammer, 2001). The strain has also been used in yogurt, where 80% of the commercial yogurts sold in the USA contain the strain (Trahan, 2008). The survival of NCFM in milk medium is considerably low (may be less than 50% survival) (Trahan, 2008) and many of the liquid fermented products in the market contain cell counts of below 10^6 cfu/mL (Tamime et al., 2005). For these reasons, *L. acidophilus* NCFM was selected for the development of yogurt dry mix in the current study. The NCFM cells are rod-shaped with rounded ends, similar to the LB, and occur singly or in short chains with optimum growth at pH 5.5-6. Unlike *Bifidobacteria* species which produce acetic acid and lactic acid, NCFM is an obligate homofermenter and produces mainly lactic acid. The bacterium does not design to

reduce the pH quickly (IDF, 2002) and requires riboflavin, pantothenic acid, folic acid, and niacin for growth (Tamime and Robinson, 2007).

4.3. Factors Affecting Survival of Probiotics

The survival of lactic cultures in bile acids, acids, and digestive enzymes in the stomach is one of the requirements of probiotics. Although probiotics NCFM have met the primary requirements, it is not uncommon that they may die during manufacture and storage before the products are consumed. Yogurt contains the essential nutrients (e.g. sugars, amino acids) required for growth of lactic bacteria, thus yogurt may serve as a food carrier for the probiotics (Lourens-Hattingh & Viljoen, 2001). Apart from the long-documented history of the consumption of yogurt by humans, the beneficial use of yogurt as food carrier of lactic bacteria may be partly due to its low pH environment. From the food safety point of view, the acid conditions reduce contamination caused by growth of other microorganisms, particularly food-borne pathogens. Moreover, studies have shown that the probiotic *L. acidophilus* La-5 demonstrated better performance in the *in-vitro* GI tract stimulator if pre-treated at pH 3.5 (Sumeri et al., 2010); while the *L. bulgaricus* adapted better survival to cold temperature (e.g. freezing) if previously introduced to acid environment (Streit et al., 2008). This may be partly attributed to the absence or low activity of stress-induced proteins which aid the bacteria to adapt to the various stressed environment. This may lead to the conclusion that the growth of lactic bacteria at optimal environmental conditions might not always give the maximum survival in stressed conditions, such as in the passage through the GI tract (Sumeri et al., 2010).

Although lactic acid bacteria have been associated with beneficial effects to the host (Kailasapathy & Rybka, 1997), *S.thermophilus* (ST) and *L.bulgaricus* (LB) are not bile resistant and may die along the GI, leaving probiotics (e.g. *L. acidophilus* and *Bifidobacterium*) as superior habitats of gut microflora. However, the metabolites produced by lactic starter bacteria in yogurt, nevertheless, may have a positive impact on pathogens inhibition and/or lactose digestion (Lourens-Hattingh & Viljoen, 2001). The challenges of maintaining the survival of probiotics in yogurt have still encountered by many manufacturers as reported by Kailasapathy and Rybka (1997) who showed that almost 50% of commercial yogurt in Australia and UK

contain unsatisfactory counts of such probiotics. The reasons for the poor viability of probiotics remain questionable. The factors which may contribute to the survival of probiotics are discussed in sections 4.3.1 – 4.3.5.

4.3.1. Exposure to Oxygen

The poor survival of probiotics in the yogurt environment is influenced by many factors such as acidity, hydrogen peroxide produced by other cultures, chemical composition of fermentation medium, solids concentration (e.g. sugars, milk solids), incubation temperature, storage condition (e.g. in fridge or at room temperature), and their interactions within the environment including yogurt starter cultures (Kailasapathy and Rybka, 1997; Talwalkar and Kailasapathy, 2004). Among the environmental factors, oxygen appears to be the most significant limiting factor for growth of *L. acidophilus* (LA). LA is microaerophilic and does not have cellular mechanisms to metabolize oxygen; hence exposure to oxygen through homogenization/agitation and packaging materials may lead to cell death (Kailasapathy & Rybka, 1997). The oxygen toxicity of probiotics however is strain dependant. In aerobic metabolism, oxygen is metabolized through the electron transport chain (ETC). As anaerobic microorganisms, they do not have the enzymes required in the ETC; thus they have to rely on fermentative mechanism such as substrate level phosphorylation reaction driven by the reduced form of nicotinamide adenine dinucleotide (NADH). When oxygen is present, NADH is bound with O_2 mediated by NADH oxidase enzyme to produce hydrogen peroxide (H_2O_2) or free radical (O_2^-) if the reduction of oxygen is incomplete. The by-products of this reaction are toxic to the cells and can cause damage to cell membranes or even the deoxyribonucleic acid (DNA) (Talwalkar & Kailasapathy, 2004). The destructive effects of O_2 up to DNA level have been reported in many studies (Andersen et al., 1999; Kurtmann et al., 2009b). To overcome lethality of LA to oxygen, gas flushing with nitrogen and low oxygen permeability packaging materials could potentially be applied. The presence of ST also aids the reduction of dissolved oxygen concentration in yogurt (Kailasapathy & Rybka, 1997).

Miller et al. (2002) analysed the level of [O_2] in commercial stirred yogurt sold in Australia. The commercial yogurt was packaged in polystyrene with permeation rate of air at 1.0-5.0

cc/package (200 mL)/day and stored for 42 days at 4°C. They reported that the initial oxygen level was 20 ppm (0.002 %) and during storage, the [O_2] increased not only in the headspace but also in the yogurt (oxygen permeates into the yogurt). After 42 days storage, the level of [O_2] was 50 ppm (0.005%). The authors indicated that although the level of oxygen was low compared to the oxygen in the atmosphere, it was sufficient to inhibit the probiotic bacteria, particularly when oxygen appeared to be mobile within the yogurt. An extended study carried out by Talwalkar et al. (2004) investigated the effects of various oxygen levels on the viability of *L. acidophilus* and *Bifidobacterium* spp in stirred yogurts. They found that (up to) 83% increase of O_2 during refrigerated storage (4°C) did not significantly (final concentration of [O_2] was 0.01%) reduce the viability of probiotics. This may be attributed to the low temperature storage which slows the metabolism of the probiotics. The metabolism of LA and *Bifidobacteria* are optimum at 37°C, thus at this temperature, oxygen would be most deleterious to the cells (Talwalkar et al., 2004). Both studies (Miller et al., 2002; Talwalkar et al., 2004) agreed that the heterogeneous oxygen distribution within the yogurt packaging may probably give an additional advantage in protecting the bacteria from oxygen. This is attributed to the protective effect of the gel network, which may not allow oxygen distribution in every part of the yogurt, therefore not all cells may have been exposed to the same levels of oxygen (Miller et al., 2002; Talwalkar et al., 2004). Although investigations on the actual oxygen levels required to reduce the viability of LAB is still scarce, Miller et al. (2002) have recommended the incorporation of N_2 to facilitate almost the complete removal of oxygen from the product and thus shelf life extension may be feasible when very low [O_2] (close to 0%) is achieved.

4.3.2. Species Interaction

While there is no doubt that the interaction between ST and LB in yogurt is symbiotic (Tamime and Robinson, 2007), they however may restrict the growth of LA in yogurt. Interestingly, LA does not inhibit the growth of ST and LB (Kailasapathy & Rybka, 1997). This may be due to the inhibitory factor of hydrogen peroxide which is mainly produced by LB (Gilliland et al., 2002). Accumulation of toxic H_2O_2 in the environment can lead to microbial cell death in regards to LA, which lacks the catalytic enzyme responsible for the breakdown of H_2O_2 into water and oxygen (Ng et al., 2011). Nevertheless, the presence of ST in yogurt is beneficial for both LA

and LB. Acting as oxygen scavenger, the mutual relationship is also due to the vigorous activity of (NADH) peroxidase, an enzyme present in ST that converts toxic hydrogen peroxide into water (Smart & Thomas, 1987).

Another factor associated with microbial loss is the lactic acid production which accumulates during storage of yogurt. LB is well-known for its ability to produce acid during yogurt fermentation and is responsible for "over-acidification" defects in yogurt. Although LA has natural tendency to survive acidic environment, the mortality however is higher at pH below 4 (Kailasapathy & Rybka, 1997; Sellars, 1991). This finding, however, is still arguable among yogurt experts as reported by Kailasapathy and Rybka (1997). These authors reported the survival of LA can be maintained at pH 3 as opposed to the traditional starter cultures in yogurt. While LB is often attributed to its inhibitory effects on LA, proteolytic activity of LB is believed to stimulate growth of LA by providing essential amino acids from casein-derived peptides (Donkor et al., 2007).

Studies on the antagonist effects of LA to other microorganisms including pathogens *S. aureus, S. typhymurium, E.coli*, and *Clostridium* species have been reported (IDF, 2002); inhibition was attributed to the production of hydrogen peroxide and organic acids (IDF, 2002). The production of bacteriocin, lactacin B, by the NCFM has been reported by many studies (Percival, 1997; Sanders & Klaenhammer, 2001). The antagonistic effects of bacteriocin were reported to be active against closely related *Lactobacillus* species of *L. bulgaricus*, *L. helveticus* (Percival, 1997), *L. fermentum* and other bacteria of *Enterococcus faecalis* and *Lactococcus lactis* (Barefoot & Klaenhammer, 1983). Tamime and Robinson (2007) are also reported to be inhibitory to *L. bulgaricus* and if this happens in the intestine, *Lactobacillus* may be dominant in the gut population. The role of the bacteriocin production and its influence to other microorganisms *in vivo*, however, is still not clear (Sanders & Klaenhammer, 2001).

4.3.3. Water activity (a_w)

Although significant studies have been conducted on the survival of probiotics in liquid fermented milks and in the gastrointestinal tract, their viability in the dry yogurt mix at various

water activities have not received much attention. The reports by Kailasapathy and Rybka (1997) and Sellars (1991) discussed that water activity is another important factor for the optimization of the microorganisms survival throughout the shelf-life (around 9-12 months at room temperature). According to Sellars (1991), maximum survival of *L. acidophilus* (LA) in dehydrated form stored at room temperature could be achieved at a_w level of less than 0.25. Freeze-dried bacteria stored below their glassy transition temperature protects its viability as suggested by Kurtmann et al. (2009b) who reported that viability of LA at 20°C was optimal at a_w 0.11 and the performance of LA steadily decreased with significant viability loss at 0.43 a_w compared to 0.11 a_w. During manufacture of cultures (freeze-dried), the loss of survival was less pronounced at the beginning but extensive degradation commenced after storage for two weeks (Kurtmann et al., 2009b). Furthermore, the concentration of dissolved oxygen seemed to greatly influence the survival of cultures and the viability improved when an oxygen radical scavenger (e.g. ascorbate) was incorporated (Kurtmann et al., 2009a). The authors concluded that water activity between 0.11 and 0.22 with less than 4% oxygen improved the storage ability of lyophilized LA. The finding also suggests that the detrimental effect of oxygen may be attributed to the sensitivity of LA towards oxidative process and chemical reactions (e.g. protein-sugar interaction).

To maintain low water activity levels, cultures are usually preserved as freeze dried products. During freeze-drying, LA cell walls may be damaged and as a consequence, cell permeability for the transfer of toxic materials increases (Sellars, 1991). This problem could be overcome by incorporating a cryoprotectant (Sellars, 1991) or embedding them in sucrose matrices (Kurtmann et al., 2009b).

4.3.4. Total Solids Content

The amounts of total solids (e.g. sugars) greatly influence the survival of probiotics due the osmotic pressure discrepancies between the outer and inner cell. Creating and maintaining cell balance of osmotic pressure requires high energy. Meanwhile, adenosine triphosphate (ATP); energy source generated through lactic acid fermentation is far less than aerobic respiration; therefore the bacteria have to generate energy faster to maintain their viability. This observation

was supported by the study of Micanel et al. (1997) which reported better survival rates of yogurt cultures in natural yogurt as opposed to fruit-flavoured yogurt. However, the effect of total solids content on lactic bacteria survival in yogurt varies depending on the type of fruit used (Kailasapathy et al., 2008).

Although milk fat contributes significantly to the creamy and rich sensation of yogurt, the effects of fat on yogurt probiotic cultures is minor. The investigation conducted by Micanel et al. (1997) on commercial yogurt produced and sold in Australia showed little or no beneficial effects towards viability probiotics *Bifidobacteria* and *L. acidophilus*. The effect, however, is more pronounced during the passage of the bacteria through the gastrointestinal (GI) tract. High fat diet promotes bile acids secretion upon consumption which results in higher death rate of LA (Kailasapathy & Rybka, 1997).

4.3.5. Inoculation Rate and Temperature of Incubation

The inocula levels of LA are significantly influenced by the degree of heat-treatment and the fermentation temperature. Although it is a common practice to incubate yogurt at 43°C for optimum growth of starter cultures, however, lower incubation temperature (37°C) favours better viability of probiotics. Moreover, survival of LA during storage is improved at lower temperature (3 - 4°C) (Kailasapathy & Rybka, 1997). The beneficial effect of low incubation temperature was attributed to lower acid development in yogurt intended for long term storage (Lourens-Hattingh & Viljoen, 2002). High temperatures reduced the microbial enzyme of β-galactosidase, which aids the bacteria efficacy during passage through the GI tract (Sellars, 1991).

Although temperature plays an important role in maintaining viability of probiotic cell counts, inoculation level has been shown to influence the viability of bacteria. Interestingly, excessive level of probiotics *L. acidophilus* (2.33 g/100 g) resulted in lower cell counts of yogurt cultures compared with yogurt inoculated with 0.0239 g/100 g and 0.238 g/100 g where similar inocula levels of *S. thermophilus* and *L. bulgaricus* were used for all products (Olson & Aryana, 2008).

4.3.6. Modified Atmosphere Packaging (MAP)

Packaging plays an important role in maintaining the viability of LAB. One of the key roles is to act as a barrier which shields the contents between the interior and exterior environment and thus prevent the ingredients from deterioration (e.g. fat oxidation, vitamin loss, airborne contamination, etc). For the LAB, alteration of the gas composition in the packaging may extend their rate of survival. Inclusion of oxygen scavengers, such as ascorbic acid or powdered iron have been recommended by many authors (Miller et al., 2002; Sarkar, 2010). The oxygen scavengers however have to be present up to the end of the shelf-life of the product (Dave & Shah, 1997b). The [O_2] in the headspace using oxygen scavenger technology can be reduced to as low as 0.01% (Miller et al., 2002).

Flushing the head-space with an inert gas such as N_2 is also plausible in yogurt manufacture (Miller et al., 2002). Another economically feasible method is through the incorporation of CO_2 or mixtures of CO_2 and N_2 (Hotchkiss et al., 2006). The combination of the gas mixtures is usually applied in food where bacteria spoilage is an issue. Vacuum packaging is also a common practice applied by food manufacturers. The method utilizes the removal of air under vacuum while at the same time sealing the package. Removal of air followed by gas flushing methods is usually preferred for O_2 sensitive products. Typically, residual [O_2] of 0.3 to 3% is achievable by gas flushing or vacuum methods. If vacuum is not to be used, injection of gas into the package followed by flushing out the air prior to sealing is another approach. This method, however yields higher residual [O_2] to a level of 2 to 5% (Miller et al., 2002).

The incorporation of these gases or the oxygen absorbers will obviously have little benefit if the gas is allowed to permeate. Sufficient gas barrier in the packaging materials therefore should not be overlooked. As in the case where only N_2 is used, the driving force for N_2 exiting the package is 1-0.79 (percentage of N_2 in the air = 0.21, which is the same driving force for O_2 (21% in the air) entering the package. If the package is selective for oxygen but not for nitrogen, then the driving force between the gases may be disrupted causing an increase in pressure inside the package (Robertson, 2005). Although the product is perfectly safe, this situation may result in product removal from sale.

4.3.7. Effect of Sorption Isotherms of Yogurt Powder Blend during Storage

As mentioned in the preceding section, water activity plays a significant role in the shelf-life of dry yogurt mix. The movement of water in foods may occur through diffusion within the components/ingredients or between the materials and the environment; therefore the properties of each material is critical. Water sorption isotherms describe the equilibrium relationship between water activity and moisture content of the food system at constant temperatures and pressures. The isotherms give information on the stability of food products during storage (Koç et al., 2010; Stencl, 2004). The dehydrated yogurt base (DYB) in the current study was produced by dry-blending various powders of different sizes (whole and skim milk powder, sucrose, yogurt cultures, flavourings, and colorants). The sorption isotherm of this mixture is therefore complex. As sorption isotherms of dry products depends on the quality of dry raw materials, the sorption behaviour is therefore unique for each food material (Ko et al., 2008). The adsorption and desorption behaviour of yogurt powder has been successfully determined using Chung-Pfost's model (Stencl, 2004), Guggenheim-Anderson-de Boer (GAB) model (Kirn & Bhowmik, 1994) and Oswin model (Koç et al., 2010; Kumar & Mishra, 2006). Irrespective of the mathematical models used to describe the sorption behaviour, these reports concluded that the adsorption behaviour of yogurt powder manufactured by either spray-drying, freeze-drying, or microwave vacuum-dried, followed type II isotherm BET (Brunauer-Emmett-Tetter). An increase in temperature causes an increase in a_w at constant moisture content. This indicates that at higher temperature, the tendency of the material to bind water is lower. This is because the hygroscopic properties of proteins and carbohydrates at high temperature is lower than at low temperatures (Koç et al., 2010). An example of the adsorption curves of milk powder (Ko et al., 2008) and dried yogurt (Koç et al., 2010) are shown in Figure A. 7.

As mentioned in preceding section, an increase in temperature results in the increase of water releases (evaporated) and hence, the moisture and a_w of the powder. Since lactose is the most abundant component in milk powders (35-40% in WMP and 45-55% in SMP), generally the storage properties of milk powders depend, to a certain extent, on the glassy temperature (Tg) of lactose. Glass transition temperature (Tg) is related to the marginal temperature at which molecular diffusion of the compounds within the system is governed by the temperature

(Baechler et al., 2005). Food systems stored below their glassy temperatures have limited mobility which is similar to solid-like products. In amorphous state (below Tg), lactose, fat, and air vacuoles of milk powders are dispersed in a continuous matrix where water is bound to both lactose and proteins (Baechler et al., 2005). An increase in temperature reduces its hygroscopic properties. The Tg of milk powders are typically around 92°C, which is similar to pure amorphous lactose (101°C).

4.4. Survival Mechanism of probiotic *L. acidophilus* NCFM in Acidic Environment

Survival of probiotic in acidic conditions is crucial not only during passage through the GI tract but also during manufacture and storage as in the case of yogurt. The mechanisms of surviving acidic environment includes proton pumps, proteins involved in repair of damaged cells, expression of regulators responding to the environment, and alteration of cell composition (Azcarate-Peril et al., 2004; Kullen & Klaenhammer, 1999; Rius et al., 1994). In yogurt, the proteolytic sytem of LA remains active during refrigerated storage as shown by high production of smaller amino acid fraction from hydrolyzed caseins (Donkor et al, 2007; Yadav et al., 2007).

4.5. Yogurt Quality Measurements

4.5.1. Viscosity and Texture

Texture is one of the most important parameters in fermented dairy products including yogurt. The methods of analyzing rheological properties of yogurt vary depending on the type of yogurt; stirred, drinking or set-yogurt and the investigated information. Many studies have been done to better understand the rheology of yogurt using the rheometer, texture analyzer, or viscometer (Bourne, 2002). Viscosity is the common term used to define “flowability” of a product. Viscosity or so called ‘absolute viscosity’ is the tendency of a product to resist flow and is defined by equation (1) (Bourne, 2002);

$$\eta = \frac{\sigma}{\gamma} \qquad (1)$$

η = viscosity (milliPascal second (mPa.s) or centipoises (cP))

σ = shear stress (Pascal or Newton meter^{-2})

γ = shear rate (s^{-1})

Absolute viscosity is used for a Newtonian fluid at which the flow of fluid is directly proportional to the applied stress and that viscosity is constant at changing shear rates. Water is the best example of Newtonian fluid and its viscosity at 20°C is 1 mPa.s (Bourne, 2002). Since yogurt is rather a complex system consisting of lactose, proteins, fats and other suspended matters, its behaviour is categorised as non-Newtonian fluid and has apparent viscosity (Bourne, 2002). The best method of measuring viscosity is by point measurements, at which viscosity is measured at various rates of shear stress and shear rate (N. Shah, personal communication with Brookfield™, August 26, 2010). This allows the investigation of the flow and the overall rheology of the product, particularly if the product of interest follows pseudoplastic behaviour (N. Shah, personal communication with Brookfield™, August 26, 2010).

As food contains various chemical components, the behaviour (flow) of non-Newtonian fluid is categorised into different classes according to its shear stress-strain relationship. Unlike the Newtonian fluid which flows freely, certain amount of pressure is required to force non-Newtonian fluid such as tomato sauces and condiments to flow. Once the fluids start to flow, the shear stress is proportional to shear rate. The minimum force required to begin the flow is called yield stress and the flow behaviour of such product follows a plastic or Bingham model (Figure 8). A slightly different model of Bingham flow is pseudoplastic fluid. The Bingham model of pseudoplastic fluid shows Newtonian behaviour at low shear rate but an increase of shear force causes an incline in shear rate, resulting in changes of viscosity (Figure 8). Typically, salad dressings fall into this category. The last flow behaviour that has been successfully quantified is dilatants or frequently called 'shear thickening' at which equal increments of shear stress gives less increments in shear rate (Figure 8). The application of this behaviour, however, is rarely applied in the food industry (Bourne, 2002). Yogurt, either in stirred or set-form, follows non-Newtonian behaviour that shows yield stress with 'shear thinning' and time dependency behaviour (Ozer & Kirmaci, 2010). Shear thinning is described as non-Newtonian behaviour where apparent viscosity decreases with time of shearing and the change is irreversible; that is, it

stays in the thinner state even if shear stress is removed. In contrast, fluid that reverts to its original state after shear stress removal is called thixotropic. The plot of apparent viscosity with time to describe these flows is shown in Figure 9.

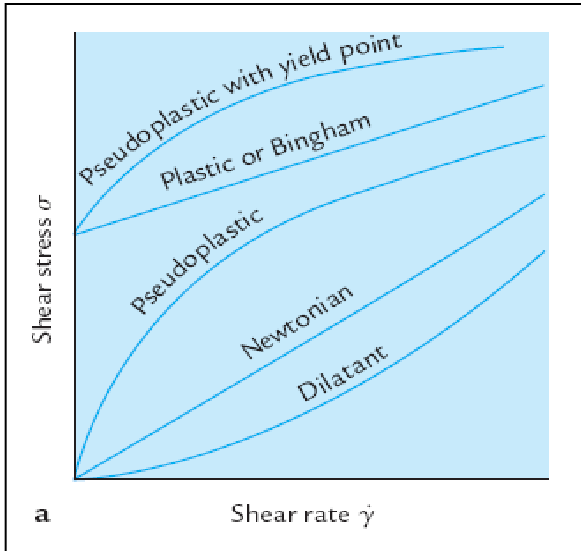


Figure 8. Different fluid flows at various shear stress and shear rate (Bourne, 2002)

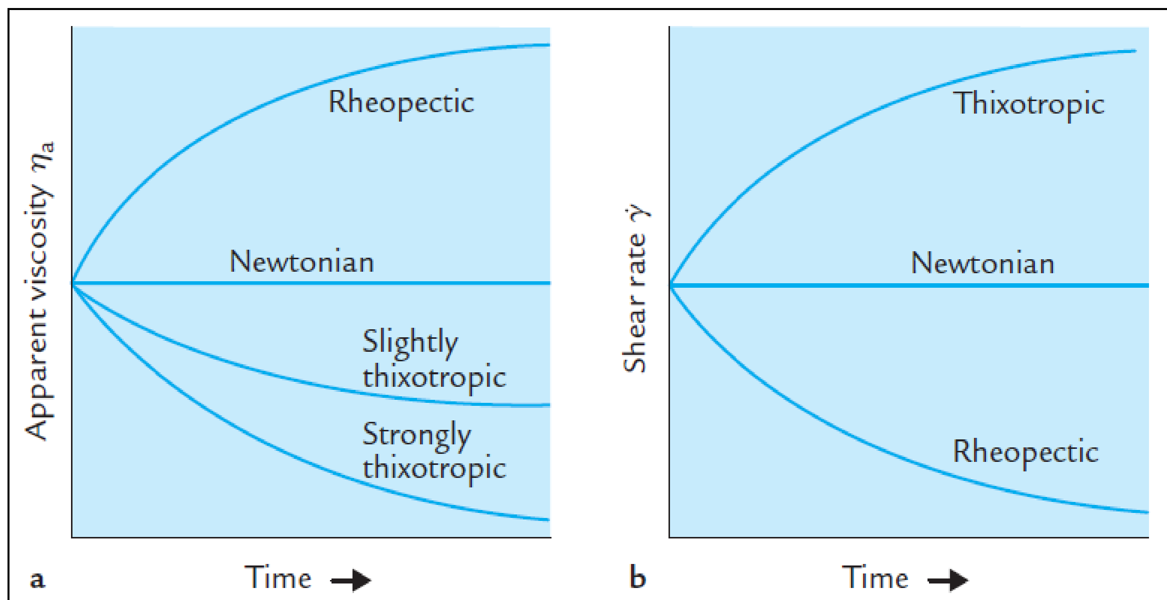


Figure 9. Apparent viscosity over time of various flow (a); shear rate versus time of various flow (b) (Bourne, 2002).

Many factors can contribute to the viscosity of a product which include temperature, solute concentration, molecular weight of the solution, and suspended matter of solution (e.g. fruit pulp,

fibre, etc) (Bourne, 2002). Temperature, however, plays the most important role in viscosity measurements. Regardless of the type of behaviour of the fluid (Newtonian or non-Newtonian), measurements of viscosity are significantly affected by an increase in temperature, where the relationship between viscosity and temperature is (typically) inversely proportional (Bourne, 2002).

Texture is an important factor in set-yogurt as it describes the strength of gel network. The strength of gel in set-yogurt contributes to its firmness, which is defined as the force attained at a given deformity of a product, hence attributed to the degree of syneresis. With many scientific studies have been done to improve textural properties of yogurt, texture is a single crucial factor in manufacturing yogurt. Such studies include improving yogurt quality by reducing oxygen level (Horiuchi et al., 2009), incorporating lentil flour (Zare et al., 2011), skim milk/starch (Tamime et al., 1996), and whey protein (Augustin et al., 2003; Guzmán-González et al., 1999).

4.5.2. Sensory Evaluation and Consumer Acceptance

The objective of this study was to develop a stable probiotic rich dehydrated yogurt base which can be used to produce acceptable liquid yogurts by potential consumers at home, restaurants, etc. Although many important factors can be measured by instruments, this is still insufficient in characterising the liquid yogurt products. Consumer acceptance tests, which require 50-100 panellists, give food manufacturers important information about consumer likeliness towards the product based on the product's sensory properties. Information can be obtained for single products without comparing to other products. A nine-point-hedonic scale is the commonly used rating method for sensory evaluation as the scales can easily be interpreted using statistical approaches of analysis of variance (ANOVA), regression and correlation analysis (Resurreccion, 1998). With many yogurt products available in the market nowadays, consumer acceptance tests provide an indication of product acceptance without the effect of other factors, such as packaging, price, and health claims, which may enhance its acceptance among target consumers thus minimizing product failure once it is on the market (Resurreccion, 1998). According to the objective of the analysis, it is not uncommon that panellists are screened and selected based on their organoleptic abilities. Panellists are then asked to generate important attributes that best

describe the products, called descriptive analysis. With so many varieties of yogurt products available in the market, descriptive attributes may differ between products. For example, descriptive attributes of peach-flavoured yogurt drink were overall acceptability, colour, flavour, and mouthfeel (Gonzalez et al., 2011). Meanwhile, for stirred yogurt, smoothness, graininess, flavour, overall acceptance, and colour (Zare et al., 2011) or taste, characteristic yogurt flavour, colour, and consistency may be considered as important attributes (Obi et al., 2010). For set-yogurt, texture and mouthfeel (Tamime et al., 2006) can be added to the appearance, colour, smoothness, sweetness, sourness, and overall acceptance (Hashim et al., 2009).

4.6. Shelf-life Determination

Food quality is defined as the assembly of properties which differentiate individual units and influence the degree of acceptability of the food by the consumer or user (Labuza et al., 1997). Food is a complex system consisting of various compounds (e.g. carbohydrate, proteins, fats, etc) which affects the biological and chemical changes of the products during manufacture and storage. The length of time at which food retains organoleptic and safety qualities under recommended storage conditions is defined as shelf-life (Labuza et al., 1997). Given that good manufacturing practice (GMP) and HACCP may be implemented during yogurt manufacture, the shelf-life of yogurt could be determined by monitoring quality changes (e.g. yogurt cultures, flavour compounds) of the product at a given time period. Determination of end of shelf-life varies, depending on the manufacturer's standard and compliance with food authorities of the country or of the country of destination (e.g. the export market). Kinetic reaction is the most widely accepted method to determine or predict shelf-life (Labuza et al., 1997). The rate of quality loss over time could be at constant rate (zero-order reaction) or exponential (first-order reaction). When quality attribute "Q" is plotted against time, the changes of quality over time determine the rate of quality loss where linear relationship shows a "zero order" while exponential line shows a "first order" reaction. Zero order reaction is generally used to describe enzyme degradation, non-enzymatic browning, lipid oxidation, and overall quality of frozen foods, whereas microbial growth/death, vitamin loss, protein deterioration, loss of colour and texture follow the first order reaction (Labuza et al., 1997; Singh, 2000). The deterioration rate of food for zero and first order reaction can be determined by equations 2 and 3:

For zero order reaction:

$$\frac{[Q_0]-[Q]}{t} = k \tag{2}$$

For first order reaction:

$$\frac{\ln[Q_0]-\ln[Q]}{t} = k \tag{3}$$

Q_0 = initial quality attributes

Q = the amount of quality attribute left after time t

t = time

By plotting a graph for the change in "Q" with "t", the reaction rate constant and thus the shelf-life of the product can be determined (Singh, 2000).

4.6.1. Accelerated Shelf-life Determination

While the determination of shelf-life described earlier is applicable for short storage periods, such a method is not applicable for food with long storage potential (e.g. more than one year). In that situation, shelf-life prediction could be achieved by the implementation of accelerated shelf-life method. Temperature is the major limiting factor in food deterioration, subjecting the product to various temperature treatments could determine the reaction rate at each temperature (Hough, 2010). The study by Hough (2010) also recommended obtaining at least six readings for at least three storage temperatures to reduce the confidence interval, hence, avoiding over or underestimation of shelf-life prediction (Hough, 2010). The effect of one factor (e.g. temperature) on microbial growth can be modelled by the Bĕlehrádek and Arrhenius models (Mataragas et al., 2011; McMeekin et al., 1993) with the latter model attempting to predict the microbial death of *L. acidophilus* in fermented dry yogurt base (Tsen et al., 2007) and starters (ST and LB) (Kumar & Mishra, 2004a). The Arrhenius law can be modelled using equation 4;

$$\ln k = \ln k_0 - \left(\frac{Ea}{2.303RT}\right) \quad (4)$$

Where k_0 is the experimental constant, T is absolute temperature (Kelvin), R is the gas constant, and Ea is the activation energy.

Due to the complexity of food systems, shelf-life of food varies considerably. Although temperature is one of the important parameters in shelf-life determination, it is important to consider other factors (e.g. fat oxidation, colour changes, vitamins degradation, etc), which may play crucial roles in the quality acceptance of the food. In applied microbiology, modelling the combined effect of temperature, water activity and other factors such as salt level and pH on microbial growth in food would be desirable (McMeekin et al., 1993). However, such work is labour-and-cost intensive which explains why many researchers, in attempt to better understand their product, use one factor (e.g. temperature) only to generate the model. However, irrespective of the model used to predict microbiological models, data validation is critical to ensure consumer safety and confidence (McMeekin et al., 1993).

The thermal death rate of LAB during storage at various temperatures usually followed first order reaction, where the effect of temperature on their survival was explained by the Arrhenius equation; $\ln k = \ln k_0 - (Ea/2.303R) * (1/T)$ (Ishibashi et al., 1985; King et al., 1998; Tsen et al., 2007) (Appendix J). The k value represents the thermal reduction rate of cells; slope of first-order microbial death (Tsen et al., 2007). As the storage temperature increased, the value of the reaction rate constant (k) becomes larger and the reduction of bacteria increased. This observation was in agreement with the accelerated storage studies of dehydrated *L. acidophilus* which showed significant reduction at higher temperature during storage at 4, 20, 50, 60, and 70°C in both studies (King et al., 1998; Tsen et al., 2007). In the study of King and Su (1993), cell decrease was not significant during storage at 5°C and -20°C suggesting the potential of using low storage temperature for short term preservation (King & Su, 1993). These reports were supported by the review of Kumar and Mishra (2004b) which cited that the survival of ST and *Lactobacillus* was better during prolonged storage at 5-10°C than at 18-25°C. In their study, lower k values at refrigeration storage (4°C) than at room temperature (18-25°C) were also observed.

5. MATERIALS AND METHODS

In the current study, experimental design was used to develop various DYB formulations. The milk powder used was then analysed thouroughly. Of the DYB formulations, three formulations were selected to be made into liquid yogurt, which was then characterised further. Shelf life of selected DYB formulations were also predicted.

5.1. Experimental Design

The experiments were conducted in two integrated phases. The first phase focused on the effect of water activity, packaging, and oxygen on the survival of the probiotic NCFM and yogurt starter cultures in dehydrated yogurt bases (DYB) during storage for 9 weeks at various temperatures. Preliminary results from phase one were then used to screen the most potential yogurt formulations to produce ready-to-eat (liquid) yogurts; these products were then analysed for viable cell counts, texture, acidity, syneresis, and sensory properties. All analyses were done in either duplicate or triplicate. All the chemicals and reagents used in this study were of reagent grade.

5.2. Characterization of Milk Powder

Commercial milk products in powders of various fat levels (whole and skim milk) were produced at Westland™, Hokitika, New Zealand. Raw milk was obtained from the local dairy farm and delivered to the plant on the same day. Milk temperature was maintained at 4°C during transportation. Upon receipt, milk composition was rapidly analysed using the NIR-based scanner (MilkoScan FT 120, UK) against the company standards. Fresh milk was then manufactured into powder as shown in Figure 2. The milk powder was analysed for protein (section 5.2.2), fat (section 5.2.3), and moisture content (section 5.2.4). The extent of denaturation of whey protein β-lactoglobulin and α-lactalbumin was determined using High Performance Liquid Chromatography (section 5.2.5) and the dye binding method (section 5.2.6). The accuracy of the methods used was checked against the NIR-based method (section 5.2.1).

The water activity of the milk powder was determined using the Novasina TH-500 (Switzerland). The specifications of the milk powder are shown in Appendix A.

5.2.1. Rapid Analysis of Milk Powder using the Near Infrared Spectroscopy (NIR)

The rapid analysis of fat, moisture, and protein content were done by measuring the vibration of the bonds within the samples using the NIR-based equipment (FOSS XDS MasterLab™, Denmark). The NIR equipment releases light energy through the samples and then milk powder which absorbs and scatters the light. The light emitted by the samples is detected by the detector (FOSS XDS MasterLab™, Denmark). Using calibration models, the detector then produces the absorbance spectrum (800-2500 nm) and expresses the data as percentages. The wavelength at which light is emitted varies depending on the composition of milk powder (e.g. wavelengths between 900 and 1400 nm detect moisture content) (Osborne et al., 1998).

5.2.2. Protein analysis by the Kjeldahl method

Measurement of protein content was conducted using the International Dairy Federation (IDF) Standard 20-1 (IDF, 2001). In this method, concentrated sulphuric acid and potassium sulphate convert organic nitrogen present in the sample to ammonium sulphate in the presence of catalyst copper (II) sulphate. Excess sodium hydroxide liberates ammonia which is condensed during distillation into boric acid and then back-titrated with standard hydrochloric acid. The amount of ammonia released represents the nitrogen presents in the milk.

Two Kjeltabs (e.g. glowed pumice, zinc dust, hard pieces of porcelains); potassium sulphate (15 g), 1 mL of copper (II) sulphate solution (5g/100 mL), 0.5 g of milk powder, and 25 mL of sulphuric acid (>95%) were added into Kjeldahl flask and gently mixed. The flask was placed onto the pre-heated digestion apparatus (FOSS Teccator™, Denmark) set to 25°C and digested at 200 °C for 15 min then at 300°C for 30 min, and finally at 425°C for 2.5 hours. After digestion, the flasks were cooled to room temperature for approximately 25 min. Fifty percent sodium hydroxide (75 mL) was added into the mixture to form ammonia gas. The mixture was then quickly distilled into 50 ml boric acid solution (40 g/l) to form ammonium borate. The amount of

ammonia present was titrated with standard HCl (0.1mol/l) until the first persistent pink was observed. The amount of nitrogen present was calculated (% N_2 = 1400.7 (V $standard_{HCl}$ – V $blank_{HCl}$) $Molarity_{HCl}$ / $m_{test\ portion\ in\ g}$). The amount of N_2 protein in milk powder was then determined by multiplying the amount of N_2 (%) with 6.38.

5.2.3. Fat Content

The determination of milk powder fat content was achieved by using the International Dairy Federation (IDF) Standard 9 (IDF, 2008). Three extractions were conducted to extract the fat in the powders. A homogenous sample (1 g) of whole milk powder or skim milk powder was transferred into the Mojonnier fat-extraction flasks. Distilled water (10 mL) and 2 mL ammonia solution were then added into the flasks to completely dissolve the sample. The flasks were heated in a water bath at 65°C ± 5°C for 15-20 min with occasional shaking. After heating, the flasks were cooled to room temperature. Once temperature equilibration had been achieved, 10 mL of ethanol (96%, Sigma Aldrich NZ) and 25 mL of diethyl ether (30 - 60) were added to the flasks with alternate shaking for each solution added for 1 min. Then the 25 mL of light petroleum pentane (30 - 60), were added to the test solution and mixed for 30 s. The homogenous test solution was then allowed to stand (on bench top) for at least 30 min until a clear separation of supernatant was observed. The supernatant of the first layer was decanted into the fat-collecting vessel containing a few boiling chips followed by the addition of 5 mL ethanol. The aqueous solution left in the Mojonnier fat-extraction flasks was extracted again using 25 mL of diethyl ether and 25 mL of petroleum pentane with shaking for 30 s - 1 min for each addition of the reagent. The flasks were then allowed to stand on bench top until a clear separation of supernatant was observed. The supernatant was then decanted into the fat-collecting vessel containing the solution from the first extraction. The third extraction of aqueous solution left in the Mojonnier flasks was repeated. This time, only 15 mL of diethyl ether and 15 mL petroleum pentane were used. Distillation was then carried out to remove any remaining solvent on the fat-collection vessel. The solution in the vessels were then heated for 1 h in the drying oven at 102°C and then cooled to room temperature and weighed on an analytical balance (AB 204-S Mettler Toledo, Switzerland). The results were expressed as percentage of fat.

5.2.4. Moisture content

The determination of moisture content of the milk powder used in the current study was carried out following the IDF Standard 26 (IDF, 2004). A representative sample of 5.0000 ± 0.3 g dried milk was weighed accurately into an aluminium dish in a drying oven at 87°C for 5 hours while dry air was passed through the test portion. The weight of sample before and after drying represented the loss of moisture, which is related to the non-chemically bound water (IDF, 2004). The result was expressed on a percentage bases by mass.

5.2.5. Determination of the Whey Protein Nitrogen Index (WPNI) by High Performance Liquid Chromatography (HPLC)

WPNI is a measure of the undenatured protein (β-lactoglobulin and α-lactalbumin) which shows the extent of heat applied to the milk (IDF, 2002). Casein and denatured whey proteins are precipitated isoelectrically at pH 4.6. The undenatured proteins present in the filtrate of milk powders were determined using the HPLC standard method (Method 162) of the IDF (IDF, 2002).

Ten grams of milk powder were dissolved in 90 ml distilled water to give total solids of 10%. The solution was mixed with magnetic stirrer for 30 min. During mixing, drop-wise addition of 1 ml of 1 M HCl was added to adjust the pH to 4.6. The mixture was left undisturbed for 15 min at room temperature to facilitate salt precipitation. The test portion (± 100 mL) was then filtered through Whatman no.2 filter paper. The filtrate was centrifuged at 14,000 x g for 5-10 min and filtered through a 0.45 µm membrane filter into vials.

Prior to use, the Shimadzu HPLC GF-250 (25 cm length and 0.94 cm internal diameters) equipped with a UV detector at 280 nm was rinsed with distilled water and conditioned using phosphate buffer (pH 6, appendix L). The HPLC column temperature was set at 30°C. Of the sample, 20 µl were injected into the HPLC at a flow rate of 1.2 ml/min. The integrator automatically calculated the peak area of whey protein which occurred at retention times

between 8 and 11 min. After analysis, the HPLC column was rinsed with 10% acetonitrile solution. All analyses were done in duplicate. The WPNI was calculated using Equation (5):

$$\%\,WPNI = \left(\frac{peak\ area}{extension\ coefficient} \Big/ \frac{0.06}{flow\ rate}\right) \Big/ \text{injected sample volume} \qquad (5)$$

Extension coefficient α-lactalbumin = 20.06; β-lactoglobulin = 9.41.

5.2.6. Determination of the Whey Protein Nitrogen Index (WPNI) by Dye Binding Method

WPNI of milk powder was analysed using the NZTM 3.15.6 method (2006). Casein and denatured whey proteins are precipitated in the presence of salt. The un-denatured whey protein present in the filtrate binds with amido black dye (Merck, NZ) and its absorbance is determined using spectrophotometer.

Of the test sample, 3 g were weighed into a test-tube and diluted with 20 mL of preheated distilled water (37°C). The solution was kept at 37°C for 30 min with mixing every 5 min. Standards (Westland™, NZ) of high-, medium-, and low heat-treated milk powder samples were also analysed. A portion of 8.5 g cheese salt (Westland™, NZ) was mixed with the solution and further heating at 37°C for 30 min was carried out with shaking at 2-min intervals in the first 15 min, and 5 min intervals for the last 15 min. This was done to allow sufficient time for salt precipitation. The warm solution was then filtered through Whatman no. 2 filter paper for approximately 60 min. A portion of 0.5 mL clear filtrate solution was mixed with 10 mL amido black and kept in a dark cupboard for 15 min using a bench top centrifuge (Beckman Coulter Inc, Switzerland). The mixture was then centrifuged at 14000 x g for 5 min. Without disturbing the precipitate, 3 mL of the solution were carefully pipetted into a 100 ml volumetric flask and diluted with distilled water up to the mark. The liquid was thoroughly mixed by inverting at least 10 times and transferred into a spectrophotometer plastic cuvette. The spectrophotometer was set at 615 nm and pre-warmed for at least 20 min. The standard curve was generated to determine the concentration of WPNI (mg/g) of the test samples. All analyses were done in duplicate.

5.3. Formulation of Dehydrated Yogurt Bases (DYB)

Source of yogurt starter cultures and the probiotic NCFM

The commercial mixed yogurt culture of ST and LB (Yo-Mix™ 305 LYO 250 DCU) and the probiotic *Lactobacillus acidophilus* NCFM (Howaru™ Dophilus LYO 100 DCU-S) were obtained from Danisco® in New Zealand. The cultures were obtained in freeze-dried from and stored at 4°C in line with the manufacturer recommendation until required for use. The cultures were added aseptically, where 0.020 g of Yo-Mix 305 and 0.0252 g of Howaru™ Dophilus were measured into the dehydrated yogurt base (DYB) packages which contain milk powder, added sugar, flavouring, colorant, and the yogurt cultures. In this report, the cultures will be referred as *S. thermophilus* (ST) and *L. bulgaricus* (LB), and *L. acidophilus* (LA) NCFM.

Preparation of the formulations for the dehydrated yogurt bases

Two different products, strawberry flavoured and no-added flavour, were formulated to produce dry yogurt mixes. Full factorial (2^3) experimental design with three factors (packaging material, fat and sugar) at two levels was selected to produce 12 unique formulations using the method of Ellekjaer and Bisgaard (1998) (Table 7). The formulations were packaged into two different packaging materials (A and B). Packaging A had 3 layers of materials (Aperio® packaging) and packaging B (Huh Tamaki®) had 4 layers of materials. Both materials contained layers of polyester, polyethylene, foil, and nylon (Table 7). The gas transmission rate of both packages were $<0.3 cc/m^2/24h$ (100% O_2) 23°C/0% RH.

Table 7. Descriptions of the 12 formulations of DYB

Formulation	Description	Package type
1	1.4% fat, no added flavour, no added sugar, and no colorant	A
2	1.4% fat, no added flavour, no added sugar, and no colorant	B
3	3.5% fat, no added flavour, no added sugar, and no colorant	A
4 (HFN)	3.5% fat, no added flavour, no added sugar, and no colorant	B
5	1.4% fat, strawberry flavour (<1%), added sugar (38%), colorant (<1%)	A
6	1.4% fat, strawberry flavour (<1%), added sugar (38%), colorant (<1%)	B
7	1.4% fat, strawberry flavour (<1%), added sugar (19%), colorant (<1%)	A
8 (LFLS)	1.4% fat, strawberry flavour (<1%), added sugar (19%), colorant (<1%)	B
9	3.5% fat, strawberry flavour (<1%), added sugar (38%), colorant (<1%)	A
10 (HFHS)	3.5% fat, strawberry flavour (<1%), added sugar (38%), colorant (<1%)	B
11	3.5% fat, strawberry flavour (<1%), added sugar (19%), colorant (<1%)	A
12	3.5% fat, strawberry flavour (<1%), added sugar (19%), colorant (<1%)	B

Notes: Package A: PE/foil/PET; Package B: PE/foil/nylon/PET

5.4. Characterization of DYB

The effects of gaseous composition, water activity, and packaging on the viability of yogurt cultures were monitored during storage of DYB samples for 9 weeks. Samples were stored at room temperature (20 °C) and a random package was withdrawn from each treatment every three weeks for nine weeks analysis. For each sachet (package), the gas composition (O_2 and CO_2) was measured using a gas puncture (PBI Dansensor, Denmark). The DYB sachet was then opened and water activity was measured at 25 °C (Novasina TH-500, Swiss). To analyse for LAB survival, 1 g DYB was serially diluted with 9 ml of 0.1 % sterile peptone water. Viable cell counts of NCFM were enumerated by the pour plate technique following the IDF Standard 192 method (section 5.4.1.2). Enumeration of ST (section 5.4.1.1) and LB (section 5.4.1.3) were done following the standard method of the IDF. All analyses were done in duplicate. Data were tested for normality and were statistically analysed using the ANOVA of the General Linear Model at 95% confidence interval. At the end of storage, the most promising formulations, one natural and two strawberry flavoured, were selected for further analyses as described in section 5.5.

5.4.1. Microbiological Analysis

5.4.1.1. *Streptococcus thermophilus* (ST)

Enumeration of *Streptococcus thermophilus* (ST) was carried on M17 agar (Oxoid, UK) following the International Dairy Federation method 117 (IDF, 2003). M17 agar (pH 7.1±0.2) is selective for *S. thermophilus* growth. Suitable serial dilutions were prepared for pour plating technique. Enumeration of cells was done on plates containing colonies between 10 and 300 (IDF, 2003). Incubation was done aerobically at 37°C for 48 hours. Gram staining was conducted to confirm the type of bacteria growing on the plates (ST is a Gram positive coccus and *Lactobacillus* is a Gram positive rods; Figure A. 1, Figure A. 2, Figure A. 3 in Appendix D).

5.4.1.2. *Lactobacillus acidophilus* (LA)

Viable cell counts of LA were enumerated following the IDF Standard 192 method (IDF, 2006) with a slight modification. MRS agar (Oxoid, UK) was prepared following the instruction of the manufacturer. Clindamycin hydrochloride (C5269-10 mg, Sigma Aldrich) (0.4 ml, 0.005%) was added aseptically to 200 mL molten MRS agar and the medium was used immediately. Incubation was done anaerobically using Aerocult oxygen absorber (Oxoid, UK) at 37°C for 72 hours. Prior to analysis, the medium had been confirmed for its ability to selectively cultivate the growth of *L. acidophilus* and inhibit the growth of ST and LB (data not shown).

5.4.1.3. *Lactobacillus bulgaricus* (LB)

The enumeration of LB was carried out following the IDF Standard 117 method (IDF, 2003) with a slight modification. MRS agar (pH 6.0) is a universal medium which supports the growth of *Lactobacillus* species. Therefore, the colonies grown on MRS agar represented combined total counts of LA and LB (Dave & Shah, 1996). Cell counts of *L. bulgaricus* were estimated by determining the difference between the cell counts obtained on the MRS agar and MRS+clindamycin (MRS agar with clindamycin allows for the selective enumeration of the *L. acidophilus* in food

products that contain other similar species (data not shown)). Incubation was done anaerobically using Aerocult oxygen absorber (Oxoid, UK) at 37°C for 72 hours.

For each sample, suitable serial dilutions were prepared for pour plating technique. Enumeration of cells was done on plates containing colonies between 10 and 300 (IDF, 2003). Gram staining was conducted to confirm the type of bacteria growing on the plates (ST is a Gram positive coccus and *Lactobacillus* is a Gram positive rods; Figure A. 1, Figure A. 2, Figure A. 3 in Appendix D).

5.5. Characterization of Yogurt (liquid form) Prepared from DYB

5.5.1. Fermentation Profiles

Growth Rate of Yogurt Starter Cultures and probiotic NCFM measurements during fermentation

Yogurt was prepared by fermenting the DYB in a water bath at 43°C (Ng et al., 2011) for a period of 8 hours to mimic the method used at Easiyo®. Measurement of pH (AOAC method 947.05) (AOAC, 2005) and analysis of cell counts (section 5.4.1) of ST, LB, and the probiotic NCFM were conducted every hour.

5.5.1.1. Growth Rates during Fermentation in Broth Medium

To study the microbial growth rates of ST, LB, and *L. acidophilus* NCFM in broth medium, the micro-titre plate (Fluostar Optima, UK) was used to measure absorbance during fermentation. Prior to the experiment, cultures were cultivated twice in sterile broth under anaerobic condition. One percent starter cultures were inoculated into M17 and MRS broth respectively to allow growth of ST and LB at 37°C for 24 hours. Probiotic NCFM (1 %) was inoculated into MRS broth at 37°C for 24 hours. On day one, appropriate dilutions of fresh stock cultures were inoculated into M17 agar and MRS agar to obtain pure colonies of ST, LB and LA, respectively. The agar plates were incubated at 37°C for 48 hours. On day two, typical colonies from each plate were propagated into respective 10 mL sterile broth and incubated at 37°C for 48 hours.

Gram stained colonies were examined under the Carl Zeis microscope (HBO 50/AC, Germany) at 100 x magnifications to confirm purity of cultures (data not shown). Cells were then harvested by centrifugation at 3250 x *g* (Heraeus Biofuge Primo R, Germany) for 10 min at 4°C. Following centrifugation, the supernatant was discarded and cells were washed twice with 10 ml of 0.1% peptone saline solution. The suspended cells (inocula) were then ready for analysis using the Fluostar Optima plate reader. Three blank (control) references were prepared as follows: 135 µl MRS broth + 15µl peptone solution; 135 µl M17 broth + 15 µl peptone; and 135 µl MRS clindamycin broth+15µl peptone. The mixtures were pipetted into the 96-wells (BMG Labtech, UK) of the micro-plate. To monitor culture growth, serial dilutions of the suspended inocula were prepared. For the yogurt starter, 0.5 mL of ST + 0.5 mL of LB were mixed with 9 mL peptone water to make 10^{-1} dilution. For the NCFM as a single strain, 1 mL of culture was mixed with 9 mL peptone water to make 10^{-1} dilution. For the mixed culture, 0.25 mL of ST + 0.25 mL of LB + 0.5 mL of the NCFM were mixed with 9 mL peptone water to make a 10^{-1} dilution. The appropriate serial dilutions procedure was prepared from the 10^{-1} dilution up to 10^{-7}. The microplate wells were subsequently filled with 15 µl of each prepared cell suspension and 135 µl of suitable broth. Following an initial reading (time 0), the microplate was incubated at 37ºC for 24 hours. The growth of cultures was monitored by measuring absorbance using a Fluostar Optima (UK) microplate reader at 595 nm (Kotikalapudi et al., 2010).

5.5.1.2. Effect of Various Inoculation Rates on Growth of Yogurt Bacteria and *L. acidophilus* NCFM

To understand the effect of initial inocula levels on growth behaviour, freeze dried cultures at various concentrations (1%, 2%, and 3%) were inoculated in sterile 10% reconstituted skim milk (RSM) to achieve initial inocula levels of 10^{10} and 10^{11} cfu/gram. The growth profile of starter and probiotic cultures in RSM during 8 h fermentation was monitored by enumeration of viable cell counts and pH measurement. Cultures were allowed to ferment 10% RSM for 8 h at 43°C using a water bath. One mL sample was withdrawn hourly and diluted with 9 ml of 0.1% sterile peptone water to make serial dilutions of up to 10^{9} cfu/mL. The cultures were plated on appropriate molten agar (IDF, 2003). The details of inocula levels are summarised in Table 8.

Table 8. Inocula levels of yogurt bacteria in sterile 10 % RSM.

Name of mixtures	Inoculum level
NCFM alone	1% (≈10^{11} cfu/g)
ST and LB in commercial starter	1% (≈10^{10} cfu/g) starter cultures which consist of ST and LB
1% mixture	0.5% starter cultures (≈$5x10^{9}$ cfu/g) + 0.5% NCFM (≈$5x10^{10}$ cfu/g)
2% mixture	1% starter cultures (≈10^{10} cfu/g) + 1% NCFM (≈10^{11} cfu/g)
3% mixture	1.5% starter cultures (≈$1.5x10^{10}$ cfu/g) + 1.5% NCFM (≈$1.5x10^{11}$ cfu/g)

5.5.2. Characterization of Ready-to-eat (liquid) Yogurt Prepared from DYBs

5.5.2.1. Syneresis

Yogurt was prepared by fermenting the dehydrated yogurt base (DYB) in water bath at 43°C for 8 hours and chilled overnight (about 16 hours) at 4°C; this was referred to as "day one" samples. The yogurt samples were stored for two weeks at 4°C and presence of syneresis was evaluated at days 1, 7 and 14. Syneresis is referred to as the amount of water expelled on the surface of set-yogurt (Salvador & Fiszman, 2004). Bottles of yogurt samples were withdrawn from the refrigerator (4°C), wiped outside using paper towels and weighed. The water expelled onto the surface of the yogurt was recovered using disposable 2.5 mL plastic pipettes (Huh Tamaki®). The weight of yogurt after removal of water was measured gravimetrically. Syneresis was calculated as follows (Salvador & Fiszman, 2004): % syneresis = 100 (Initial weight – Final weight) / Weight of yogurt container. Yogurt samples were analysed in duplicate and the experiment was replicated twice.

5.5.2.2. Rheology

Gel firmness of the RTE yogurts stored for 2 weeks was measured using the TA-XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK) at room temperature (20°C) using a flat base

cylinder probe (1cm diameter) at 1 mm/s to a depth of 20 mm; the TA-XT was equipped with a 5 kg load cell (Salvador & Fiszman, 2004). The strength of the gel was recorded as firmness. Firmness (in Newton) is the force required to produce first significant peak in the curve during penetration of displacement of 20 mm.

Viscosity measurements were done using a T-bar spindle S96 (Donkor et al., 2007) attached on the Brookfield DV II+ (UK). Plastic container (500 mL) supplied Easiyo was used for all measurements. Viscosity was first carried out on un-disrupted yogurt gel followed by manual stirring of the yogurt for 30 s prior to measurement. For an unbroken gel, the speed was set to 1 rpm. For a disrupted gel, the speed was set to 10 rpm (Guzmán-González et al., 1999). The temperature of yogurts during viscosity measurements were 9 ± 2 °C. The spindle continuously penetrated the gel up and down throughout the sample. The viscosity readings were recorded after 35, 40, 50, and 60 s. The mean readings of the recorded viscosity at various times through the sample were compounded (Guzmán-González et al., 1999). Analyses were done in duplicate with two replications and readings were expressed as mPa.s.

5.5.2.3. Titratable acidity

Titratable acidity was measured following the IDF (1997) standard method 11869. Twenty grams of yogurt samples were diluted with 40 ml of distilled water. Titration of the diluted samples was carried out against standard 0.1 M NaOH (Sigma Aldrich, NZ) until first persistent pink (for natural flavour) and bluish colour (for strawberry flavour) or until pH 8.3 was observed using indicator phenolphthalein and bromothymol blue, respectively. The NaOH was initially standardised against potassium hydrogen phthalate (KHP) (Meat Research Corporation, 1997). The volume of NaOH used in the titration was recorded and the concentration of lactic acid was calculated using equation (6). Analyses were done in duplicate and the experiment was repeated twice.

$$\%\ \text{lactic acid} = \frac{\text{volume of NaOH required (ml)x } 0.009}{\text{sample weight (g)}}\ x\ 100 \tag{6}$$

1 mL 0.1M NaOH = 0.009 g lactic acid; 1 mL of sample ≈ 1 g of sample

5.5.2.4. pH Measurement

The pH was obtained by direct measurement of yogurt samples using a digital pH meter (Sartorius, Japan) equipped with a glass electrode following the AOAC method 981.12 (AOAC, 2005). Prior to each measurement, the pH meter was calibrated using standard buffer solutions at pH 4.0 and 7.0. Measurements were done in duplicate and the experiment was repeated twice.

5.5.2.5. Sensory Analysis

Sensory analysis of consumer acceptance was performed on freshly made yogurts (day 1 storage) of the three (selected) formulations following the method of Hashim (2009). Prior to serving, yogurt was stirred using a metal spoon for 30 s (similar to treatment of broken gel during viscosity measurements described in section 5.5.2.2). The evaluation was conducted two times in a sensory booth under white light environment and under normal light (white) at the Sir Neil Waters Foyers (similar to retail conditions). During analysis, distilled water was used for cleaning the palate between samples. A 1-9 hedonic rating (1 = dislike extremely and 9 = like extremely) was used to evaluate each attribute (appearance, texture, flavour, taste) and overall acceptance of the products (Hashim et al., 2009) (Appendix K). The participants who participated in yogurt sensory evaluation were selected on the familiarity and consumption of the products. Yogurt samples were served in transparent plastic cup coded with random 3-digit codes and monadically served. Time lapse between evaluation of samples was fixed at 30s (Bayarri et al., 2011).

5.6. Determination and Prediction of LAB Survival in DYB at Various Temperatures

To study the shelf-life of the dehydrated yogurt base (DYB), selected formulations of the products were stored at 4, 22, 35, 45, and 55 °C. Cell counts of ST, LB, and NCFM were determined for each storage temperature. The numbers of microorganisms were plotted on a graph against time and the best-fit line was used to determine the order of the reaction, which showed a linear relationship of ln (Q) *vs* time (days) indicating first order reaction ($\frac{\ln[Q_0]-\ln[Q]}{t} = k$). The slope obtained from the graph indicates the reaction rate constant (k), which differs

between temperatures (Labuza et al., 1997). Using the Arrhenius equation ($\ln k = \ln k_0 - (Ea/2.303R) * (1/T)$; where k_0 is the experimental constant, T is absolute temperature, R is the gas constant, and Ea is the activation energy, the ln of each k value was plotted against the reciprocal of absolute temperature (1/T) gives information about k_0, an experimental constant and *Ea,* the activation energy. The model predicted using Arrhenius equation, therefore it can be used to predict shelf-life of the DYB (*t*) at any storage temperature ($\frac{\ln[Q_0]-\ln[Q]}{t} = k$) (King et al., 1998; King et al., 1993; Tsen et al., 2007). Storage temperatures of 35, 45, and 55 °C were used as experimental data while temperatures of 4 and 22 °C were used to validate the model except for formulation #HFHS where 22, 35, and 55 °C were used as experimental observations and the 4 °C storage for model validation. The survival of ST, LB, and NCFM in the DYB was carried out for each formulation and storage temperature.

5.7. Data Analysis

The General Linear Model (GLM) of the analysis of variance (ANOVA) was carried out to analyse the data obtained in the analyses of DYB and liquid yogurt. The assumption for using ANOVA is that data are normally distributed, which is shown by a bell-type curve. However, in the case of sensory data arising from hedonic scales, skewiness of the curve is common as consumer panellists may rate low or high scores for their degree of likeliness of the products. For sensory analysis, the Kolmogorov-Smirnov test was used to determine the normality of data distribution (O'Mahony, 1986). A simple normality test of Kolmogorov-Smirnov confirmed that the data distribution of each sensory profile from each sample was not normal ($p>0.05$) except for data on texture and sweetness (Table 11). Therefore, both parametric and non-parametric tests were used. One-way ANOVA was performed for the parametric consumer data whereas the Kruskall-Wallis was used for the non-parametric data. Tukey's and Mann-Whitney tests were applied to separate significantly different comparisons of the treatments at 95% confidence interval (O'Mahony, 1986). Principal Component Analysis (PCA) was applied to the mean intensity values of sensory attributes. To identify panellist behaviour during rating the samples, subgroups of panellists were segmented based on overall acceptability using Cluster Analysis (Euclidean). To determine the relationship between consumer sensory and instrumental data, regression analysis was used (Bayarri et al., 2011; Lyon et al., 1992). All analyses were carried

out using Minitab® (Minitab Ltd. version 15.1, USA) except for non-parametric data which were analysed using the SPSS 18 (IBM ™ Company, USA).

6. RESULTS

6.1. Characterization of Milk Powder

The HPLC results of milk characterization are shown in Table 9. The amounts of undenatured α-lactalbumin and β-lactoglobulin in milk powders used in the current study were considerably low, below 2% (Figure 6) and in line with the company requirement for making the DYB. Using the dye-binding protein method, the values of WPNI for skim and whole milk powders were 2.5, 2.9, 3, and 3 mg/g respectively indicating the powder had received medium-high heat treatments. Such milk powders produce liquid yogurts with high viscosity (Ozer, 2010; Spreer, 1998; Tamime and Robinson, 2007). Full data on the characteristics of the powders are given in Appendix A.

Table 9. α-lactalbumin and β-lactoglobulin milk powders were measured by HPLC.

Milk powders type	α-lactalbumin (%)	β-lactoglobulin (%)
SMP batch 1	0.43	0.88
SMP batch 2	0.68	1.06
SMP batch 3	0.66	1.01
WMP	0.52	0.48

6.2. Characterization of DYBs during Ambient (20°C) Storage

6.2.1. Viability of Yogurt Cultures in DYBs

The cell counts of *S. thermophilus* during storage were shown in Figure 10 while the counts of *L. acidophilus* NCFM and *L. bulgaricus* are shown in Figures 11 and 12. Overall, the counts of the probiotic and yogurt starter bacteria in the packaged DYB at 20°C for 9 weeks storage were 10^8-10^9 cfu/g and 10^6-10^7 cfu/g respectively. The cell counts of the LAB varied significantly between formulations and storage time (p-value<0.05; Appendix Table A. 2). Similarly, the level of water activity was significantly variable during storage ($p<0.05$; Appendix Table A. 4).

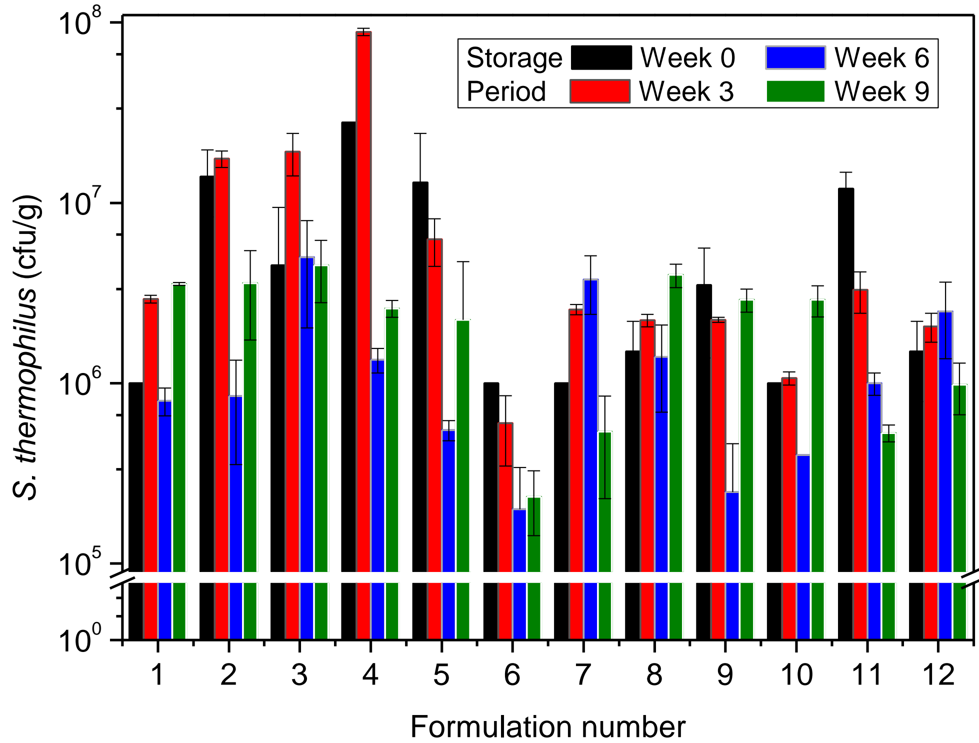


Figure 10. Cell counts of *S.thermophilus* in dehydrated yogurt bases. Each bar represents average values of two samples. Error bars are ± standard deviation. Descriptions of the formulations are shown in Table 7, section 5.3.

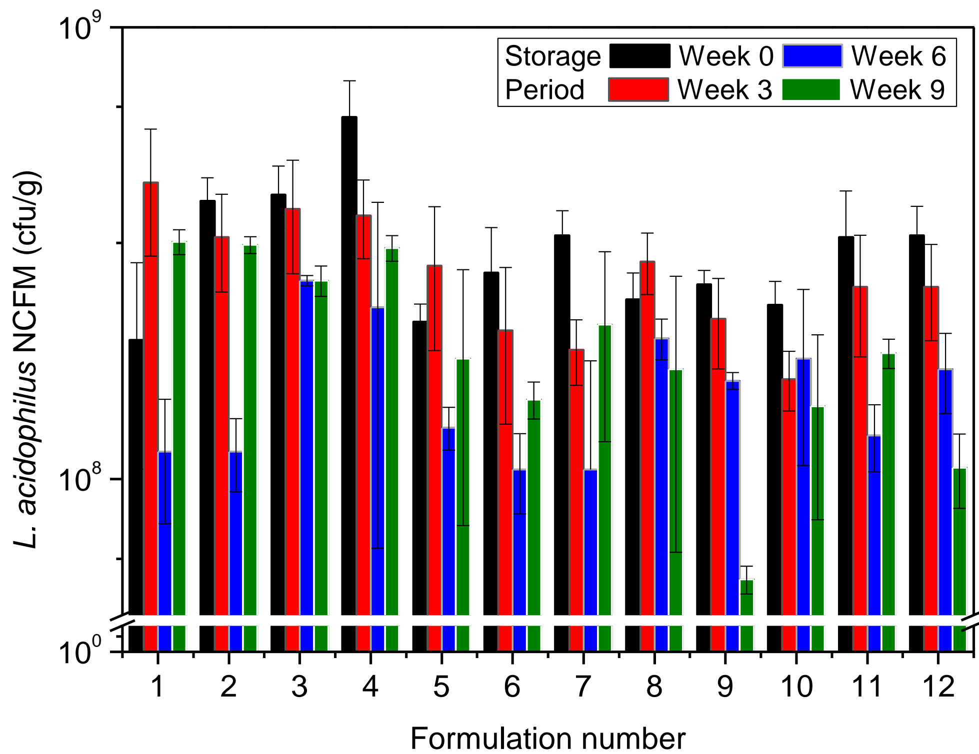


Figure 11. Cell counts of *L. acidophilus* NCFM in dehydrated yogurt base. Each bar represents average values of two samples. Error bars are ± standard deviation Descriptions of the formulations are shown in Table 7, section 5.3.

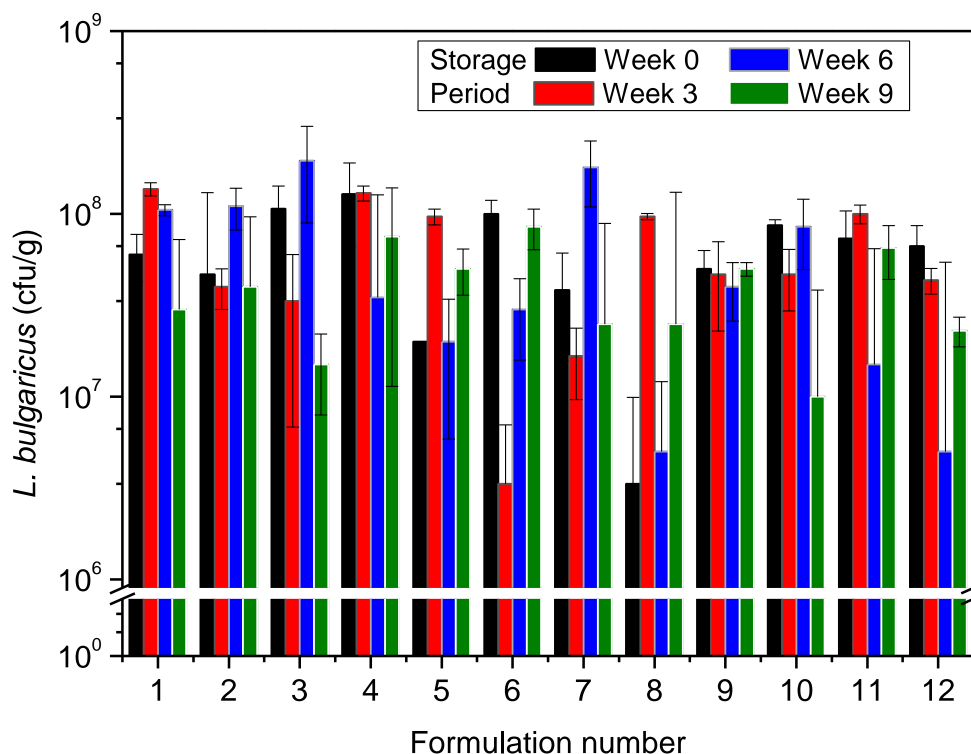


Figure 12. Cell counts of *L. bulgaricus* in dehydrated yogurt bases at 20°C for 9 weeks. Each bar represents average values of two samples. Error bars are ± standard deviation Descriptions of the formulations are shown in Table 7, section 5.3.

Among the natural yogurt formulations (formulations 1-4), cell counts of ST, LB, and NCFM in formulations 3 and 4 were more than 10^6, 10^7, and 10^8 cfu/g, respectively. Statistically, there was no significant ($p>0.05$) reduction of the counts of LAB (Table A. 3, Appendix) during storage in formulations 3 and 4. In terms of [O_2] and a_w level, formulations 3 and 4 showed <11% [O_2] and 0.1 a_w. Based on these preliminary results, formulation 4 was selected for further characterization.

Among the strawberry samples (formulations 5-12), cell counts of NCFM were stable during storage at approximately 10^8 cfu/g (Figure 11) although significant decreases ($p<0.05$ Table A. 2, Appendix) in cell counts were observed in formulations 9, 11 and 12 (Table A. 3, Appendix). For formulation 6, the survival of ST (Figure 10) was very poor (less than 10^6 cfu/g, $p<0.05$; Table A. 3 Appendix) although the viability of LB (Figure 12) was ≈10^7 cfu/g. Additionally, the a_w (Figure 14) of formulation 6 increased (around 38%) during storage ($p<0.05$; Table A. 4, Appendix) although the levels of [O_2] (Figure 13) and [CO_2] were stable during storage ($p>0.05$;

Table A. 5 & Table A. 6). Similar patterns of a_w and the $[O_2]$ were also observed in the strawberry flavour of formulation 5. Thus, based on these results, these two formulations (5 and 6) were excluded from further characterization.

Of formulations 7-12, the cell counts of NCFM decreased significantly in formulations 11 and 12 ($p<0.05$; Table A. 3) during storage (Figure 11). The loss of viability by the ST (Figure 10) was found to be significant in formulation 11 ($p<0.05$; Table A. 3) although loss of survival in LB was fairly low ($p>0.05$; Table A. 3) (Figure 12). The decrease in cell counts of ST was probably due to increases in a_w (Figure 14) and $[O_2]$ (Figure 13). Therefore, formulations 11 and 12 were excluded from further studies.

The remaining formulations (#7 - #10) were comparable in terms of LAB cell counts, where the counts ranged between 10^6 and 10^8 cfu/g for 9 weeks. Similar results of a_w and $[O_2]$ were also observed in the remaining formulations where the levels were below 0.16 and 16%, respectively. In formulation 9, the stability of starter cultures was satisfactory, however, reduction in the NCFM during storage was significant (Figure 11; Table A. 3), thus it was not selected for further analysis. Due to the similarity of formulation #7 and #8 and the preference for packaging B, formulation 8 was therefore selected along with formulation 10. The selected formulations for further studies were then 4, 8, and 10 which coded #HFN, #LFLS, and #HFHS in subsequent references. The nutritional information of the three formulations is shown in Table A. 1.

6.2.2. Water activity (a_w) and Gas Analysis in DYBs Packages

The concentration of oxygen in the DYB packages during 9 weeks storage at 20°C is shown in Figure 13. The level of oxygen content in the DYB packages was between 8 and 16%. Slight changes ($p>0.05$) in oxygen concentration during storage were observed in all formulations (Table A. 5). Although the level of $[O_2]$ was comparable at the time of packaging and at the end of storage, the values were however far too high than the values suggested in many reports reviews (Miller et al., 2002; Miller et al., 2003; Talwalkar et al., 2004). For significant reduction of bacterial death, O_2 concentration in the MAP yogurt packaging should be less than 4% as suggested by Robertson (2005). The detrimental effects of oxygen on LAB survival were due to

the oxidation and free radical generation which may be destructive to DNA level of bacteria (Andersen et al., 1999; Kurtmann et al., 2009b). The presence of O_2 in DYB may have contributed to the reduction of LAB as observed with lower cell counts of yogurt microorganisms as (Figures 10, 11, and 12). As the concentration of O_2 during storage in the DYB did not significantly differ, the loss of cell viability in DYB could not be associated with packaging materials where the rate of oxygen transmission was very low (<0.3 cc/m^2/24 hours at 23°C/0-80% RH) and thus (initial) high O_2 concentration in the package may be responsible. The presence of [O_2] in the current study may also be attributed to the mixing step (dry-blending) involved in the manufacture of DYB. The possibility of incorporation of O_2 during the agitation and mixing steps has been reported in other studies (Miller et al., 2002; Talwalkar et al., 2004).

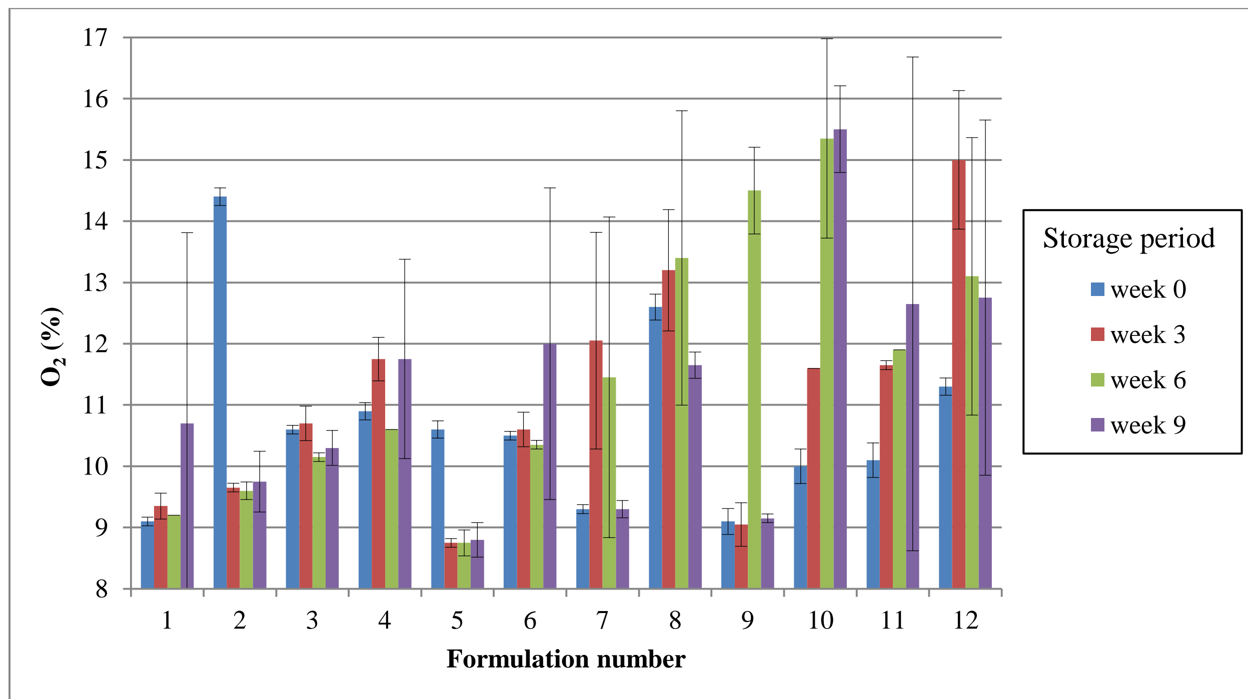


Figure 13. Concentration of oxygen in DYBs packages during storage at 20°C for 9 weeks. Descriptions of the formulations are shown in Table 7, section 5.3. Each bar represents average of two measurements. Error bars are standard deviation of two measurements.

The amount of free water available for bacterial growth, or so-called water activity (Early, 1998) in the products is shown in Figure 14. The values for a_w were below 0.2 for all formulations. The level of a_w was fairly constant during storage indicating the efficiency of packaging material which keept the gas transmission to a minimum (Miller et al., 2002). Water activity plays a crucial role in maintaining the survival of lactic acid bacteria (LAB) during storage

(Kailasapathy & Rybka, 1997; Sellars, 1991). The declined cell counts of LAB in this study might be attributed to an increase (p-value<0.05) of a_w (Table A. 4) during storage. Many studies have reported an inverse relationship between survival and a_w (Kailasapathy & Rybka, 1997; Sellars, 1991; Wirjantoro & Phianmongkhol, 2009). The study carried out by Ishibashi et al. (1985) demonstrated maximum survival of *Streptococcus, Bifidobacteria*, and *Lactobacillus* species in the dry mix (similar to the current study) when a_w level was 0.1. According to the report, freeze-dried bacteria in the DYB system had maximum viability and minimum susceptibility to elevated temperature during storage at a_w below 0.2. While the relationship between oxygen and water activity in a dehydrated food system is beyond the scope of this study, further study on this issue may nurture the knowledge of optimising LAB viability in the system as no studies have been done on this area.

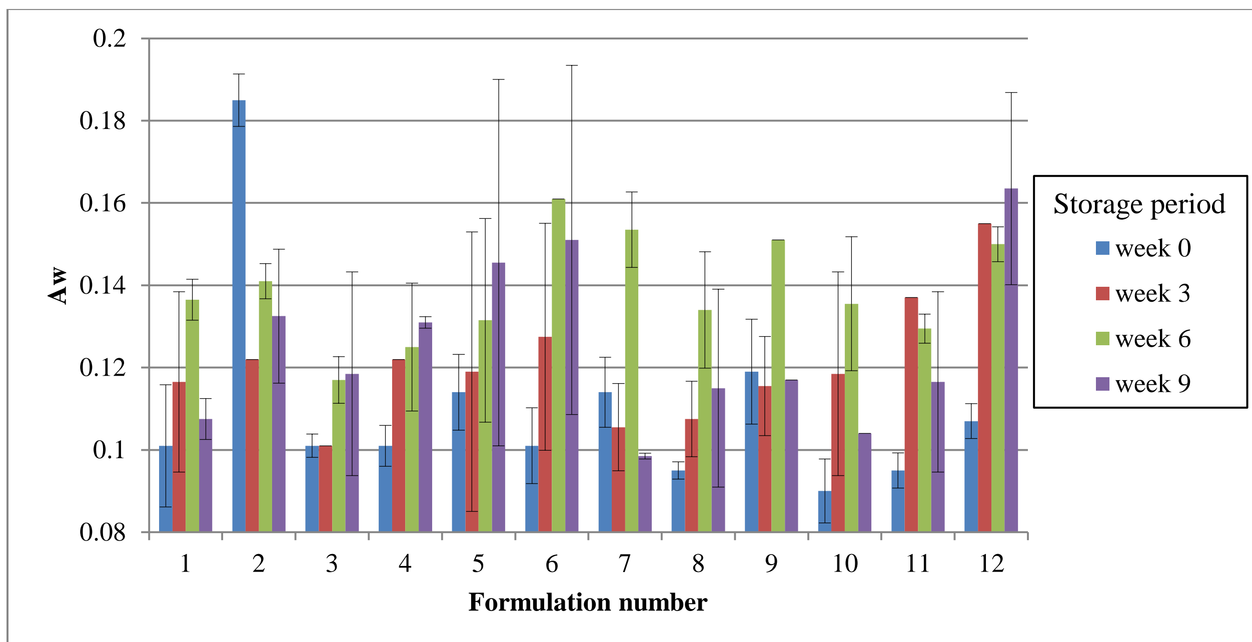


Figure 14. Water activity of dehydrated yogurt bases during storage at 20°C for 9 weeks. Measurements of a_w were carried out at 25°C. Description of the formulations is shown in Table 7, section 5.3. Each bar represents average of two measurements. Error bars are standard deviation of two measurements.

6.3. Characterization of RTE Yogurt (Liquid-Base)

6.3.1. Fermentation Profile of Yogurt Bacteria in Broth Media

The growth of yogurt bacteria in broth media (M17, MRS, and MRS+clindamycin) during 24 hours incubation is shown in Figure 15. The growth of all bacteria cultures showed a typical sigmoidal curve divided into lag, log, and stationary phases. The growth patterns of the cultures

were similar irrespective of the type of culture present in the medium. The growth pattern of ST and LB were similar irrespective of the presence of the probiotic NCFM. The activity of ST was more prononounced at the beginning of the growth curve and decreased when it entered the stationary phase, which occurred after 9 hours of incubation. The sigmoidal growth of ST indicated prolonged stationary phase after the exponential growth. This suggests that the strain used in the current study may not be classified as a rapid autolytic bacterium. Rapid autolytic growth of ST was characterised by a bell-shaped curve where autolysis of the bacterium occurred at the end of the exponential growth due to limited sources of lactose (<5 g/l) and/or at neutral pH between 6 and 7 (Selma et al., 2007).

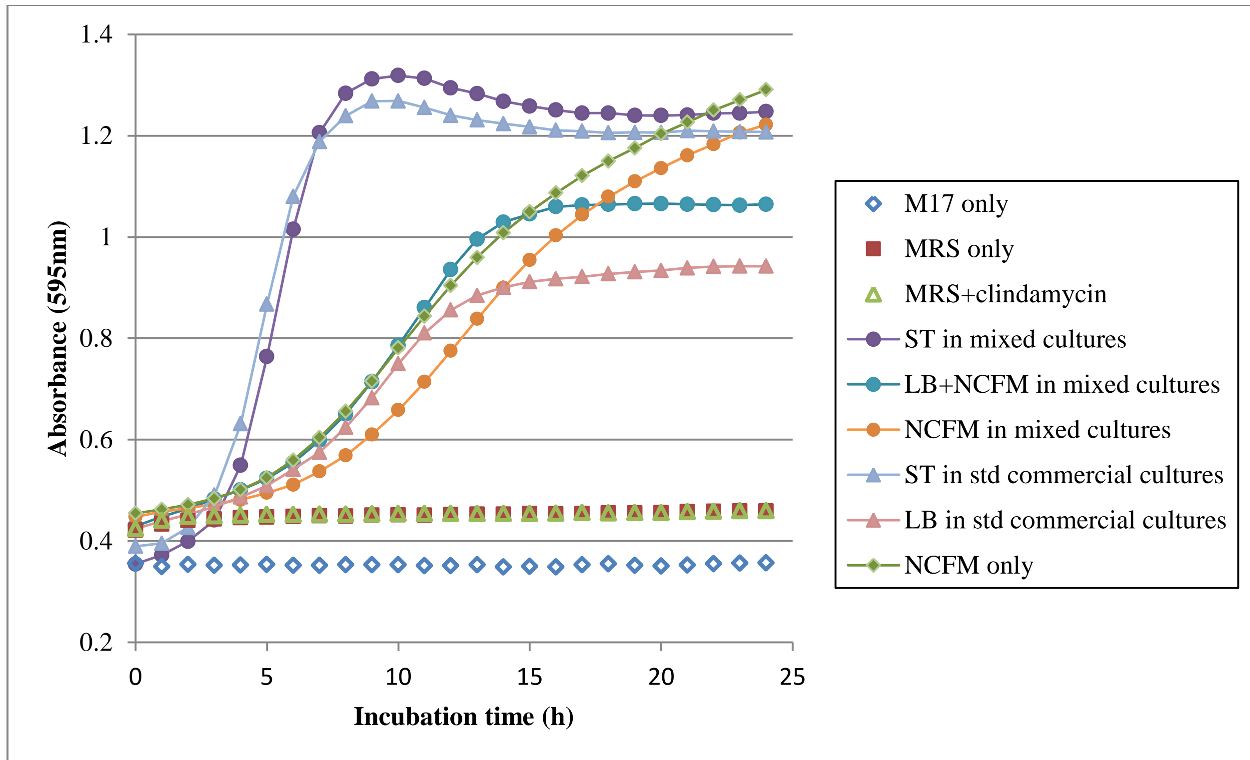


Figure 15. Growth rates of yogurt cultures measured using Fluorostar Optima micro-plate reader absorbance technique at 37°C. Each point represents average values of (at least) 2 samples. A mixture culture indicates the presence of ST, LB, and NCFM. Standard (std.) cultures indicate the presence of ST and LB.

As the environment gradually becomes more anaerobic due to ST activity, the growth rate of the *Lactobacillus* started to increase markedly. The growth pattern was similar to reports by Chandan (2006) and Tamime & Robinson (2007) on the synergistic effects between starter cultures ST and LB. The synergistic effects between the yogurt starter cultures and the NCFM are clearly observed. The exponential growth of NCFM (log phase) was observed after 4 h and

continued to grow until the bacterium reached stationary phase which was observed after 20 h of incubation (Figure 15). The activity of NCFM was more pronounced as a single culture than in the mixed cultures. However, it should be noted that the initial concentration of NCFM as a sole fermenter was higher than the concentration in the mixed population (section 5.5.1.2).

6.3.2. Effects of Inoculation Rates on Growth of Yogurt Bacteria

To determine the growth pattern of the probiotic strain, various concentrations of NCFM and starter cultures at 1:1 ratio were inoculated into 10% RSM. For each inoculation level, the number of cells on the incubated RSM were analysed as well as the measurements of pH. Details of inocula levels are shown in Table 8 (section 5.5.1.2).

The growth of ST and LB at 1% inocula ($\approx 10^{10}$ cfu/g) in the absence of the NCFM ranged between 10^8 - 10^9 cfu/g in 8 hours (Figure 16) and 10^7 - 10^8 cfu/g in 8 hours respectively (Figure 17). When 0.5% NCFM (~$5x10^{10}$ cfu/g) was used, the number of ST (ST in 1% mixture, Figure 16) and LB (LB in 1% mixture, Figure 17) ranged from 10^7 - 10^8 cfu/g. The synergistic growth of ST and LB in the presence and absence of NCFM was demonstrated in this study. The high number of ST and LB in the presence of NCFM confirmed that these cultures could be used together as mixed cultures.

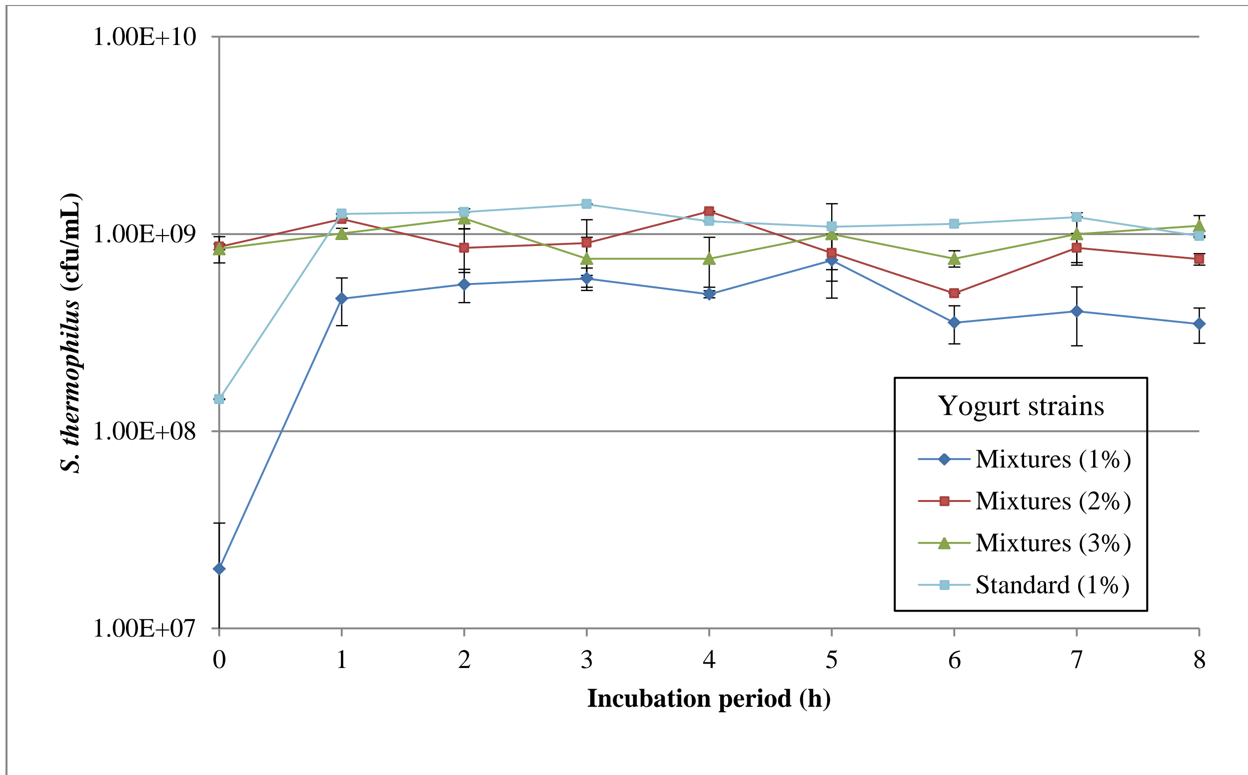


Figure 16. Growth of *S. thermophilus* (ST) when inoculated into 10% RSM as mixed freeze-dried starter cultures and incubated at 43°C for 8 h. Each point represents average of two samples. Error bars are ± S. D. Note: Standard (starter) cultures contained ST and LB; mixtures of cultures contained ST, LB, and NCFM.

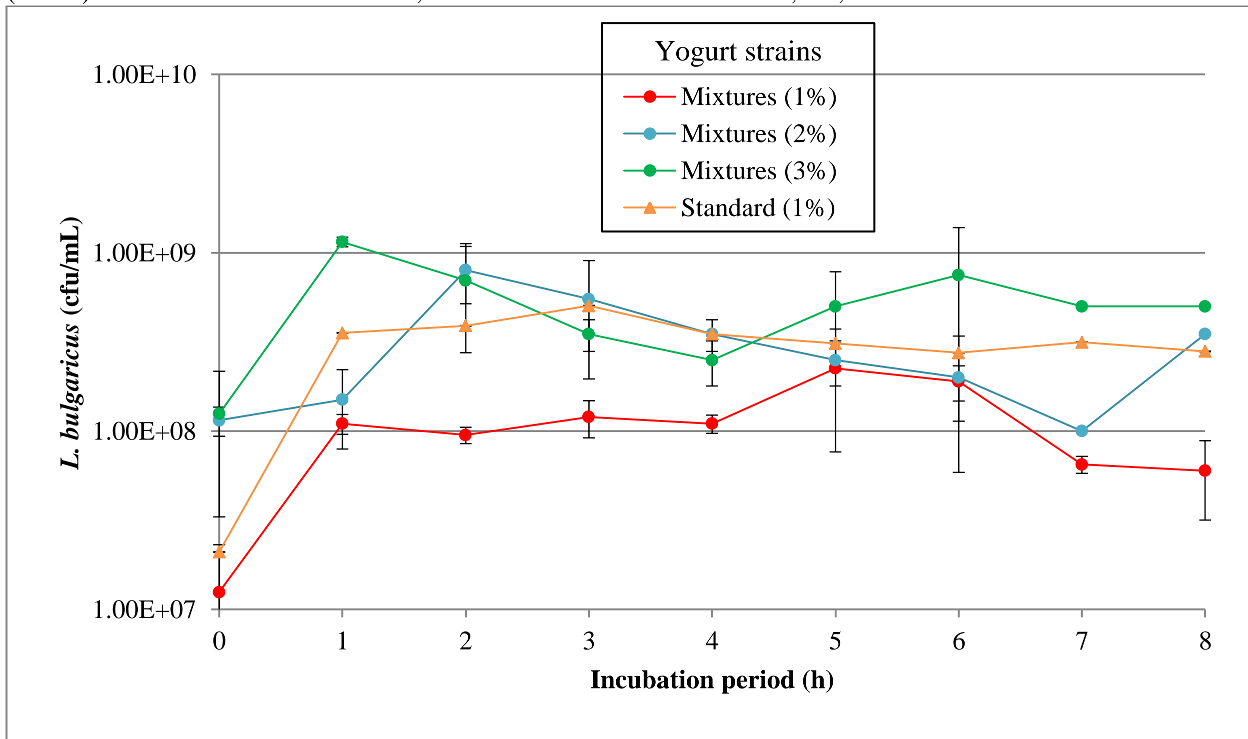


Figure 17. Growth of *L. bulgaricus* (LB) when inoculated into 10% RSM as mixed freeze-dried starter cultures and incubated at 43°C for 8 h. Each point represents average of two samples. Error bars are ± S. D. Note: Standard (starter) cultures contained ST and LB; mixtures of cultures contained ST, LB, and NCFM.

As a single culture, the growth of NCFM increased from 10^8 cfu/g to 10^9 cfu/g (Figure 18). Similar growth pattern of this bacterium was observed in the presence of the yogurt starter cultures (ST and LB) at 1% mixtures (Figure 18). In 1% mixed cultures, the inoculum level of NCFM was 0.5% (10^{10} cfu/g) and the growth of the probiotic in milk increased from 10^7 cfu/g to 10^9 cfu/g. Although the cell counts of NCFM in the absence of the yogurt starter cultures was about 0.4 logs cfu/g higher than the counts in 1% mixtures, such discrepancies may be attributed to the presence of higher inocula levels of NCFM at the beginning of fermentation. This suggests that an initial inoculum level of 0.5% starter cultures (10^9 cfu/g) supported growth of the probiotic NCFM at inoculation level 0.5%.

When the inoculation rate of the probiotic was increased to 2% and 3% mixtures, the growth of NCFM (Figure 18), LB (Figure 17) and ST (Figure 16) neither increased nor decreased, although higher cell counts of LAB were obtained due to initial higher level of inoculum. Interestingly, inocula rates of 2% and 3% had similar yogurt bacteria counts during fermentation irrespective of the bacteria type used. Therefore, it would not recommend to increase the initial inocula rates of each bacterium to more than 0.5% as the recovery levels of the yogurt bacteria were comparable at higher rates (e.g. 1 and 1.5%). This observation was similar to the findings of Dave and Shah (1997a) which showed that starter cultures and probiotic LA grew faster in yogurt at lower levels of inocula than at higher levels.

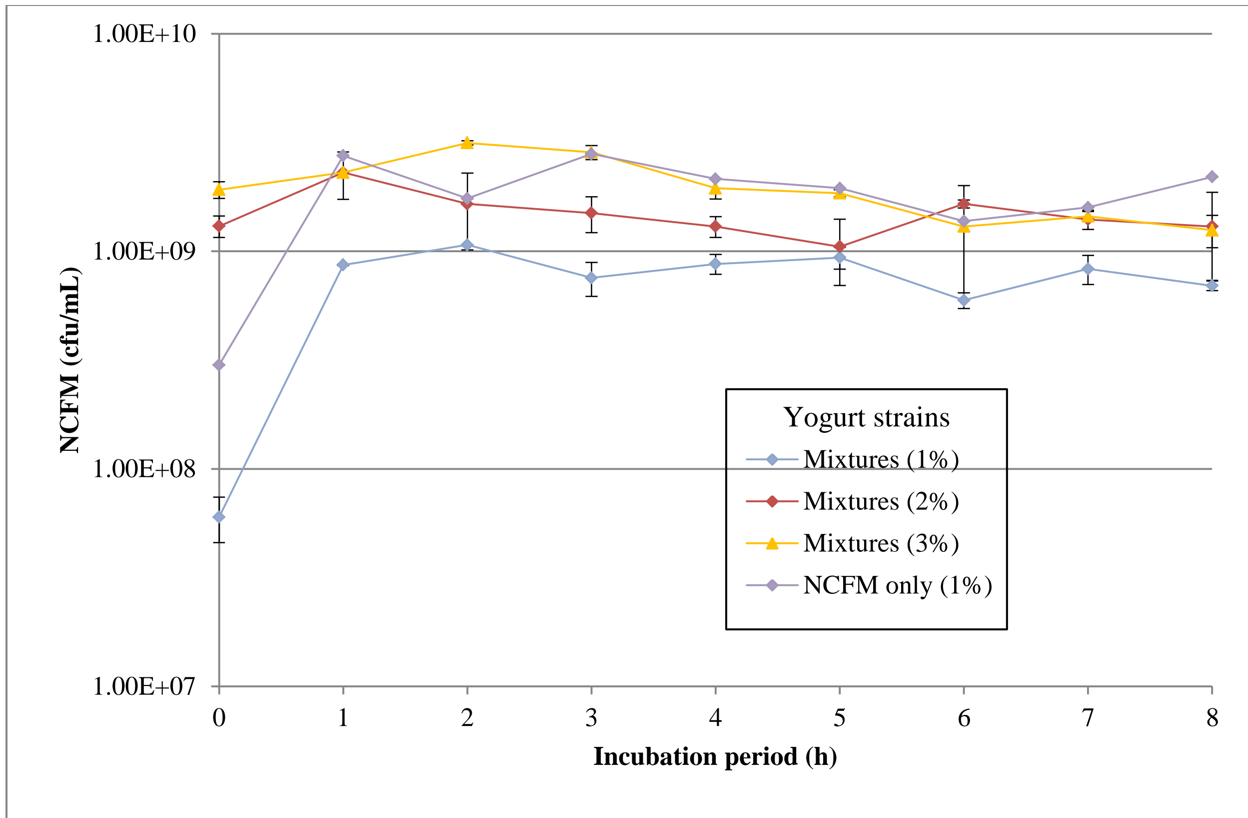


Figure 18. Growth of *L. acidophilus* (LA) NCFM when inoculated into 10% RSM as mixed freeze-dried starter cultures and incubated at 43°C for 8 h. Each point represents average of two samples. Error bars are ± S. D. Note: mixtures of cultures contained ST, LB, and NCFM.

The pH of milk inoculated with 1% NCFM (~10^{11} cfu/g) gradually decreased from 6.7 to nearly pH 4 (Figure 19). Similarly, the pH decreased from 6.5 to nearly 4 in yogurt fermented with mixtures of NCFM and starter bacteria at 1% inocula levels (Figure 19). In 1% mixtures of LAB, the pH rapidly declined within 3 h of incubation and gradually slowed down as fermentation progressed towards the end (Figure 19). The rapid decline in pH in mixed cultures could be attributed to the presence of ST which actively utilizes lactose to produce lactic acid (Dave & Shah, 1997a). While this report (Dave & Shah, 1997a) suggested that the decrease in pH was faster in yogurt made using lower inocula rates, this was however contrary to the finding from this study which observed no difference in acidification rates irrespective of the inocula rates used.

While the performance of ST, LB, and NCFM during fermentation is strain dependent, the growth of these bacteria was (in general) rapid at the beginning of fermentation (2 hours). Similarly, the drop in pH was fast initially (2 hours) and slow down as fermentation progress.

The high growth of LAB at the beginning of fermentation may be attributed to the abundant nutrients which becomes exhausted as the population of bacteria increase. Furthermore, the slow growth of bacteria after 2 hours incubation may also be attributed to the sensitivity of these bacteria to acid and other metabolites compounds which may be inhibitory (Kailasapathy & Rybka, 1997; Sellars, 1991).

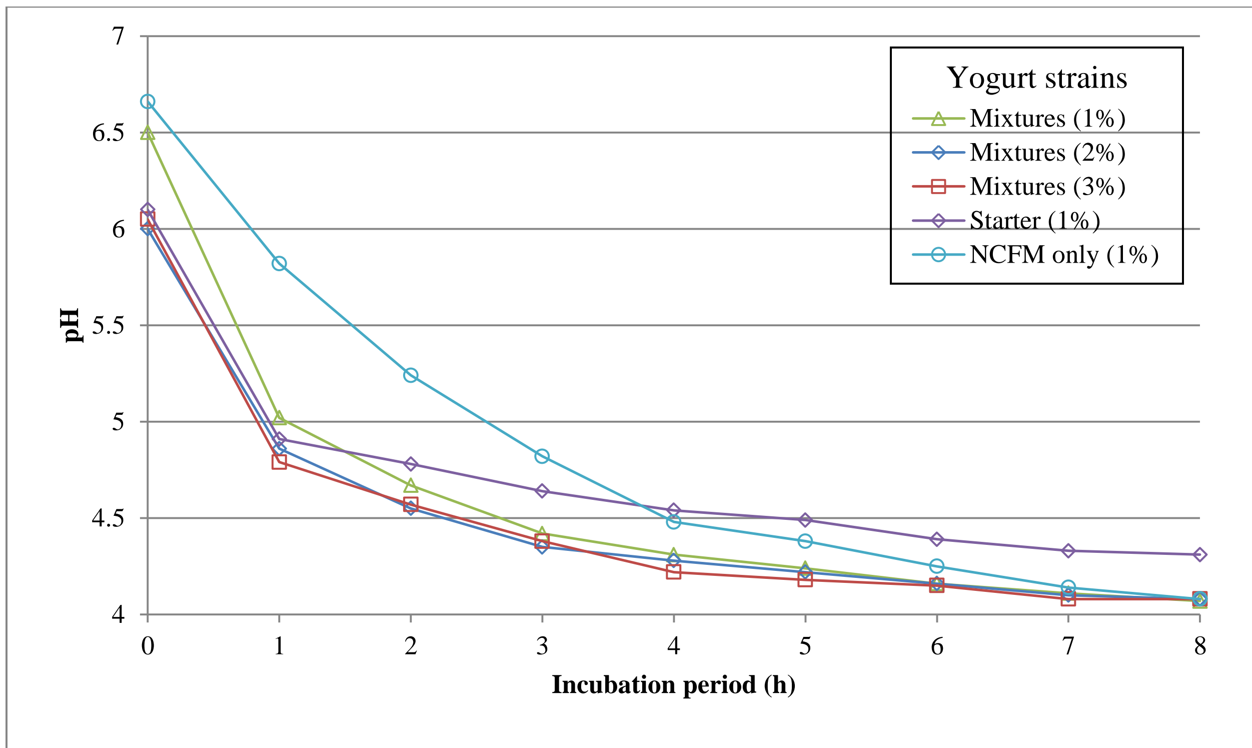


Figure 19. pH of LAB when inoculated in 10% RSM as mixed freeze-dried starter cultures and incubated at 43°C for 8 h. Note: Standard (starter) cultures contained ST and LB; mixtures of cultures contained ST, LB, and NCFM.

6.3.3. Characteristics of RTE Yogurt Prepared from Selected DYB Formulations

6.3.3.1. Yogurt Fermentation at 43°C

The fermentation profiles of three DYB formulations are shown in Figure 20. The fermentation profile of the LAB in yogurt samples was similar for all formulations despite lower inocula levels of ST ($p<0.05$, appendix E). This suggests that addition of sugar at a concentration applied in these formulations did not affect bacteria growth during fermentation. This may be attributed to the ability of LA and ST to metabolise sucrose (Table 5) (Haukioja et al., 2008). The rapid

growth (up to 1 log cfu/mL per hour) of ST (up to 4 log cfu/mL) was more pronounced at the beginning of fermentation and slowed down when LB growth had increased towards the end of fermentation (after 5 hours). This confirms the synergistic relationship between starter organisms in the milk medium as reported in many scientific studies (Spreer, 1998; Tamime and Robinson, 2007). As for the NCFM, the level of growth increased by about a log ($p<0.05$, appendix E) from 10^7 cfu/mL to 10^8 cfu/mL during 8 hour fermentation. Although the growth of LB during fermentation was not as rapid as ST, the cell counts increased significantly ($p<0.05$, appendix E) from 10^6 to 10^8 cfu/mL in all the formulations ($p>0.05$, appendix E). The pattern of growth by LAB was fairly similar in all formulations suggesting that neither the presence of sugar nor fat affected their growth during yogurt fermentations. While the interaction of species between the LAB during fermentation is beyond the scope of this study, the observation from this investigation showed good growth of the NCFM during fermentation in the presence of ST and LB. This indicated the potential use of these cultures in the formulations used in the DYB system.

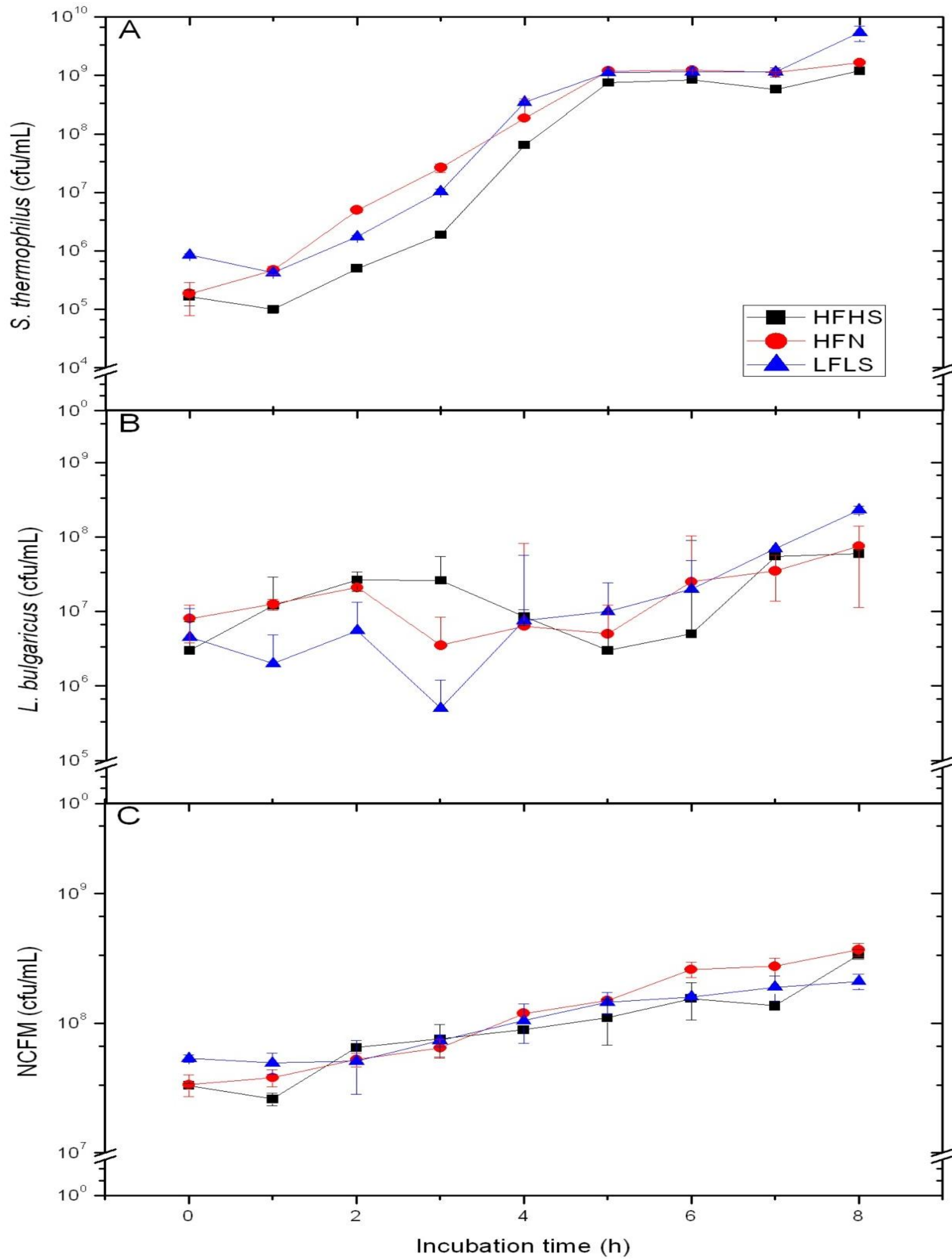


Figure 20. Culture growth of LAB at 43 °C for 8 h. Each point represents mean $\log_{10}$ cfu mL^{-1} of two independent analyses. Descriptions of the formulations are shown in Table 7, section 5.3. Error bars are ± S.D.

The acidification of yogurt during fermentation is presented in Figure 21. The effect of sugar (#HFHS and #LFLS) and fat levels (#HFN) on the acidification profile of yogurt during 8 hours of incubation was not significant ($p>0.05$, appendix E). The initial pH values (pH 6.5) decreased to 4.4 by the end of fermentation irrespective of the formulations. The rapid decline in pH was due to the conversion of lactose to lactic acid and the rate of milk acidification depends on the buffering capacity of the food system (Zare et al., 2011). Although the acidity levels shown in Figure 21 and Figure 19 of the products decreased to about pH 4.2 after 8 hour incubation, the acidity did not impact negatively on the sensory evaluation (section 6.3.5), the mean score for the acceptability of the products (#HFHS and #LFLS) was above 6 of 9-hedonic scale. This may be attributed to the optimal balance of high quality raw materials used, volatile (acetaldehyde, ethanol, and CO_2) and non-volatile (lactic acid and acetic acid) sensory compounds produced by the LAB in yogurts during fermentation and storage (Imhof et al., 1994; Kneifel et al., 1992; Routray & Mishra, 2011). The volatile compounds (acetaldehyde) although are present in low amounts (µg/kg) can significantly influence the flavour and aroma perception of the products (Hugenholtz, 1993; Imhof et al., 1994; Imhof et al., 1995).

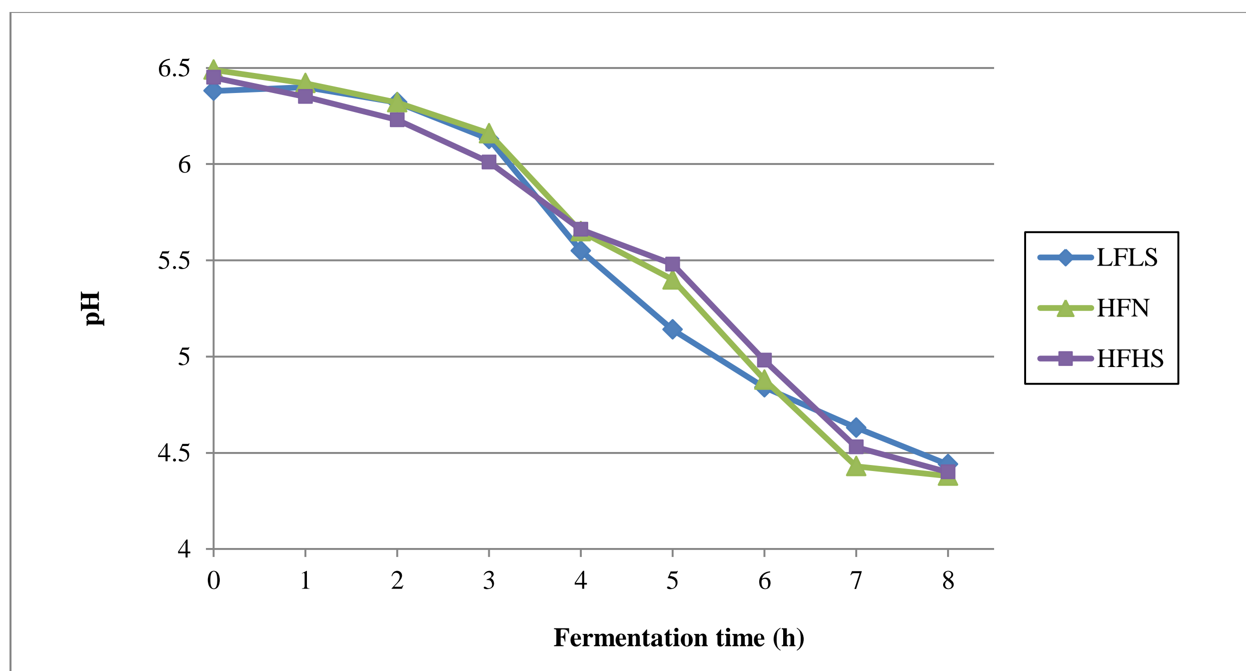


Figure 21. Changes in pH during yogurt fermentation at 43°C for 8 h. Yogurt was fermented from DYBs. Descriptions of the formulations are shown in Table 7, section 5.3.

6.3.3.2. Fermentation of LAB Cultures during Refrigerated Storage of Yogurt

Chemical and physical changes of the three liquid yogurts during refrigerated storage for 2 weeks (4°C) are shown in Figures 22 and 23. The decrease in pH ($p<0.05$, Table A. 7) and concomitant increase in levels of lactic acid were observed during storage of 3 yogurt formulations. The pH and lactic acid levels of yogurt in the current study ranged between 4.2-4.55 and 0.7-1.5% respectively. The increase in lactic acid was mainly due to the conversion of lactose to lactic acid by LAB (Özer, 2010). As expected, #HFHS was the least acidic of all of the samples during storage presumedly due to the presence of added sugar (descriptions of the formulations are shown in Table 7, section 5.3). However, the effect of sugar in the formulations on acid development was not investigated in this study. After 11 days of storage, #LFLS which contained added sugar had lower pH value than #HFN which had no added sugar; the rapid decrease in pH in #LFLS was more pronounced after 7 days of storage. This may be attributed to the ability of the bacteria strains to utilize sucrose (Barrangou et al, 2006; Haukioja et al., 2008; Wang et al., 2003) and their (particularly LB) respective proteolytic activity which are still active during chilled storage (Chryssanthopoulos and Maridaki, 2010) as shown by the increase of LB in Figure 23. In general, yogurt organisms of ST and LB species are proteolytic in yogurt (Christensen et al., 1999) and the production of amino acids is greater towards the end of cold storage than during the initial stages (Donkor et al., 2007). The proteolytic activity of LAB in milk is crucial for their growth and survival. This may explain the stability of yogurt bacteria during cold storage in the current study (Figure 23).

Despite the significant decrease in acidity ($p<0.05$, Table A. 7), initial NCFM counts of $>10^8$ cfu/mL were stable during storage of three samples. The decrease of NCFM counts was more pronounced ($p<0.05$, Table A. 8) in the sugar added formulations (#HFHS) than natural yogurt (#HFN). This may be due to the sensitivity of the probiotic NCFM towards acid (Kailasapathy & Rybka, 1997; Sellars, 1991) and the added sugar (Özer, 2010). The inhibitory effects of added sugar in formulation #HFHS was evident by lower cell counts (10^7 cfu/mL) of LB compared to their growth in product #LFLS which contained 50% less of added sugar. This outcome indicated that the added sugar in product #LFLS may be stimulatory for the growth of LB while sugar addition of twice the level (#HFHS) may not significantly affect their growth during

storage. The decrease however may not be attributed to the level of fat as shown by comparable level of the ST and NCFM cell counts in the products. This finding was similar to the study of Obi et al. (2010) which reported similar viability profile of the *L. acidophilus* and yogurt starter in whole and skim milk yogurt. Although the decrease of NCFM during storage was significant ($p<0.05$, Table A. 8), the concentration of this bacterium remained high (Figure 23) and above the level ($>10^6$ cfu/mL) recommended by EC (2003).

The loss of viability by the ST ($p<0.05$, Table A.8) in the three formulations was similar during yogurt storage which only showed about 0.5 log^{-1} decrease (Figure 23). The initial ST counts in the products was $>10^9$ cfu/mL for three formulations and decreased to $>10^8$ cfu/mL by the end of storage. The high counts of ST were attributed to its high growth during fermentation (Figure 20, Figure 22) and was responsible for the increase of lactic acid and decrease in pH (Walstra et al., 2006). The high counts of ST compared to LB counts (Figure 23) have been reported in many commercial yogurts where the proportion of ST could be between 10-100 times higher than LB (IDF, 2002). While significant decreases ($p<0.05$, Table A. 8) of cell counts were observed in ST and NCFM during 2 weeks storage at 4°C, on the contrary, LB showed significant ($p<0.05$,Table A. 8) growth irrespective of the formulations used with the exception of the #HFN which showed a decrease in cell counts at the end of storage (Figure 23). The cell counts were however maintained between 10^7 and 10^8 cfu/mL throughout storage irrespective of the formulations used. This indicates that the growth of LB was appreciable in the yogurt formulations without challenging the probiotic growth in contrast to the finding by Gilliland et al. (2002) who reported growth inhibition of *L. acidophilus* (LA) in the presence of LB. The increase of LB concentration during storage could be attributed to the synergistic mechanism between ST and LB as reported by Ozer (2010). Moreover, the ability of yogurt bacteria and LA to utilize sucrose in fermented milk products has been reported in many studies (Haukioja et al., 2008; Vinderola et al., 2002; Wang et al., 2002; Wang et al., 2003).

The degree of syneresis of the yogurts is shown in Figure 22. Syneresis gives an indication of the non-homogeneity in the gel system of the yogurt, thus higher whey separation is related to gel instability which is also related to the pH of the yogurt system (Zare et al., 2011). A significant difference in syneresis values was observed between the three formulations ($p<0.05$, Table A. 9).

Yogurt with low fat level (#LFLS) was characterised by high homogeneity (lowest syneresis) during storage, followed by naturally flavoured yogurt (#HFN), and #HFHS which showed less wheying-off after 14 days storage at 4°C. This finding was in agreement with the study of Zare et al. (2011) which showed improvement of texture and syneresis indices in yogurt with higher protein content. The syneresis indices were stable during storage for 14 days in all samples although slight decreases ($p>0.05$, Table A. 9) were observed in #HFN and #HFHS after 14 days. An improvement in syneresis in yogurt has been reported in yogurts stored for longer storage period (after 20 days) (Tamime et al., 1996). The authors reported that the decrease in wheying-off may be due to the hygroscopic property of whey protein and its interaction with casein as described earlier in sections 3.6 and 4.1.7.

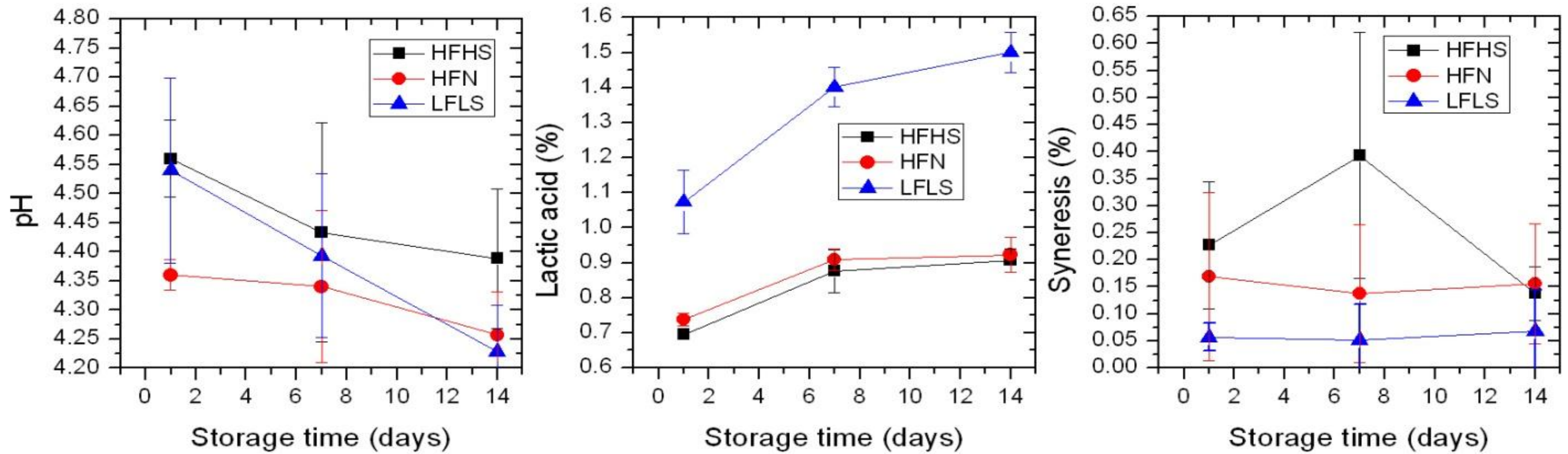


Figure 22. Changes in pH, lactic acid, and syneresis in liquid yogurts during storage at 4°C. Each point represents four independent analyses. Error bars are ±S.D. Descriptions of the formulations used are shown in Table 7, section 5.3.

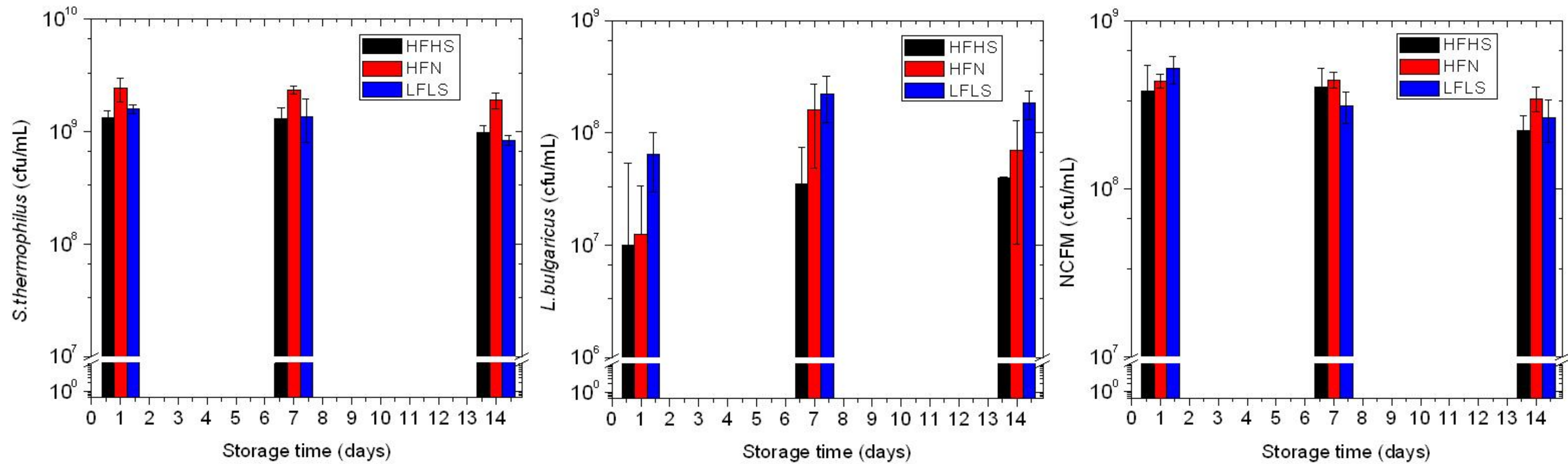


Figure 23. Cell counts of yogurt bacteria and *L. acidophilus* NCFM in yogurt during storage for two weeks at 4°C. Each bar represents four independent analyses. Error bars are ±S.D. Descriptions of the formulations used are shown in Table 7, section 5.3.

6.3.4. Rheology

The viscosity of the three yogurt formulations during refrigerated storage for 2 weeks at 4°C measured in their original state (undisturbed gel) are shown in Table 10. The viscosity of the unbroken gel during storage changed significantly ($p<0.05$, Table A. 12, Appendix) and varied ($p<0.05$, Table A. 12) between formulations. The increase in viscosity (using Brookfield) of the unbroken gel was shown by an increased firmness during storage (Table 10). The viscosity of the products ranged between 4.9-5.6E5 mPa.s for the #HFHS, 5-5.3E5 mPa.s for #HFN, and 6.3-6.8E5 mPa.s for #LFLS during storage.

The high values of viscosity in the #LFLS were probably due to the high protein content in the product which corresponded to its gel network in the final product as shown by the differences of up to 1.2 mPa.s in viscosity ($p<0.05$) of #LFLS and #HFN on day 1 whereas no evidence of variation ($p>0.05$) in viscosity between #HFHS and #HFN during the same storage period (Table A. 14). Although a decline in viscosity ($p<0.05$) was observed in #HFHS during 7 days of storage, the yogurt, however, re-gained its viscosity as shown by an increase of 0.6 mPa.s in viscosity ($p<0.05$, Table A. 13) even though the values were lower than the initial readings. Overall, the increase of viscosity of the unbroken yogurts gel during storage was significant ($p<0.05$, Table A. 12). Yogurt fermentation continues at low temperatures, thus producing acid, which then increases syneresis, giving a stronger gel.

The viscosity of stored yogurts after mixing for 30 s is shown in Table 10. The viscosity of the stirred yogurt samples differed significantly ($p<0.05$, Table A. 15) between formulations. A significant effect of the storage period ($p<0.05$, Table A. 15) on the viscosity of the yogurts was also observed in the three formulations. The trend of the viscosity of the broken gel was similar to the initial state. Yogurt formulation (Table 7, section 5.3) with high protein content (#LFLS) was the most viscous of all the products which ranged from 1.59E4 to 2.13E4 mPa.s. For sample #HFHS, viscosity decreased during storage ($p<0.05$, Table A. 15) from 1.66E4 to 1.1E4 mPa.s. A significant increase ($p<0.05$, Table A. 15) in viscosity during storage was observed in the #HFN. The effect of casein and β-lactoglobulin interactions during fermentation and storage may partially explain the higher viscosity values of high protein formulation observed in the current

study (Ozer, 2010). The network of protein interaction can prevent the disruption of gel firmness (Walstra et al., 2006).

Table 10. Values for viscosity and texture analyses of three yogurt formulations during refrigerated storage for 2 weeks at 4°C.

Formulation	Storage time (days)	Unbroken gel (mPa. s)	Broken gel (mPa. s)	Gel firmness (N)
HFHS	0	5.62E+05	1.66E+04	0.1074
HFHS	7	4.27E+05	9.40E+03	0.12966
HFHS	14	4.91E+05	1.10E+04	0.1414
HFN	0	4.97E+05	1.19E+04	0.12306
HFN	7	5.79E+05	9.99E+03	0.12966
HFN	14	5.27E+05	1.31E+04	0.1224
LFLS	0	6.27E+05	1.59E+04	0.15544
LFLS	7	6.00E+05	2.18E+04	0.1966
LFLS	14	6.82E+05	2.13E+04	0.1968

Notes: Viscosity values are averages of 4 independent analyses at intervals of 35, 40, 50, and 60 s. Gel firmness is average of 5 independent analyses. Descriptions of the formulations are shown in Table 7, section 5.3. Statistical analysis is shown in Appendix.

The firmness measurements of three yogurt formulations during storage at 4°C for 2 weeks are shown in Table 10. The firmness of the yogurt is determined from the first highest peak of the texture analyzer graph as shown in appendix F. The gel firmness profiles were similar in the three yogurt formulations during which the gel deformed during the penetration test and further deformed as the probe moved further into the gel resulting in considerable damage to its structure and then broke as reported by Salvador and Fiszman (2004). The gel firmness varied between the formulations ($p<0.05$) with lower values obtained in the formulations with higher levels of fat (#HFHS and #HFN) (Table A. 10). This may be attributed to the lower protein content which plays an important role in the formation of gel firmness during fermentation (Walstra et al., 2006). The yogurt firmness of formulations #HFHS and #HFN at day 1 were similar ($p>0.05$, Table A. 11) ranging between 0.11 and 0.15 N (Table 10). However, the viscosity of the two products (#HFHS and #HFN) significantly differed from #LFLS at the beginning of storage period ($p<0.05$, Table A. 11), indicating the importance of protein in gelation of the product after fermentation. During storage, yogurt gel firmness increased significantly ($p<0.05$) except for HFN which showed an increase during storage for 7 days but decreased on day 14 (Table 10). The decrease in gel strength of #HFN formulation was

correlated to the slight increase in the syneresis index at the end of storage (Figure 22). An increase in firmness during storage ranging from 0.01-0.02 N was observed in #HFN and #HFHS after 14 days storage while the changes were more pronounced in #LFLS (Table 10). Gel firmness (0.2 N) of the product #LFLS (Table 10) was considerably lower than the expected level of firmness (0.4 N) as suggested by Horiuchi et al. (2009) who reported that firmness of 0.4 and higher was sufficient to withstand the impact of shaking which occurs during transportation. For this reason, stabilisers such as gelatine, starch, and whey protein may be added to the products to increase firmness (Ozer, 2010). However, yogurts produced in the current study did not contain any stabilisers.

6.3.5. Sensory

The results shown in Table A. 16 (Appendix) indicate that the responses of the consumer panellists for each sensory attribute (appearance, texture, sweetness, sourness, flavour, and overall acceptability) was not affected ($p>0.05$) by the random order of serving samples (randomized block design). This confirms that the experimental design for sensory evaluation was appropriate for the evaluation of the yogurt samples.

The overall consumer acceptance towards the products is shown in Figure 24. Of the 77 sensory panellists, about 41 % indicated their acceptance of product #HFHS, about 35 % of product #LFLS and about 24 % of product #HFN (Figure 24). In terms of the overall acceptance, scores of #HFHS, #LFLS, and #HFN were 7, 6 and 4 respectively (Table 11). Therefore this indicates that product #HFHS was the most accepted by the sensory panellists followed by #LFLS and #HFN.

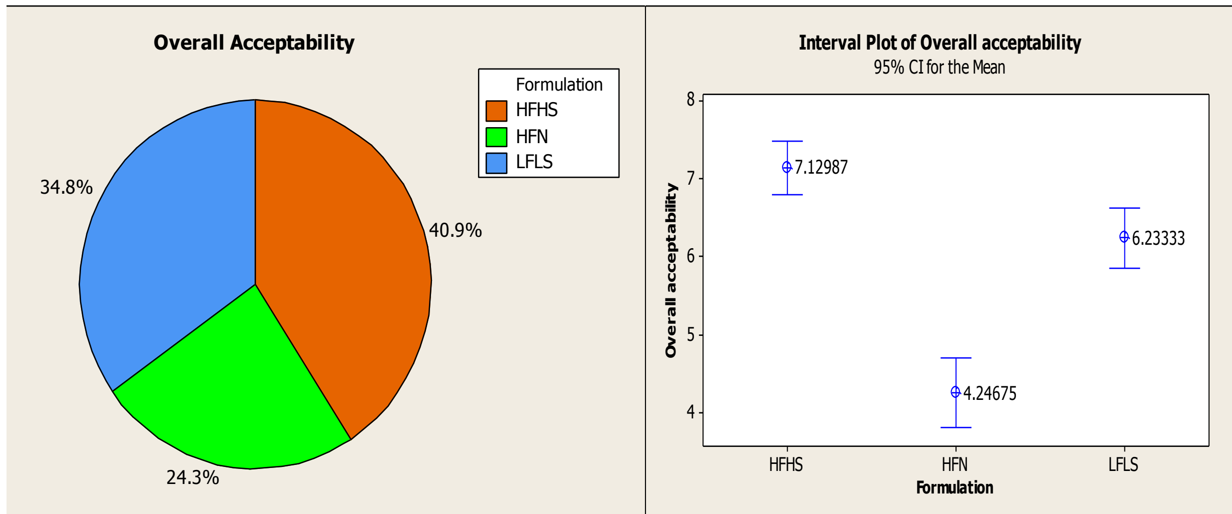


Figure 24. (Left) Percentage of overall sensory acceptability of product by 77 panellists and their respective mean scores values (right) of the liquid yogurt formulations.

The results of the sensory attributes of each yogurt sample are shown in Table 11. The appearance and texture of three samples obtained mean high score (6 to 7). Variation in mean score of sensory attributes sweetness, sourness, and flavour was observed between yogurt samples (Table 11). For all the sensory attributes, product #HFHS obtained mean score of 7. Meanwhile, mean scores of 6 ("like slightly") for the sensory attributes were obtained for #LFLS. Lower mean score values for the sensory attributes, however was observed for product #HFN which was given mean score of 4. The mean score of more than 6 in the 9-hedonic scale for sensory attributes in any sensory evaluation indicate that the samples were well-accepted by the consumers panellists attributed to the high market potential of the high fat high sugar product (Behrens, Roig, & Da Silva, 2004).

The distribution of the sensory data in the current study is shown in Table 11. A p-value of less than 0.5 indicated that the data was normally distributed (parametric) while a p-value above 0.5 indicated that the data was not normally distributed (non-parametric). The results (Table 11) indicated that the data were not normally distributed except for sensory attribute of texture and sweetness. For this reason, the data were analysed using non-parametric and parametric tests.

Table 11. Mean values of sensory attributes obtained with 9-hedonic scale. Results in brackets are S.D.

Attribute	**HFHS**	**HFN**	**LFLS**
Appearance	7.26 (1.15)	5.97 (2.06)	6.91 (1.33)
Texture	6.97 (1.31)	*5.80 (2.11)	6.84 (1.51)
Sweetness	*7.08 (1.77)	4.11 (2.20)	6.35 (1.80)
Sourness	6.79 (1.68)	4.37 (2.18)	6.19 (1.85)
Flavour	7.08 (1.76)	3.89 (2.02)	5.93 (1.88)
Overall acceptability	7.13 (1.53)	4.23 (1.97)	6.25 (1.68)

Notes: values with (*) indicated that the data was normally distributed ($p<0.05$) using Kolmogorov-Smirnov test; n=77.

Using the non-parametric tests, the acceptance of each sample in term of each descriptive factor outlined in the sensory questionnaire varied significantly ($p<0.05$, Table A. 19). Sample #HFN differed significantly ($p<0.05$, Table A. 19) from all formulations in all sensory attributes wherein for #HFHS and #LFLS, sweetness and flavour ($p<0.05$, Table A. 19) were the most important attributes which influence the difference in overall acceptability of the two products.

Using the parametric test of ANOVA method, similar result was obtained. The overall acceptability of yogurt samples differed significantly between formulations (Table A. 20). Tukey's analysis (Table A. 20) showed that #HFN differed significantly ($p<0.05$) from the other formulations, while the overall acceptability for #HFHS and #LFLS was significantly influenced by the sweetness and flavour of the two products (Table A. 20). It therefore seems that sweetness and flavour significantly contributed to the acceptance of the two products (#HFHS and #LFLS).

Principal Component Analysis (PCA) showed that the drivers for the degree of likeliness of the panellists towards the products were based on flavour, sweetness, sourness, appearance, and texture (Figure 25. A & B). The PCA identifies the smallest number of latent variables (principal component) that explain the highest amount of observed variability of data using as little as 2 or 3 principal components (Meilgaard et al., 1991). In this study, the PCA explained 84.6% of total variance in the two principal components, PC 1 (72.2%) and PC 2 (12.4%) (Table 12). The first and second principal components are shown in Table 12 and may be summarised as follows:

PC1 = 0.379*appearance + 0.34*texture + 0.431*sweetness + 0.411*sourness + 0.429*flavour + 0.45*overall acceptability

PC2 = 0.469*appearance + 0.722*texture – 0.246*sweetness – 0.329*sourness – 0.251*flavour – 0.167*overall acceptability

From the two PCA equations shown here, it is evident that consumer ratings for the yogurt attributes in PC 1 (72.2%) were affected by all variables while PC 2 (12.4%) was mainly influenced by texture and appearance. This means that about 72% of the ranking was due to all variables and that they were all correlated.

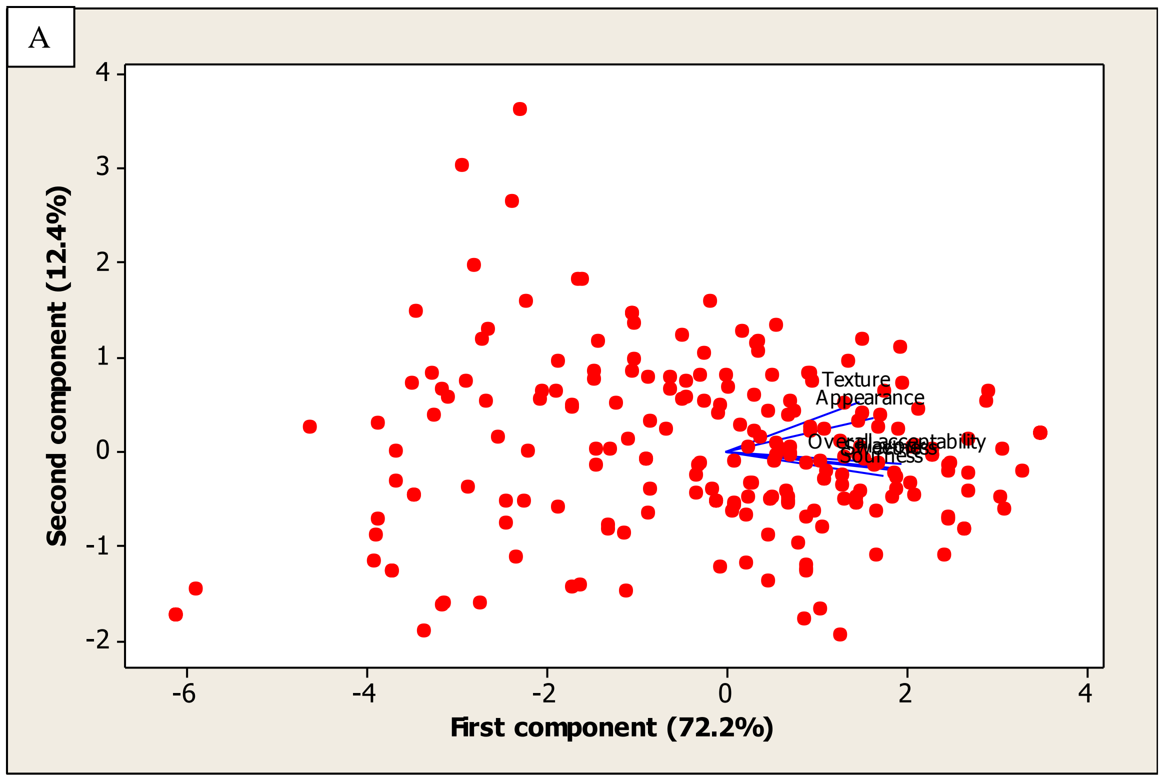
Figure 25C shows that the positive scores of the first principal component along the x-axis was dominated by formulation #HFHS and #LFLS, whereas #HFN was mainly located at the top-left of the second component which was further apart from #HFHS and #LFLS. The results agreed with the comments given by consumer sensory panellists who indicated that yogurt without added flavour and sweetener (#HFN) was not a preferred choice. The most preferred product by the sensory panellists was the #HFHS which had balanced sweetness and flavour; the panellists (n = 6) further indicated their willingness purchase the product, although many indicated (n = 10) that the texture of this product could be improved to increase the viscosity.

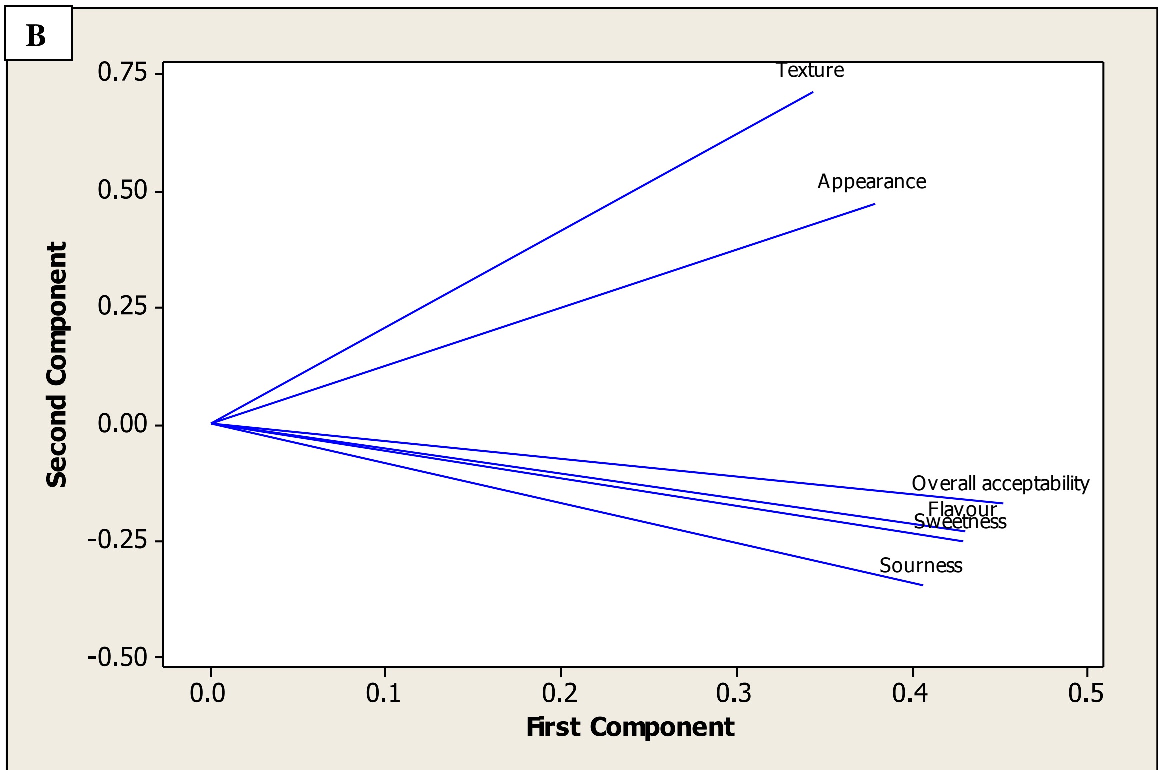
Table 12. Principal component analysis for sensory data

Eigenanalysis of the Correlation Matrix

Eigenvalue	4.3300	0.7453	0.3673	0.2587	0.1955	0.1033
Proportion	0.722	0.124	0.061	0.043	0.033	0.017
Cumulative	0.722	0.846	0.907	0.950	0.983	1.000

Variable	PC1	PC2
Appearance	0.379	0.469
Texture	0.340	0.722
Sweetness	0.431	-0.246
Sourness	0.411	-0.329
Flavour	0.429	-0.251
Overall acceptability	0.450	-0.167


A
Second component (12.4%)
First component (72.2%)
Texture
Appearance
Overall acceptability
Sourness
4
3
2
1
0
-1
-2
-6
-4
-2
0
2
4


B
Second Component
First Component
Texture
Appearance
Overall acceptability
Flavour
Sweetness
Sourness
0.75
0.50
0.25
0.00
-0.25
-0.50
0.0
0.1
0.2
0.3
0.4
0.5

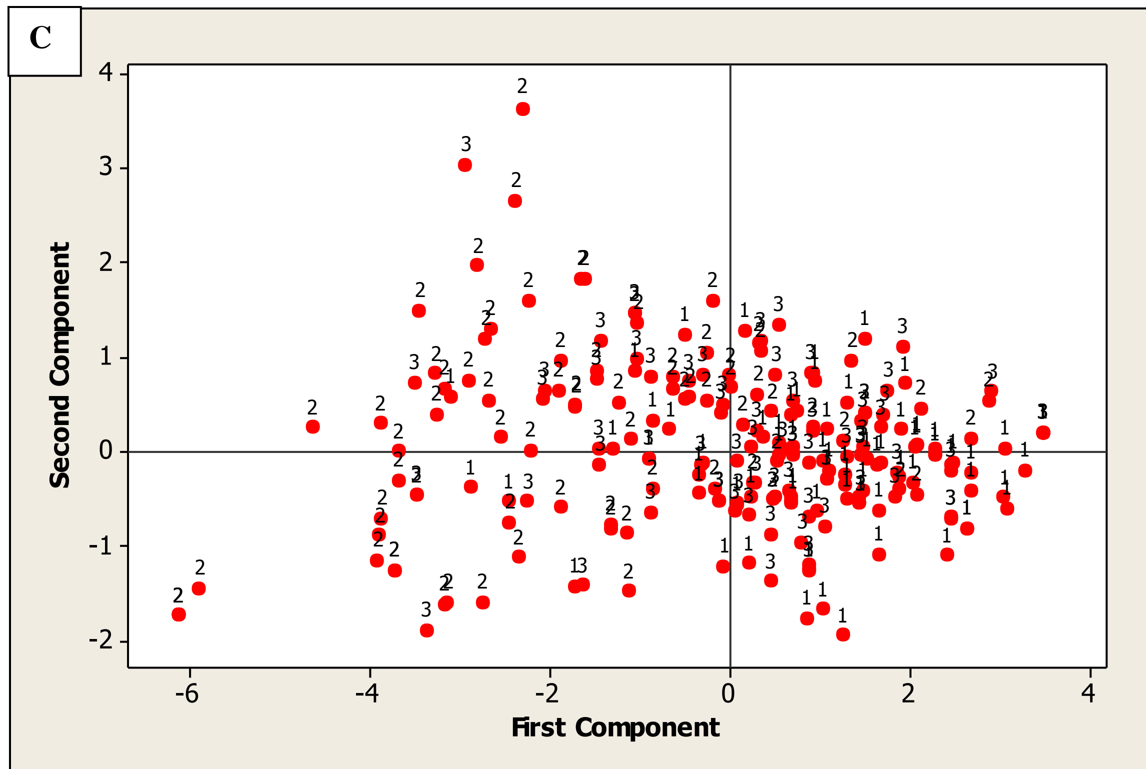


Figure 25. Principal component analysis plots of sensory properties of the yogurts (A & B) and their acceptance (C): (1) = HFHS; (2) = HFN; (3) = LFLS.

6.3.5.1. Consumer Segmentation

Unlike the PCA which groups attributes based on their correlation behaviour, cluster analysis identifies groups of observations based on the degree of similarity among their ratings (Meilgaard et al., 1991). When Cluster Analysis was applied to the consumer acceptability data, there were initially two clusters of consumers identified (Figure 26). One cluster (n=85) representing 38% of total panellists did not show clear differences in sample acceptability. The cluster scores of hedonic ratings ranged between 6 and 7 for the three sample formulations (Figure 27). The remaining cluster (n=140), representing 62% of total panellists, showed clear differences in hedonic ratings of sample acceptability (from 2.5 to 9) (Figure 27). The segmentation of consumers in the current study was similar to the cluster analysis reported by Bayarri et al. (2011) who observed two clusters of 120 consumers. In their study (Bayarri et al., 2011) one group showed clear variation in the hedonic scales for the yogurt and yogurt-like products while the other group did not. In our study, the consumer segmentation was further

identified into two clusters in the second segment of consumers. In this segment, a small number of consumers (cluster 2, n=62) disliked the products (Figure 27). This cluster (cluster 2) gave a mean score 2.5 using the 9-point hedonic scale ratings for the overall acceptability of #HFN while the mean score for the overall acceptability of #HFHS and #LFLS was 4. Cluster 3 (n=78) expressed their likeness for the products, particularly the sweet and creamy strawberry flavoured yogurt with 3.5 % fat (#HFHS). The mean sensory acceptance score was very high (close to 9). Meanwhile, slightly lower scores for the overall acceptability of #LFLS (mean = 7.5) and #HFN (mean = 7) were given by Cluster 3.

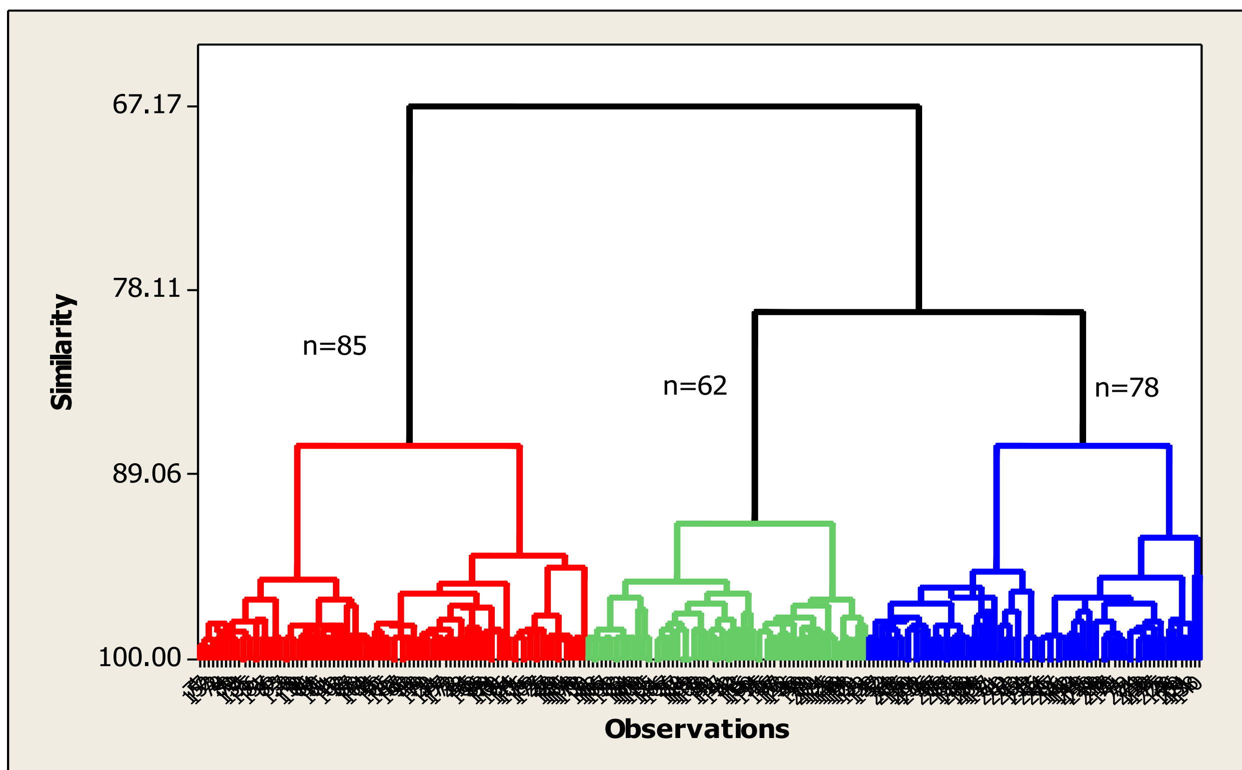


Figure 26. Cluster Analysis of consumer segmentation (n=225). Red, green, and blue represent clusters 1, 2, and 3.

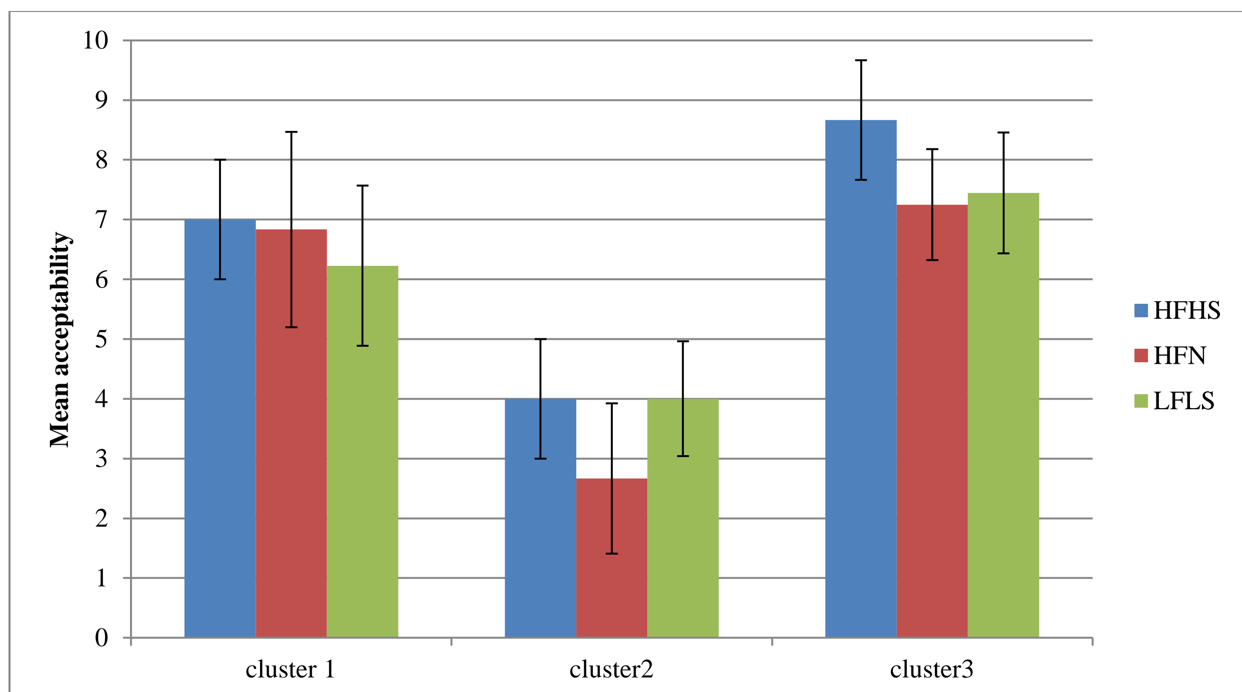


Figure 27. Mean acceptability scores of yogurts for the three clusters of consumers. Note: Error bars are ± S.D.; 1-9 hedonic scale was used with 1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely.

6.3.5.2. Relationship between Sensory Acceptability and Instrumental Analysis Data

Regression analysis was used to determine the relationship between the instrumental data and the acceptability of the consumer panellists' subgroup. As different subgroups (cluster 1, 2, and 3) were discovered among consumer panellists, only cluster 3, which liked the products, would be further evaluated in the regression analysis. Cluster 1 which is the "non-distinguisher" and cluster 2 which disliked the products therefore would not be discussed further.

In this study, the overall acceptability of consumer panellists towards the products were significantly influenced by sensory attributes flavour, sweetness, and sourness as shown in Figure 25A & B. Regression analysis showed that yogurt samples which were flavoured and sweetened gained more popularity among panellists compared to yogurt sample which was not sweetened (Figure 29). The preference for flavoured and sweeter yogurts among the sensory consumers might be attributed to their age which ranged from 20 to 35 years. The high Pearson coefficient (R^2 about 0.90) shown in Figure 29 was evident that strong correlation between sensory results (mean acceptability of sweetness and sourness) and instrumental data (pH

measurements) were observed in this study. Furthermore, strong correlation ($R^2 = 0.617$) was also observed between viscosity and panellists acceptance towards the products (Figure 28). The texture of the yogurt samples which ranged from 1-2 x 10^4 mPa.s was given score of 7-8 in the 1-9 point hedonic scale. This indicated that regression analysis using the Pearson coefficient may be used to determine the relationship between sensory and instrumental data.

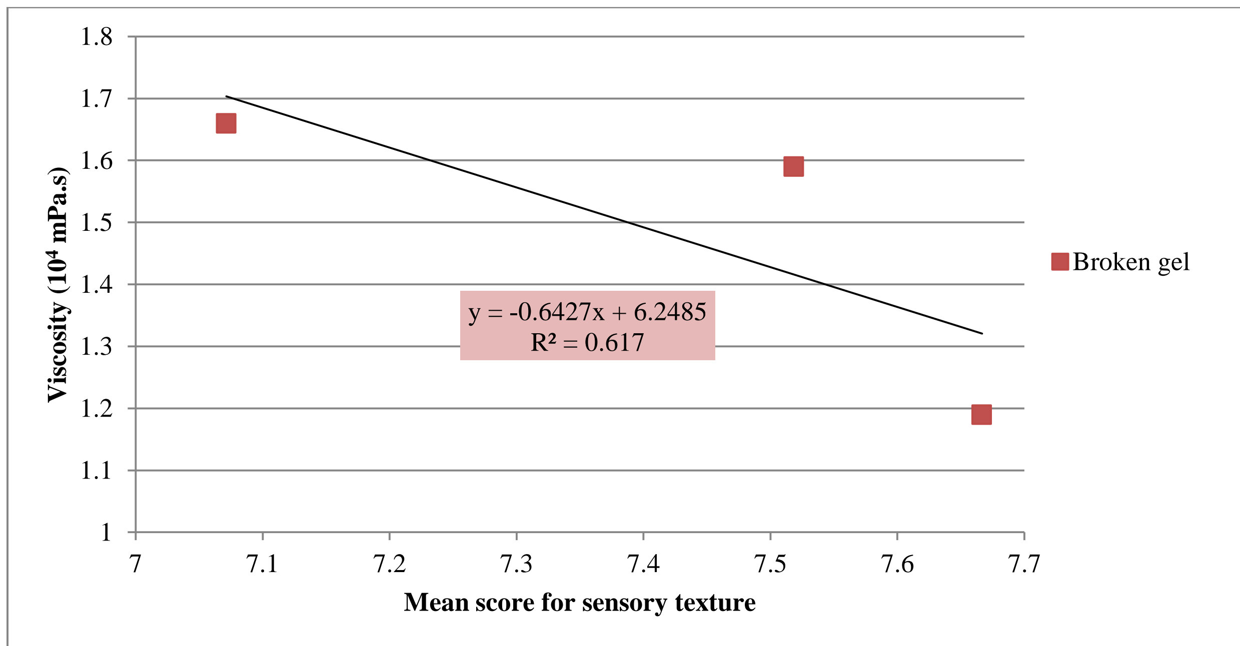


Figure 28. Regression analysis of sensory attribute texture compared to Brookfield viscosity values.

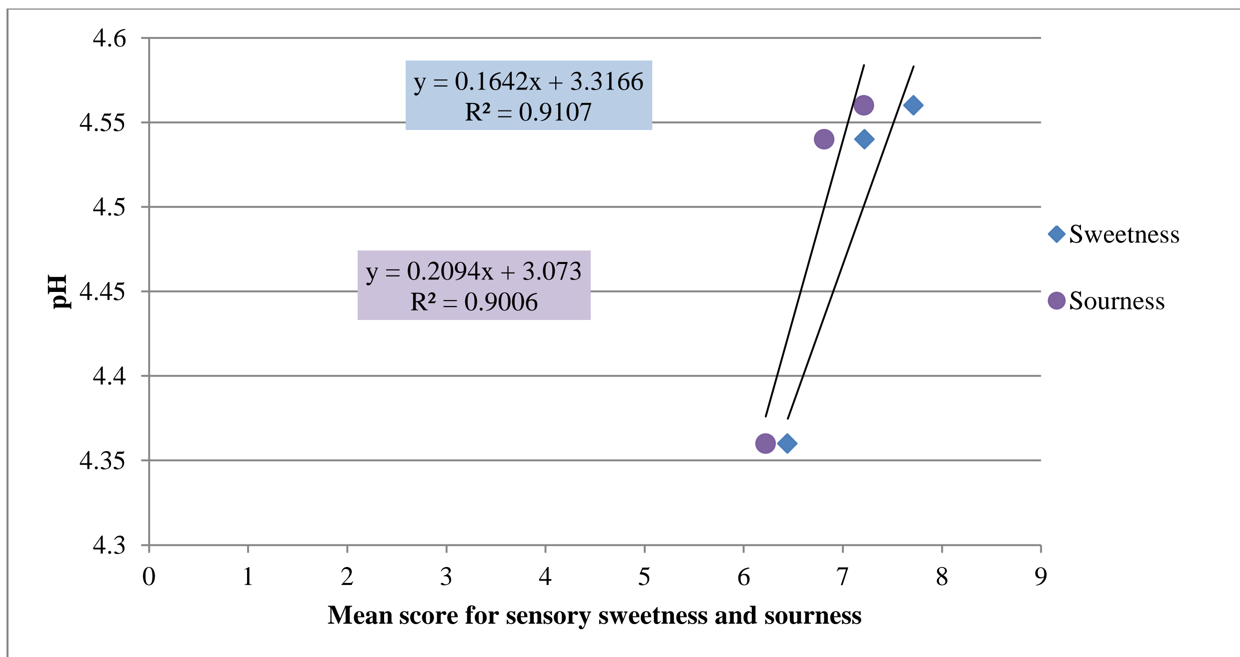


Figure 29. Regression analysis of sensory attributes sweetness and sourness compared to pH values.

6.4. Shelf-life Determination and Prediction of DYBs

The thermal death rate of LAB in the package during storage at various temperatures followed first order reaction where the *k* values at 4°C were at least ten times lower than the values at 22°C (Tables 13, 14, and 15).

The predicted and experimental measured death rate of all LAB bacteria at 4°C and 22°C in this work were comparable (Tables 13, 14, and 15). Since the storage life of a product is depedent on the reaction rate (*k*), shelf-life prediction of *k* is of importance, thus data validation plays an important role in predicting shelf-life of products. Obtaining the value of *k* allows for the prediction of the shelf-life using the first order kinetics reaction ($At=Ao.e^{-kt}$).

Decrease in cell counts was higher for ST (Table 13) compared with the LB (Table 15) and the NCFM (Table 14) for the three formulations. The predicted shelf-life of DYBs in the current study is shown in Table 16. It was observed that ST was the limiting factor for shelf-life of mixed yogurt cultures (ST, LB and NCFM) at ambient temperature storage, in spite of which all the strains in the three formulations could have more than 18 months shelf-life when stored at 4°C. The loss of viability of the ST would however decrease to <18 months if stored at 22°C as shown in Table 16. The detrimental effect of using elevated temperature was also reported in the study by Wang et al. (2004) who showed a difference of 20% in the survival rate of freeze-dried ST stored at 4 and 25°C.

It is noteworthy to mention that the ST strain used in the current study may be different from the strains that have been reported in literature review elsewhere. As the characteristics of each bacterium are strain specific, the difference in survival rate between the same species is not uncommon. A recent study by Grześkowiak et al. (2011) showed that even the properties of the same strain of *L. rhamnosus* GG may differ significantly due to different manufacturing process involved in the production of the bacterium.

The difference in the survival rates between ST, LB and NCFM may be attributed to the effect of different carriers used in the preparation of cultures prior to freeze drying. Kurtmann (2009b)

reported that *L. acidophilus* embedded in sucrose + maltodextrin matrix gave a higher recovery than lactose + maltodextrin matrix during storage at 20°C. Yogurt cultures embedded in ascorbic acid and monosodium glutamate prior to spray-drying had shelf-life of up to 6 months at 21°C (Porubcan & Sellers, 1975). Freezing matrix (e.g. saccharides) acts as protectants which shield both cell membranes and cystosolic protein of the bacterium through linkage to lipophilic site of phospholipids (Andersen et al., 1999). The reports (Andersen et al., 1999; Grzeskowiak et al., 2011) indicated that the storage stability of starter ST was highly related to the freezing matrix, storage temperature, and presence of air, particularly oxygen. The study indicated that at the same storage temperature (30°C), the survival of the bacterium was higher when [O_2] was low (<2% oxygen) compared to air-packaging (normal atmosphere) giving a shelf-life difference of 8 weeks. However, the storage stability was poorer at 5°C than in normal atmosphere packaging, indicating the sensitivity of ST to temperature (Andersen et al., 1999). Our results on the survival of ST in the packaged DYBs were similar. The current study observed that low temperature storage (4°C) prolonged the shelf-life of DYBs (Table 16). This observation agrees with Nikolova (1975) and Karadimov et al. (1975) who reported that yogurt bacteria in reconstituted yogurt powder survived better during storage at 5-10°C than at room temperature. Karadimov et al. (1975) suggested that refrigeration temperatures of 4-6°C were ideal for prolonged storage in polyethylene/Al foil package. Under refrigerated storage of freeze dried ST and LB for 6 weeks, a cell recovery of about 86.3% was obtained. Cabrini et al. (1982) suggested that a shelf-life of up to 2 years could be achieved if the products are stored at 4°C. The significant effect of low temperature storage may be related to the glass transition temperature (T_g) of the dried cultures as reported by many authors (Andersen et al., 1999; Kurtmann et al., 2009b; Selma et al., 2007). Glassy temperature is the temperature at which molecular mobility and enzymatic activity effectively reduced such that chemical reactions and oxygen diffusion through bacterial cell is limited. However, their viability may not be affected, therefore, cultures are more stable when stored at or below their T_g. The importance of storage temperature was also emphasized by Kumar and Mishra (2004b). In the case of reconstituted yogurt powder, considerations should be given to incubation temperature which may reduce vitamins and induce bacterial loss. More importantly, lactase activity of freeze-dried cultures may decline by 6.3% after 24 months storage at 10°C (Kumar & Mishra, 2004b) and such factors should be considered when predicting shelf-life. The protective effect of freezing matrix and low temperature storage may

equally be important in keeping the moisture content to a minimum. The hydrogen bonding between the protective agent and the bacteria limits the unbound water content thus lowering the a_w content of freeze-dried cultures (Selma et al., 2007). Interestingly, while freezing matrices were found to be beneficial to ST, Champagne et al. (1996) showed detrimental effect of some polymers (gelatin, xanthan gum, and maltodextrins) on the survival of bacteria during storage at 20°C. Up to 99% viability loss was observed in the control sample after 12 months storage, whereas the loss was evident in less than 6 months in ST-embedded in the polymers mentioned here.

The viability of ST during storage was not as high as the two *Lactobacillus* species in the current study. This finding was however contrary to the other studies (Kalugin, 1979; Nikolov & Vitanov, 1969) that found better viability of the ST than the *Lactobacillus* species in elevated storage condition (37°C). The study by Nikolov and Vitanov (1969) reported that dry yogurt powder stored at 4°C under vacuum or N_2 environment lasted up to 9 months but their survival decreased markedly within one month when stored in dry air at 37°C. According to Gyosheva (1995), lyophilized cells of potential *S. thermophilus* strains stored at 6°C can last up to 10 years without changes in cell morphology, biochemical, and technological characteristics. The review from Kumar and Mishra (2004b) reported that dehydrated sweet yogurt and fruit berry yogurt had shelf life of 12 and 18 months at 20 ± 5°C and 4 ± 3°C where 1 log decrease was observed in freeze dried yogurt starter bacteria (ST and LB) during storage at 4°C for 15 months.

It is however important to mention that the storage life of the cultures predicted by the Arrhenius model was specific to the formulations described in this study. The samples in the current study were stored in controlled storage temperatures of ± 3ºC without modification of the humidity. Hence, the use of these prediction models would be only valid under similar storage conditions.

Table 13. The k ($days^{-1}$) values for thermal reductions of *S. thermophilus* in selected DYBs formulations.

Formulation	Temperature (°C)	-k (experimental)	-k (predicted)	Arrhenius equation
LFLS	4.00	0.0020	0.00180	$\ln k = -14573(1/T) + 46.35$
LFLS	22.00	0.0360	0.04640	
HFN	4.00	0.0030	0.00288	$\ln k = -13886(1/T) + 44.28$
HFN	22.00	0.0210	0.04120	
HFHS	4.00	0.0001	0.00022	$\ln k = -18861(1/T) + 59.72$

Table 14. The k ($days^{-1}$) values for thermal reductions of *L. acidophilus* NCFM in selected DYBs formulations.

Formulation	Temperature (°C)	-k (experimental)	-k (predicted)	Arrhenius equation
LFLS	4.00	<0.0001	0.00008	Ln k = -19039(1/T) + 59.25
LFLS	22.00	<0.001	0.00503	
HFN	4.00	<0.0001	0.000014	Ln k = -22107(1/T) + 68.65
HFN	22.00	<0.001	0.001850	
HFHS	4.00	<0.0001	0.000150	Ln k = -17206(1/T) + 53.31

Table 15. The k ($days^{-1}$) values for thermal reductions of *L. bulgaricus* in selected DYBs formulations.

Formulation	Temperature (°C)	-k (experimental)	-k (predicted)	Arrhenius equation
LFLS	4.00	<0.0001	0.000699	Ln k = -14780(1/T) + 46.09
LFLS	22.00	<0.001	0.001806	
HFN	4.00	<0.0001	0.000011	Ln k = -22074(1/T) + 68.23
HFN	22.00	<0.001	0.001359	
HFHS	4.00	<0.0001	0.000159	Ln k = -17371(1/T) + 53.96

Table 16. Shelf-life of DYB at different storage temperature calculated using predicted *k* values.

Formulation	Shelf-life at 4°C	Shelf-life at 22°C
LFLS	>18 months	6 months
HFN	>18 months	10 months
HFHS	>18 months	14 months

Notes: maximum counts of $<10^5$ cfu/g were used. Shelf-life calculation was solely based on the *S. thermophilus* viability as the *Lactobacillus* species counts were predicted to be more than $>10^7$ cfu/g after 18 months irrespective of storage temperature, either 4°C or 22°C. Descriptions of the formulations are shown in Table 7, section 5.3.

7. GENERAL DISCUSSION

7.1. Stability of LAB in DYB

The Arrhenius equation of first order kinetics was successfully applied to monitor the changes in the survival of LAB during storage at various temperatures (section 6.4). The loss of viable cells was evident as temperature increased irrespective of the type of formulation and starter strain. Reaction rate constant, which is the key parameter in predicting the rate of cell death, was higher for *S.thermophilus* (ST) than the *Lactobacillus.* Interestingly, formulations with higher fat and sugar contents performed better than the those with lower fat and sugar. While the effect of sugar (Akin et al., 2007) and fat (Obi et al., 2010) levels in products containing probiotics in its liquid form has been reported, no reports have been published on the current issue. The difference in the viability of the bacteria between formulations may be attributed to strain manufacture and storage as described below.

Effect of Freeze Drying

Freeze-drying and spraying-drying are common methods of manufacturing cultures, with the latter being seldom used due to potential damage of the technology to the bacteria. Freeze-drying is a process of removing a solvent (usually water) by sublimation. Since it involves low temperature drying, chemical changes are minimal thus minimizing damages to the cultures. However, during freezing and drying, cell injuries are unavoidable. During drying, bacteria lose large amounts of water and thus may result in structure collapse (Fonseca et al., 2004). The bacterial death may continue following drying and during storage. Many studies have been done to reduce loss of viability in bacterial cultures during freeze-drying using various freezing matrices, and reduction in temperatures, humidity, and water activity. These methods however may only improve their survival. This may explain the discrepancies between the proposed initial inocula levels and the actual number obtained from the experiment in the current study as described in section 6.3.2. For instance, the proposed inoculum level of NCFM was $\approx 10^{10}$ cfu/g while the experimental number showed cell counts of $\approx 10^{8}$ cfu/g (Figure 17). The extent of cell injuries that occur during freeze-drying and storage varies from the cell wall to its RNA (Castro et al., 1997). In their study, Castro et al. (1997) showed that membrane damages were the main

site of damage as evidenced by loss of ability in maintaining pH, β-galactosidase activity, and changes in lipid ratio (unsaturated:saturated). These changes can continue during storage at 20°C resulting in membrane collapse, thus leakage of ions maybe inevitable as shown by a decrease of ATPase activity which leads to cell death (Castro et al., 1996).

Effect of Blending

The DYBs used in the current study were dry-blended using ribbon-type blender (150 kg capacity) at ambient temperature (20°C). The collision between the impeler and particle during blending may damage the structure of particles resulting in the degradation of the properties. This may explain the survival of LAB during storage in DYB package during the screening phase (section 6.2). Further, particle collision during blending may break, agglomerate or coat the particle (Maynard, 2008). If particle coating is not optimal, agglomeration of particles with dissimilar size may desegregate after blending and during storage, thus creating problems in the homogeneity of samples of the same batch (Earle, 1983); this may partially explain the variation in LAB cell counts in DYB packages (section 6.2.1, Table A. 2). More importantly, increase in temperature during storage may enhance the disintegration of the particles (Maynard, 2008), and resulting in the decrease of the functional properties as described in section 6.4. It is noteworthy to mention that there has been limited studies on the effect of impeller on microorganisms.

Effect of Modified Atmosphere Packaging

The performance of LAB in DYB during storage at 20°C (section 6.2) differed between formulations in terms of viable counts, a_w, and O_2 level. The initial [O_2] in the current products was considerably high and this may explain the lower counts of ST during storage. Talwalkar and Kailasapathy (2003) reported that the presence of oxygen at concentrations higher than 0% can induce the metabolic (lactic acid production) and biochemical (NADH oxidase and NADH peroxidase, enzymes responsible for removing toxic chemical of hydrogen peroxide) changes of *L. acidophilus* (LA) in culture mixture resulting in a reduction of cell growth. The sensitivity of probiotics towards oxygen is strain specific. High production of the enzyme NADH oxidase to scavenge H_2O_2 does not however always correlate with the survival of probiotics in the presence of oxygen (Yamamoto et al., 2011). The importance of [O_2] for the survival of dried cultures was

clearly shown by Wang et al. (2004). These authors showed that the performance of ST was better in a vacuum sealed laminated pouch (nylon/aluminium/retort-coated polypropylene) than in the oxygen-absorber and desiccant-containing glass and PET packaging irrespective of storage temperature. This was attributed to the relatively high oxygen permeability of PET bottle and thus was not suitable for yogurt packaging. The positive impact of packaging materials in maintaining low a_w level and high LAB counts were in agreement with the study of Wirjantoro & Phianmongkhol (2009) which stressed that good packaging material was important in maintaining the freshness and viability of yogurt powder, especially when it is stored at elevated temperature for long periods. While the effect of [O_2] on LAB performance is rather a complex phenomenon, other factors may also contribute to their survival during storage in DYB as described in preceding sections.

Effect of Sorption Isotherms of Yogurt Powder Blend during Storage

An increase in temperature causes an increase in a_w at constant moisture content. This indicates that at higher temperature, the tendency of the material to bind water is lower. This is because the hygroscopic properties of proteins and carbohydrates at high temperature is lower than at low temperatures (Koç et al., 2010). The sorption behaviour may therefore explain the storage stability of DYB (sections 6.2 and 6.4) where at the same moisture content or RH (due to low gas permeability of packaging materials used in the current study), an increase in temperature caused an increase in a_w. The recommended a_w values of between 0.1 and 0.2 (section 6.2) may be therefore explained by the sigmoid curve of water adsorption model at which food products fall above this range showing marked changes on the a_w values as well as moisture content at dynamic storage temperatures.

It is noteworthy to mention that the milk composition may also explain the shelf-life difference between the DYBs used in the current study. An increase in a_w decreased the glass transition temperature (Tg) of lactose (Figure 30). Since lactose is the most abundant component in milk powders (35-40% in WMP and 45-55% in SMP), generally the storage properties of milk powders depend, to a certain extent, on the glassy temperature (Tg) of lactose. Since the Tg of SMP and WMP were similar (Figure 30), their behaviour during storage therefore undergoes similar alterations (Thomas et al., 2004). When temperature rises above the lactose Tg, the a_w

level of SMP is higher than WMP because SMP contains more lactose than WMP, thus lactose crystallization which changes the anyhdrous lactose from “glassy” to “rubbery” state is more pronounced in SMP (Kessler, 1981). More importantly, the equilibrium moisture content rises with increasing protein and carbohydrate contents of the foods where the presence of fat in the foods lowers the moisture content. The protective effect of fat extends to its hydrophobic properties (Kessler, 1981). The LAB, which may presumably bind to the WMP during dry blending, would be surrounded by fats and protected against the increasing moisture in the matrix due to the hydrophobic properties of the milk fat (Kessler, 1981). Moreover, the content of free fat may increase during elevated temperature storage due to the formation of lactose crystals (Baechler et al., 2005) thus supporting this postulation. As the level of free fat and its rate of oxidation during storage is not investigated in the current study, therefore future studies on the role of fat in DYB may be useful.

The formation of the β-lactose crystal is induced at high storage temperature (Vuataz, 2002). If the dry products packaged in low gaseous permeability packaging were stored at elevated temperature as such the case in the accelerated shelf-life trial in the current study, the water released cannot escape out of the package and will accumulate within the package. Under such conditions, large amounts of water may be released from the powder, resulting in the crystallisation of the components (Vuataz, 2002). The increase of water content into the food matrix system (in our case, it is the DYB powders) would induce a significant increase in water activity, which may be responsible for the reduction in the lactic bacteria counts during storage. Hence, water activity measurement during storage of DYB would be recommended in future studies.

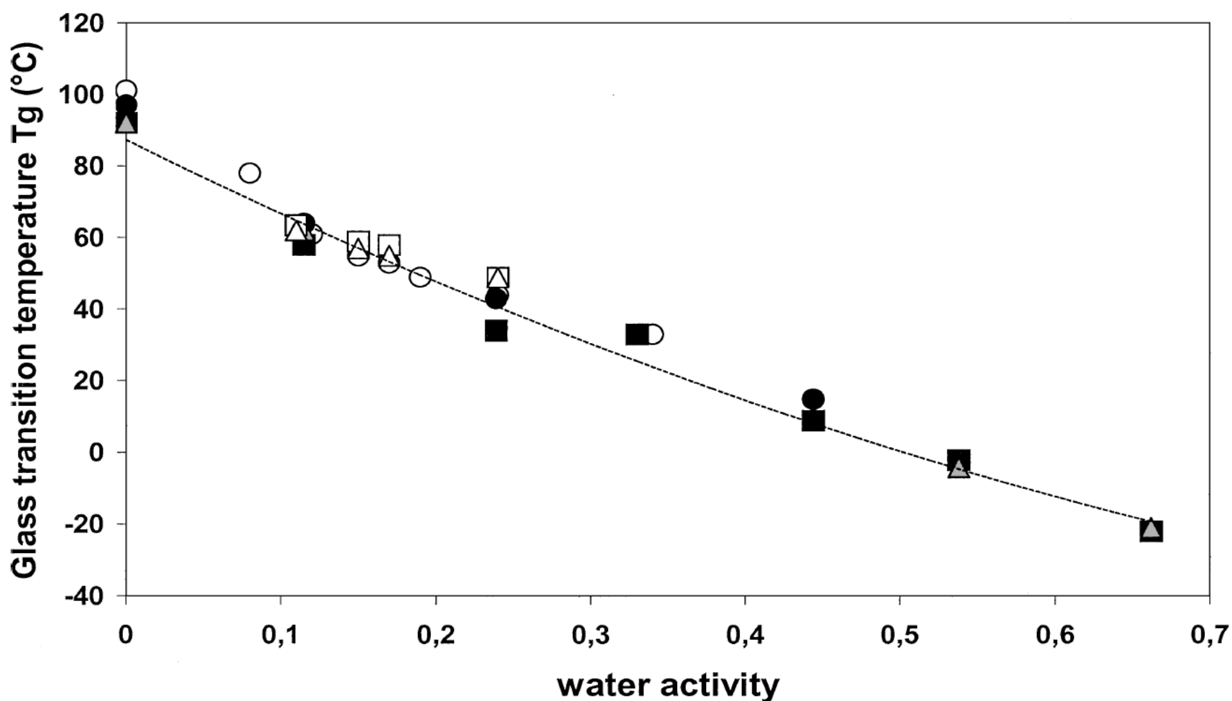


Figure 30. Glass transition temperature of dehydrated milk products as a function of water activity. Notes: circles represent Tg of lactose; triangles represent Tg of SMP; squares represent Tg of WMP. A unique curve describes dehydrated milk products containing lactose (Thomas et al., 2004).

7.2. Stability of LAB in Liquid Yogurts

The heat-treatment applied to milk indicates the degree of whey protein denaturation which is an important aspect in gelation properties of yogurt as described in chapter 3. About 98% or (97 mg/g) of the whey proteins were denatured in the medium-high heat treated milk powder (section 6.1). The medium-high heat treatment received by the milk can be confirmed by the high viscosity of the yogurts as noted in section 6.3.4. The performance of probiotic NCFM in conjunction with starter bacteria ST and LB in liquid yogurts was shown to be synergistic (sections 6.3.1 and 6.3.3.1). This finding contradicts the concern regarding the antagonistic effects of bacteriocin and hydrogen peroxide produced by the NCFM towards *L. bulgaricus* (Percival, 1997; Sanders & Klaenhammer, 2001). The latter study indicated that the bacteriocin (lactacin B) produced by the NCFM inhibited growth of *L. bulgaricus, L. lactis*, and *L. fermentum in-vitro*. Bacteriocins are proteinaceous antimicrobial compounds which are produced by lactic acid bacteria and exhibit a bactericidal effect against taxonomically closely-related bacteria with generally no effect against other microorganisms (Bernet-Camard et al., 1997).

Although the antimicrobial substance was not identified in the current study, *L. acidophilus* has been reported to produce non-bacteriocin antimicrobial against a wide range of Gram-positive and Gram-negative pathogens. The antimicrobial substance was insensitive to various human gut enzymes, independent of lactic acid production, and was not antagonistic against other human normal gut microflora such as *Lactobacilli* and *Bifidobacteria* species (Bernet-Camard et al., 1997). In the current study, the synergistic effects of ST, LB, and NCFM were more evident at lower initial inocula rates (1%) (section 6.3.2). The appreciable growth of LAB during fermentation (section 6.3.3.1) and refrigerated storage (section 6.3.3.2) in spite of the decrease in pH level demonstrated that the product formulations (e.g. presence of sugar and flavour) supported their survival in the liquid yogurts. The proteolytic activity which increased the liberation of amino acids from milk proteins may therefore partially attributed to the viability of yogurt cultures in the products (Christensen et al., 1999; Chryssanthopoulos and Maridaki, 2010; Donkor et al., 2007).

While the addition of sucrose can reduce the survival of LAB in liquid yogurts (Micanel et al., 1997), however, the presence of sucrose in the current study did not significantly influence their survival. This may be attributed to the ability of LAB to utilize sucrose. Srinivas et al. (1990) investigated the influence of various sugars (lactose, galactose, sucrose, glucose, and fructose) at 2% on fermentation rate of *L. acidophilus*. The study (Srinivas et al., 1990) reported that the utilization of sucrose was slightly faster than lactose. This outcome may be attributed to the difference in the activities of β-galactosidase and β-fructofuranosidase which are responsible for the hydrolysis of lactose and sucrose respectively (Srinivas et al., 1990). The hydrolysis of sucrose during fermentation in soy milk has been reported by Wang et al. (2003). A decrease in sucrose level was observed in soy milk yogurt inoculated with *L. acidophilus* and *S. thermophilus.* As a result, increases in the content of glucose and fructose were observed (Wang et al., 2003). The ability of the strain NCFM to utilize sucrose was reported by Haukioja et al. (2008) and Barrangou et al. (2006). The former study reported the utilization of glucose, lactose and sucrose by the NCFM strain which was incubated at 37°C for 30 min. The latter study (Barrangou et al., 2006) showed that increases in expression of genes, La399, La400, and La401 were observed in the presence of sucrose. The study by Barrangou et al., (2006) reported that sucrose can be transported into the cell by the sucrose transporter, which was significantly

expressed by the gene La401, and hydrolysed to sucrose-6-phosphate which is further converted into glucose-6-phosphate and fructose by the ScrB transporter induced by the gene La400. The effects of food additives (sucrose, flavouring, and colorant) on the viability and growth kinetics of LAB (ST, LB, and LA) have been reported by Vinderola et al. (2002). The study showed that probiotic LA was not significantly affected by the addition of sucrose (<15%), natural colourings (<0.088%), and flavouring (<0.16%) at the level commonly used by dairy manufacturers. The presence of such additives at <15%, 0.088%, and <0.16% for sucrose, colorants, and flavourings respectively, however, decreased the viability of ST and LB in their study, suggesting that their effects on the commercial yogurt starter bacteria were strain (Vinderola et al., 2002) and temperature dependent (Wang et al., 2002). The latter study reported that a greater reduction of ST and LA was found in yogurts stored at elevated temperature (25°C) than at refrigeration storage (4°C) (Wang et al., 2002).

The viscosity, firmness, and syneresis index of yogurt products during chilling were significantly influenced by the concentration of proteins, particularly β-lactoglobulin which form a casein matrix gel which traps water, thus improving the structure (Ozer, 2010).

The sensory properties of the RTE yogurts were significantly influenced by sensory attributes flavour and taste and to a lesser extent to appearance and texture as shown by PCA analysis on the overall liking of consumer acceptance tests (section 6.3.5). Although 38% of the consumer panellists did not show a clear differentiation of the products, however the remainder (35%) population quite liked the products with the highest score given to #HFHS (8.5, on the 1-9-point hedonic scale) followed by #LFLS (7.5) and #HFN (7) (section 6.3.5.1). Although sensory acceptance is not indicative a market success for a product, however the results of the sensory analysis in the current study indicate the potential of the DYB formulations to be commercialised.

8. CONCLUSION

The amount of undenatured whey proteins in SMP and WMP used in the current study was <3%, with protein levels of 36% and 26%, respectively. The fat levels in the respective products were <1 % and 28 %, with <4% moisture content in both milk products. The components of the products mentioned here were in agreement with the regulations of the Food Standards Australia New Zealand. Twelve dehydrated yogurt base formulations containing lactic yogurt starter cultures (*S. thermophilus* and *L. bulgaricus*) and *L. acidophilus* NCFM were investigated for the stability of the LAB, water activity and oxygen concentration. Three formulations (#HFHS, #HFN, #LFLS) containing >10^6 cfu/g of the LAB cell counts, <0.15 a_w, and <16% [O_2], during storage for 9 weeks at 20°C were selected for further characterization. No significant variations ($p<0.05$) in levels of O_2 in the DYB packages were observed during storage indicating that the packaging materials used (PE, foil, PET) were efficient in preventing gaseous exchange through the package. Using the Arrhenius model, the predicted shelf-life of the products #HFHS, #HFN, and #LFLS stored at 22°C were 14, 10, and 6 months, respectively. When the packaged products were stored at 4 °C, the predicted shelf-life increased to >18 months. The Arrhenius model demonstrated that the cell counts of the yogurt cultures would decrease significantly ($p<0.05$) with increase in storage temperature.

The growth of ST, LB, and NCFM in broth media (24 h/37°C) and in yogurts made from the DYBs (8 h/43°C) was synergistic, following a sigmoidal curve, where the ST increased rapidly at the onset of fermentation followed by the lactobacilli as the fermentation progressed. The results showed that the growth of NCFM was not affected by the presence of ST and LB.

The mean cell counts of the three types of liquid yogurts were >10^7 cfu/mL when the products were stored for two weeks at 4 °C. The pH of the yogurts ranged from 4.2 to 4.5 and the lactic acid from 0.7 to 1.5%. Significant increases ($p<0.05$) in viscosity and texture of the products were observed. Yogurt containing higher protein content (#LFLS) was more viscous and had less degree of wheying-off than the other two products (#HFHS and #HFN) during storage. Principal component analysis (PCA) evaluation showed that the main drivers for the degree of likeliness of the products by the consumer sensory panellists were flavour, sweetness, and sourness. Of the

consumer panellists (n=77), 35% indicated their likeness for the products, 27% disliked the products, while the remainder (38%) were indifferent, that is they did not indicate whether they liked or disliked the products. However, product #HFHS (high fat high sugar) was the most liked by the consumer panellists, followed by #LFLS (low fat low sugar), and lastly #HFN (high fat no sugar no added flavour). The mean sensory scores for the overall acceptability of products #HFHS and #LFLS were >6 using the 9-point hedonic scale, demonstrating the potential of the formulations for commercialization.

9. RECOMMENDATION

- The level of O_2 in DYB in the current study was considerably high; flushing the packages with N_2 a few times may help to reduce the O_2 content as recommended by many researchers working on LAB starter cultures (Dave and Shah 1997b; Hotchkiss et al., 2006; Miller et al., 2002).
- The effect of water activity on the concentration of oxygen would provide information on the viability of LAB in the dehydrated food system.
- The sorption behaviour of dried products is useful to determine and predict the shelf-life of a product during storage. Since the sorption isotherms of each food material are unique, therefore an understanding on water sorption of the product may be useful.
- The increase in temperature alters the physical and chemical properties of milk powders. When temperature rises above Tg, milk powders change from glassy to rubbery state (Kessler, 1981); this may result in particles collapsing, caking, and browning. These changes affect the powder quality. The shelf-life prediction in the current study was solely based on temperature; however, factors such as fat oxidation, colour and a_w can also affect the quality of the products.
- *In-vitro* studies to mimic the condition of the passage through GI tract is advisable to further confirm the performace of the bacterium NCFM.
- Longitudinal studies on the long-term effect of consumption of probiotic NCFM may be useful to investigate the benefits of probiotic NCFM.

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APPENDIX

A. **Milk powder specification**

Westland Milk Products
Hokitika • New Zealand

56 Livingstone Street
Hokitika, New Zealand
ph +64 3 756 9800

PRODUCT ANALYSIS CERTIFICATE

Product Description: Instant Wholemilk Powder

Customer: Easiyo
Buyer Order No.
Letter of Credit No.

Factory: 143
Spec No: 3412
Batch/Cypher: BU29
Manufacture Date: 29 September 2010
Unit Range: N01459-N01507

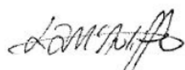
Parameter	Unit of Measure	Mean Result	Standard Deviation	Test Method
Protein (6.38 x N) as is	% m/m	26.6	0.1	NZTM 3.8.2
Fat	% m/m	28.5	0.1	NZTM 3.8.2
Moisture	% m/m	2.9	0.1	NZTM 3.8.2
Titratable Acidity	% m/v	0.097	0.002	NZTM 3.2.3
WPNI	mg/g	3.0	0.2	NZTM 3.15.6
Insolubility Index	ml	0.48	0.04	NZTM 4.6.3
Bulk Density	g/ml	0.47	0.00	NZTM 4.2.3
Wettability	secs	7	4	NZTM 5.4.10
Ash		5.6	0.0	NZTM 3.4.2
Foreign Matter		Absent	0	NZTM 4.3.3
Scorched Particles	/50g	A	0	NZTM 4.3.17
Flavour		Typical	0	WMP in house
Aerobic Plate Count at 30^{0}C	cfu/g	108	52	NZTM 2.43.1
Escherichia coli and Coliforms; detection usingLST-MUG	/g	Not Detected	N/A	NZTM 2.48.5
Yeast and Moulds; count	cfu/g	<1	0	NZTM 2.61.1
Coagulase-positive Staphylococci; Detection (Staph aureus)	/g	Not Detected	N/A	NZTM 2.47.2
Thermophilic Bacteria; count at 55^{0}C	cfu/g	50	37	NZTM 2.60.1
Pesticides[1,2]		Not Detected	N/A	
Heavy Metals[1,2]	%m/m	Not Detected	N/A	
Salmonella[1]	/750g	Not Detected	N/A	ISO 6579
Inhibitory Substances	iu/ml	<0.005	N/A	NZTM 2.51.1

[1] Tests are subcontracted [2] Tested periodically

The product supplied against this Certificate is manufactured from New Zealand origin milk.
The finished product is manufactured and tested in premises registered under statutory requirements by the New Zealand Food Safety Authority. All premises are subjected to regular audit to ensure compliance with the terms of and conditions of registration.
Samples have been examined and subjected to laboratory analysis using internationally recognised procedures.
Copies of test methods and sampling plans are available on request.

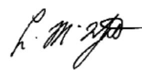
I hereby declare that this is a true, complete and accurate copy of this record.

Prepared by: Lynda McAuliffe
Title: Laboratory Administration



Date: 13 October 2010

Released by: Leo McIntyre
Title: Quality Manager



Date: 13 October 2010

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8:33 a.m. 23/12/2010

Westland Milk Products
Hokitika · New Zealand

56 Livingstone Street
Hokitika, New Zealand
ph +64 3 756 9800

PRODUCT ANALYSIS CERTIFICATE

Product Description:	Instant Skimmilk Powder	Factory:	143
		Spec No:	2407
Customer:	Easiyo	Batch/Cypher:	CU11
Buyer Order No.		Manufacture Date:	11 October 2010
Letter of Credit No.		Unit Range:	M00499-M00508

Parameter	Unit of Measure	Mean Result	Standard Deviation	Test Method
Protein (6.38 x N) as is	% m/m	36.8	0.1	NZTM 3.8.2
Fat	% m/m	0.6	0.0	NZTM 3.8.2
Moisture	% m/m	3.8	0.1	NZTM 3.8.2
Titratable Acidity	% m/v	0.106	0.003	NZTM 3.2.3
WPNI	mg/g	2.9	0.0	NZTM 3.15.6
Insolubility Index	ml	0.13	0.03	NZTM 4.6.3
Wettability	secs	70	0	NZTM 5.4.10
Bulk Density	g/ml	0.53	0.00	NZTM 4.2.3
Ash		7.8	0.0	NZTM 3.4.2
Foreign Matter		Absent	0	NZTM 4.3.3
Scorched Particles	/50g	A	0	NZTM 4.3.17
Flavour		Typical	0	WMP in house
Aerobic Plate Count at 30^{0}C	cfu/g	132	52	NZTM 2.43.1
Escherichia coli and Coliforms; detection using LST-MUG	/g	Not Detected	N/A	NZTM 2.48.5
Yeast and Moulds; count	cfu/g	<1	0	NZTM 2.61.1
Coagulase-positive Staphylococci; Detection (Staph aureus)	/g	Not Detected	N/A	NZTM 2.47.2
Thermophilic Bacteria; count at 55^{0}C	/g	76	15	NZTM 2.60.1
Pesticides[1,2]		Not Detected	N/A	
Heavy Metals[1,2]	%m/m	Not Detected	N/A	
Salmonella[1]	/750g	Not Detected	N/A	ISO 6579
Inhibitory Substances	iu/ml	<0.005	N/A	NZTM 2.51.1

[1] Tests are subcontracted [2] Tested periodically

The product supplied against this Certificate is manufactured from New Zealand origin milk.
The finished product is manufactured and tested in premises registered under statutory requirements by the New Zealand Food Safety Authority. All premises are subjected to regular audit to ensure compliance with the terms of and conditions of registration.
Samples have been examined and subjected to laboratory analysis using internationally recognised procedures.
Copies of test methods and sampling plans are available on request.

I hereby declare that this is a true, complete and accurate copy of this record.

Prepared by: Lynda McAuliffe
Title: Laboratory Administration

Date: 26 October 2010

Released by: Leo McIntyre
Title: Quality Manager

Date: 26 October 2010

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8:35 a.m. 23/12/2010

Westland Milk Products
Hokitika · New Zealand

56 Livingstone Street
Hokitika, New Zealand
ph +64 3 756 9800

PRODUCT ANALYSIS CERTIFICATE

Product Description:	Instant Skimmilk Powder	Factory:	143
		Spec No:	2407
Customer:	Easiyo	Batch/Cypher:	CU12
Buyer Order No.		Manufacture Date:	12 October 2010
Letter of Credit No.		Unit Range:	M00509-M00569

Parameter	Unit of Measure	Mean Result	Standard Deviation	Test Method
Protein (6.38 x N) as is	% m/m	36.6	0.3	NZTM 3.8.2
Fat	% m/m	0.6	0.1	NZTM 3.8.2
Moisture	% m/m	3.9	0.2	NZTM 3.8.2
Titratable Acidity	% m/v	0.105	0.003	NZTM 3.2.3
WPNI	mg/g	3.0	0.0	NZTM 3.15.6
Insolubility Index	ml	0.13	0.03	NZTM 4.6.3
Wettability	secs	65	8	NZTM 5.4.10
Bulk Density	g/ml	0.53	0.00	NZTM 4.2.3
Ash		8.0	0.0	NZTM 3.4.2
Foreign Matter		Absent	0	NZTM 4.3.3
Scorched Particles	/50g	A	0	NZTM 4.3.17
Flavour		Typical	0	WMP in house
Aerobic Plate Count at 30^{0}C	cfu/g	318	243	NZTM 2.43.1
Escherichia coli and Coliforms; detection using LST-MUG	/g	Not Detected	N/A	NZTM 2.48.5
Yeast and Moulds; count	cfu/g	<1	0	NZTM 2.61.1
Coagulase-positive Staphylococci; Detection (Staph aureus)	/g	Not Detected	N/A	NZTM 2.47.2
Thermophilic Bacteria; count at 55^{0}C	/g	110	46	NZTM 2.60.1
Pesticides[1,2]		Not Detected	N/A	
Heavy Metals[1,2]	%m/m	Not Detected	N/A	
Salmonella[1]	/750g	Not Detected	N/A	ISO 6579
Inhibitory Substances	iu/ml	<0.005	N/A	NZTM 2.51.1

[1] Tests are subcontracted [2] Tested periodically

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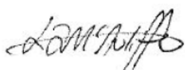
Prepared by: Lynda McAuliffe
Title: Laboratory Administration



Date: 26 October 2010

Released by: Leo McIntyre
Title: Quality Manager

Date: 26 October 2010

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8:34 a.m. 23/12/2010

Westland Milk Products
Hokitika · New Zealand

PRODUCT ANALYSIS CERTIFICATE

Product Description: Instant Skimmilk Powder

Customer: Easiyo
Buyer Order No.
Letter of Credit No.

Factory: 143
Spec No: 2407
Batch/Cypher: IU20
Manufacture Date: 20 April 2010
Unit Range: M04468-M04477

Parameter	Unit of Measure	Mean Result	Standard Deviation	Test Method
Protein (6.38 x N) as is	% m/m	36.2	0.1	NZTM 3.8.2
Fat	% m/m	0.8	0.0	NZTM 3.8.2
Moisture	% m/m	4.0	0.2	NZTM 3.8.2
Titratable Acidity	% m/v	0.104	0.003	NZTM 3.2.3
WPNI	mg/g	2.5	0.2	NZTM 3.15.6
Insolubility Index	ml	0.10	0.00	NZTM 4.6.3
Wettability	secs	20	12	NZTM 5.4.10
Bulk Density	g/ml	0.52	0.00	NZTM 4.2.3
Ash		8.0	0.0	NZTM 3.4.2
Foreign Matter		Absent	0	NZTM 4.3.3
Scorched Particles	/50g	A	0	NZTM 4.3.17
Flavour		Typical	0	WMP in house
Aerobic Plate Count at 30^{0}C	cfu/g	122	69	NZTM 2.43.1
Escherichia coli and Coliforms; detection using LST-MUG	/g	Not Detected	N/A	NZTM 2.48.5
Yeast and Moulds; count	cfu/g	<1	0	NZTM 2.61.1
Coagulase-positive Staphylococci; Detection (Staph aureus)	/g	Not Detected	N/A	NZTM 2.47.2
Thermophilic Bacteria; count at 55^{0}C	/g	40	14	NZTM 2.60.1
Pesticides[1,2]		Not Detected	N/A	
Heavy Metals[1,2]	%m/m	Not Detected	N/A	
Salmonella[1]	/750g	Not Detected	N/A	ISO 6579
Inhibitory Substances	iu/ml	<0.005	N/A	NZTM 2.51.1

[1] Tests are subcontracted [2] Tested periodically

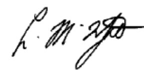
The product supplied against this Certificate is manufactured from New Zealand origin milk.
The finished product is manufactured and tested in premises registered under statutory requirements by the New Zealand Food Safety Authority. All premises are subjected to regular audit to ensure compliance with the terms of and conditions of registration.
Samples have been examined and subjected to laboratory analysis using internationally recognised procedures.
Copies of test methods and sampling plans are available on request.

I hereby declare that this is a true, complete and accurate copy of this record.

Prepared by: Lynda McAuliffe
Title: Laboratory Administration

Date: 30 April 2010

Released by: Leo McIntyre
Title: Quality Manager



Date: 30 April 2010

B. Nutritional Information of Selected DYB

Table A. 1. Nutritional information of selected DYB

Sample #LFLS		
Servings per package:	5	
Serving size:	200	g
Average quantity per:	serving	100 grams
Energy (kJ)	798	399
Energy (kcal)	191	95
Protein (g)	13.2	6.6
Carbohydrate (g)	28.9	14.4
Sugars (g)	28.6	14.3
Fat, total (g)	3.0	1.4
Fat, saturated (g)	2.1	1.1
Fibre (g)	0.0	0.0
Sodium (g)	0.115	0.058
Calcium (mg)	462	231

Sample #HFHS		
Servings per package:	5	
Serving size:	200	g
Average quantity per:	serving	100 grams
Energy (kJ)	912	456
Energy (kcal)	218	109
Protein (g)	8.4	4.2
Carbohydrate (g)	30.8	15.4
Sugars (g)	30.5	15.3
Fat, total (g)	7.1	3.5
Fat, saturated (g)	5.0	2.5
Fibre (g)	0.0	0.0
Sodium (g)	0.076	0.038
Calcium (mg)	301	150

Sample #HFN		
Servings per package:	5	
Serving size:	200	g

Average quantity per:	serving	100 grams
Energy (kJ)	560	280
Energy (kcal)	134	67
Protein (g)	7.7	3.8
Carbohydrate (g)	11.1	5.5
Sugars (g)	11.1	5.5
Fat, total (g)	7.0	3.5
Fat, saturated (g)	4.9	2.5
Fibre (g)	0.0	0.0
Sodium (g)	0.069	0.034
Calcium (mg)	273	137

C. Statistical Analysis of DYB during Storage at 20°C

Table A. 2. General linear model of the viability of yogurt bacteria in DYB formulations during storage at 20°C for 9 weeks periods.

Factor	Type	Levels	Values
Formulation	random	12	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12
Replication	random	2	1, 2
Time	random	4	0, 3, 6, 9

Source	DF	p-value		
		ST	NCFM	LB+NCFM
Formulation	11	0.000	0.000	0.000
Replication	1	0.971	0.079	0.777
Time	3	0.003	0.000	0.000

Table A. 3. One way ANOVA (Tukey's analysis) of the viability of yogurt bacteria in DYB formulations during storage at 20°C for 9 weeks periods.

Factor	Type	Levels	Values
Time	random	4	0, 3, 6, 9

Source	p-value		
Formulation	ST	NCFM	LB
1	0.000	0.112	0.697
2	0.016	0.008	0.487
3	0.088	0.227	0.249
4	0.000	0.058	0.704
5	0.295	0.137	0.060
6	0.011	0.059	0.333
7	0.032	0.093	0.068
8	0.033	0.379	0.366

9	0.135	0.011	0.933
10	0.004	0.471	0.021
11	0.006	0.033	0.668
12	0.274	0.016	0.728

Table A. 4. General Linear Model analysis of a_w of DYB formulations during storage at 20°C for 9 weeks periods.

Factor	Type	Levels	Values
Formulation	random	12	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12
Replication	random	2	1, 2
Time	random	4	0, 3, 6, 9

Source	DF	p-value
Formulation	11	0.037
Time	3	0.003
Replication	1	0.791

Table A. 5. General Linear Model analysis of $[O_2]$ of DYB formulations during storage at 20°C for 9 weeks periods.

Analysis of Variance for Oxygen, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Formulation	11	136.423	135.637	12.331	3.17	0.002
Time	3	6.898	6.641	2.214	0.57	0.638
Replication	1	0.042	0.042	0.042	0.01	0.918
Error	59	229.639	229.639	3.892		
Total	74	373.001				

S = 1.97286   R-Sq = 38.43%   R-Sq(adj) = 22.78%

Table A. 6. General Linear Model analysis of $[CO_2]$ of DYB formulations during storage at 20°C for 9 weeks periods.

Analysis of Variance for Carbondioxide, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Formulation	11	42.8815	42.9608	3.9055	8.04	0.000
Time	3	0.1051	0.1830	0.0610	0.13	0.945
Replication	1	0.1740	0.1740	0.1740	0.36	0.552
Error	59	28.6652	28.6652	0.4859		
Total	74	71.8259				

S = 0.697031   R-Sq = 60.09%   R-Sq(adj) = 49.94%

D. Gram Staining of LAB

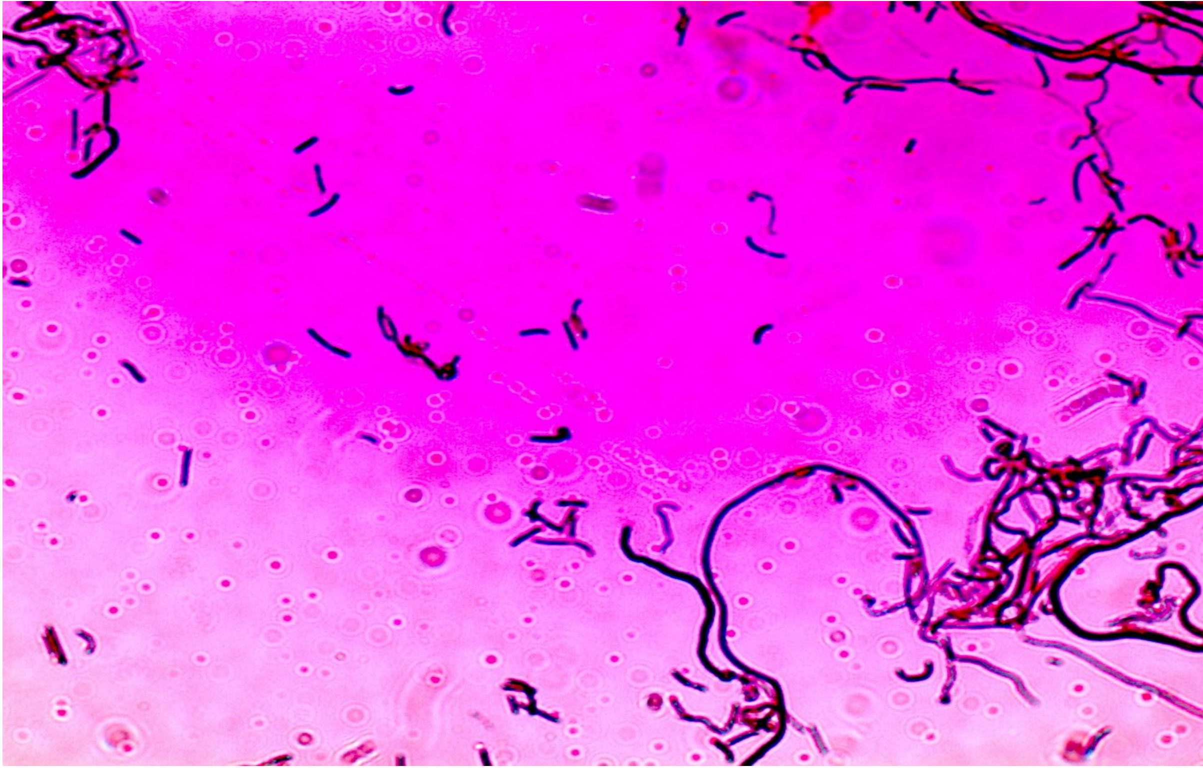


Figure A. 1. Gram staining of *Lactobacillus acidophilus* grown on MRS-clindamycin agar under oil immersion (100 x magnifications) using Carl Zeis (model HBO 50/AC, Germany) transmission light microscope.

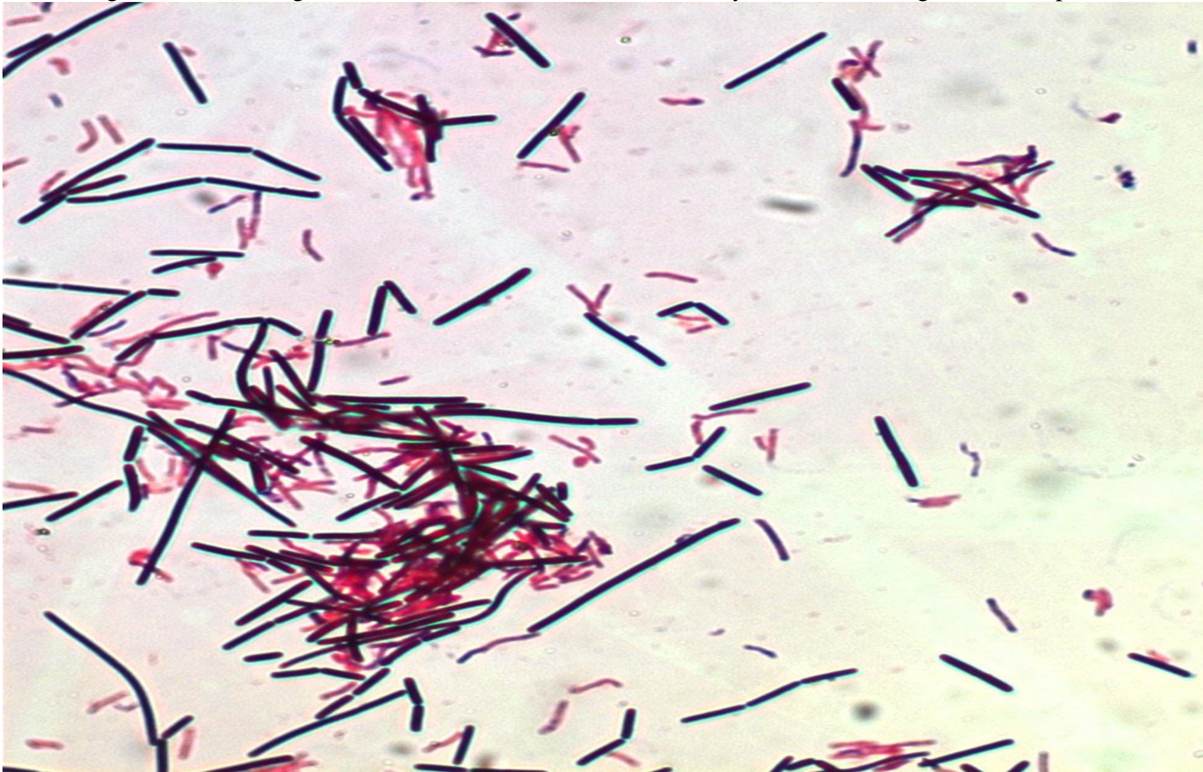


Figure A. 2. Gram staining of Lactobacillus bulgaricus grown on MRS agar under oil immersion (100 x magnifications) using Carl Zeis (model HBO 50/AC, Germany) transmission light microscope.

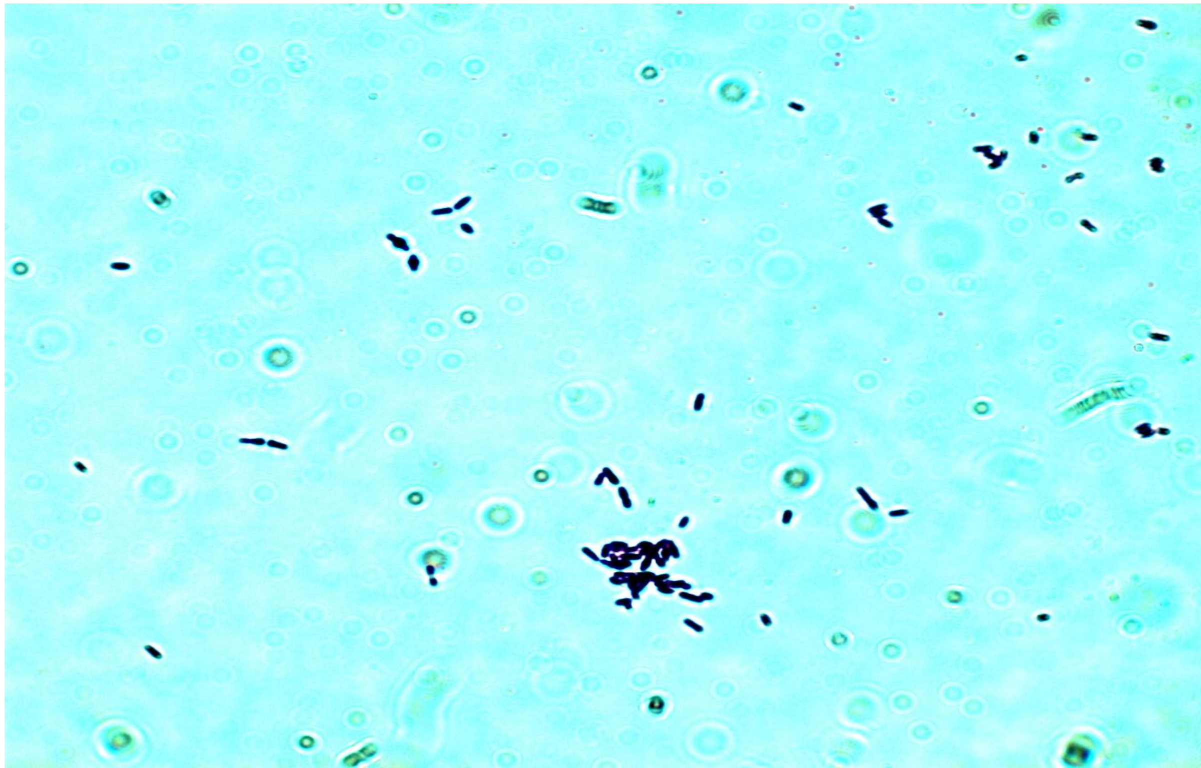


Figure A. 3. Gram staining of *Streptococcus thermophilus* grown on M17 agar under oil immersion (100 x magnifications) using Carl Zeis (model HBO 50/AC, Germany) transmission light microscope.

E. Statistical Analysis of Fermentation of Selected Formulation

pH of yogurt during 8 hour fermentation

Two-way ANOVA: pH versus Formulation, Time (h)

Source	DF	SS	MS	F	P
Formulation	2	0.0059	0.00295	0.38	0.689
Time (h)	8	15.4993	1.93741	250.45	0.000
Error	16	0.1238	0.00774		
Total	26	15.6289			

S = 0.08795   R-Sq = 99.21%   R-Sq(adj) = 98.71%

Cell counts of yogurt bacteria during fermentation

General Linear Model: ST versus Time, Replicate, Formulation

Factor	Type	Levels	Values
Time	fixed	9	0, 1, 2, 3, 4, 5, 6, 7, 8
Replicate	fixed	2	1, 2
Formulation	fixed	3	HFHS, HFN, LFLS

Analysis of Variance for ST, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P

Time	8	4.00993E+19	4.00993E+19	5.01242E+18	10.10	0.000
Replicate	1	1.01259E+17	1.01259E+17	1.01259E+17	0.20	0.654
Formulation	2	3.78687E+18	3.78687E+18	1.89343E+18	3.81	0.030
Error	42	2.08494E+19	2.08494E+19	4.96414E+17		
Total	53	6.48369E+19				

S = 704566773   R-Sq = 67.84%   R-Sq(adj) = 59.42%

Unusual Observations for ST

Obs	ST	Fit	SE Fit	Residual	St Resid	
18	6500000000	3145711852	332135962	3354288148	5.40	R
54	1200000000	2507340741	332135962	-1307340741	-2.10	R

R denotes an observation with a large standardized residual.

General Linear Model: LB versus Time, Replicate, Formulation

Factor	Type	Levels	Values
Time	fixed	9	0, 1, 2, 3, 4, 5, 6, 7, 8
Replicate	fixed	2	1, 2
Formulation	fixed	3	HFHS, HFN, LFLS

Analysis of Variance for LB, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	8	3.46003E+16	3.46003E+16	4.32503E+15	2.11	0.056
Replicate	1	5.16463E+12	5.16463E+12	5.16463E+12	0.00	0.960
Formulation	2	9.10341E+15	9.10341E+15	4.55170E+15	2.22	0.121
Error	42	8.61897E+16	8.61897E+16	2.05214E+15		
Total	53	1.29899E+17				

S = 45300505   R-Sq = 33.65%   R-Sq(adj) = 16.27%

Unusual Observations for LB

Obs	LB	Fit	SE Fit	Residual	St Resid	
9	250000000	105611111	21354863	144388889	3.61	R
18	210000000	104992592	21354863	105007408	2.63	R
43	160000000	50777778	21354863	109222222	2.73	R

R denotes an observation with a large standardized residual.

General Linear Model: NCFM versus Time, Replicate, Formulation

Factor	Type	Levels	Values
Time	fixed	9	0, 1, 2, 3, 4, 5, 6, 7, 8
Replicate	fixed	2	1, 2
Formulation	fixed	3	HFHS, HFN, LFLS

Analysis of Variance for NCFM, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	8	3.84486E+17	3.84486E+17	4.80608E+16	25.72	0.000
Replicate	1	2.94000E+14	2.94000E+14	2.94000E+14	0.16	0.694
Formulation	2	1.60176E+16	1.60176E+16	8.00880E+15	4.29	0.020

Source	DF	Seq SS	Adj SS	Adj MS
Error	42	7.84721E+16	7.84721E+16	1.86838E+15
Total	53	4.79270E+17		

S = 43224793   R-Sq = 83.63%   R-Sq(adj) = 79.34%

Unusual Observations for NCFM

Obs	NCFM	Fit	SE Fit	Residual	St Resid
18	190000000	292574074	20376363	-102574074	-2.69 R
34	300000000	213685185	20376363	86314815	2.26 R
42	80000000	166407407	20376363	-86407407	-2.27 R

R denotes an observation with a large standardized residual.

F. Statistical Analysis of RTE during Refrigerated Storage

Table A. 7. pH measurements during storage of 2 weeks at 4°C

General Linear Model: pH versus Formulation, Replication, Time

Factor	Type	Levels	Values
Formulation	random	3	1, 2, 3
Replication	random	2	1, 2
Time	random	3	1, 7, 14

Analysis of Variance for pH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Formulation	2	0.13254	0.12760	0.06380	4.97	0.017
Replication	1	0.04476	0.01430	0.01430	1.11	0.303
Time	2	0.13599	0.13599	0.06799	5.30	0.013
Error	22	0.28231	0.28231	0.01283		
Total	27	0.59559				

S = 0.113279   R-Sq = 52.60%   R-Sq(adj) = 41.83%

Unusual Observations for pH

Obs	pH	Fit	SE Fit	Residual	St Resid
7	4.65000	4.42003	0.05903	0.22997	2.38 R
19	4.49000	4.27803	0.05903	0.21197	2.19 R
26	4.66000	4.43256	0.05393	0.22744	2.28 R

R denotes an observation with a large standardized residual.

Table A. 8. Cell counts changes during refrigerated storage of 2 weeks

Cell counts during storage of 2 weeks
General Linear Model: ST versus Formulation, Time

```
Factor        Type    Levels  Values
Formulation   random       3  1, 2, 3
Time          random       3  1, 7, 14


Analysis of Variance for ST, using Adjusted SS for Tests

Source        DF       Seq SS       Adj SS       Adj MS
F      P
Formulation    2  8.16023E+18  7.85467E+18  3.92734E+18
37.10  0.000
Time           2  1.72933E+18  1.72933E+18  8.64666E+17
8.17  0.002
Error         29  3.07009E+18  3.07009E+18  1.05865E+17
Total         33  1.29597E+19
S = 325369069   R-Sq = 76.31%   R-Sq(adj) = 73.04%
```

General Linear Model: NCFM versus Formulation, Time

```
Factor        Type    Levels  Values
Formulation   random       3  1, 2, 3
Time          random       3  1, 7, 14


Analysis of Variance for NCFM, using Adjusted SS for Tests

Source        DF       Seq SS       Adj SS       Adj MS     F
P
Formulation    2  4.36034E+16  3.75740E+16  1.87870E+16  2.21
0.128
Time           2  1.39292E+17  1.39292E+17  6.96462E+16  8.19
0.002
Error         29  2.46716E+17  2.46716E+17  8.50745E+15
Total         33  4.29612E+17


S = 92235834   R-Sq = 42.57%   R-Sq(adj) = 34.65%


Unusual Observations for NCFM

Obs       NCFM        Fit    SE Fit    Residual  St Resid
  4  190000000  402051282  35416146  -212051282     -2.49 R
 10  590000000  402051282  35416146   187948718      2.21 R
 25  590000000  413461538  40448089   176538462      2.13 R

R denotes an observation with a large standardized residual.
```

General Linear Model: LB versus Time, Formulation

```
Factor        Type    Levels  Values
Time          random       3  1, 7, 14
Formulation   random       3  1, 2, 3


Analysis of Variance for LB, using Adjusted SS for Tests

Source        DF       Seq SS       Adj SS       Adj MS     F
```

```
P
Time          2  8.98081E+16  8.22176E+16  4.11088E+16  4.16
0.026
Formulation   2  3.15376E+16  3.15376E+16  1.57688E+16  1.60
0.220
Error        29  2.86681E+17  2.86681E+17  9.88554E+15
Total        33  4.08026E+17


S = 99426072   R-Sq = 29.74%   R-Sq(adj) = 20.05%


Unusual Observations for LB

Obs         LB        Fit    SE Fit    Residual  St Resid
 26  480000000  245769231  38177009   234230769      2.55 R
 35   40000000  245769231  38177009  -205769231     -2.24 R
 36  420000000  143269231  38177009   276730769      3.01 R

R denotes an observation with a large standardized residual.
```

Table A. 9. Syneresis index during refrigerated storage of 2 weeks

General Linear Model: Syneresis versus Time, Replication, Formulation

```
Factor       Type   Levels  Values
Time         fixed       3  0, 7, 14
Replication  fixed       2  1, 2
Formulation  fixed       3  HFHS, HFN, LFLS


Analysis of Variance for Syneresis, using Adjusted SS for
Tests

Source       DF   Seq SS   Adj SS   Adj MS     F      P
Time          2  0.03256  0.03256  0.01628  0.67  0.523
Replication   1  0.00003  0.00003  0.00003  0.00  0.973
Formulation   2  0.19687  0.19687  0.09844  4.03  0.031
Error        24  0.58644  0.58644  0.02444
Total        29  0.81590


S = 0.156317   R-Sq = 28.12%   R-Sq(adj) = 13.15%


Unusual Observations for Syneresis

Obs  Syneresis       Fit    SE Fit  Residual  St Resid
  3   0.882706  0.296707  0.066931  0.585999      4.15 R

R denotes an observation with a large standardized residual
```

Table A. 10. General linear model of texture analyzer measurements during refrigerated storage for 2 weeks period

General Linear Model: Texture versus Formulation, Time, Replication

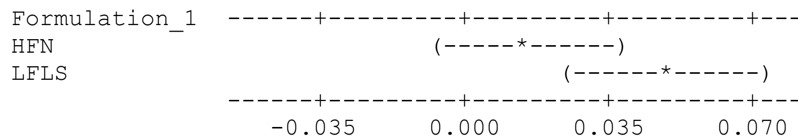
```
Factor       Type    Levels  Values
Formulation  random       3  HFHS, HFN, LFLS
Time         random       3  0, 7, 14
Replication  random       2  1, 2


Analysis of Variance for Texture, using Adjusted SS for
Tests

Source       DF     Seq SS     Adj SS     Adj MS      F
P
Formulation   2  0.0357287  0.0357287  0.0178644  78.43
0.000
Time          2  0.0050349  0.0050349  0.0025174  11.05
0.000
Replication   1  0.0000979  0.0000979  0.0000979   0.43
0.516
Error        39  0.0088831  0.0088831  0.0002278
Total        44  0.0497446


S = 0.0150921   R-Sq = 82.14%   R-Sq(adj) = 79.85%


Unusual Observations for Texture

Obs   Texture       Fit    SE Fit   Residual  St Resid
 17  0.145100  0.111727  0.005356   0.033373      2.37 R
 32  0.125200  0.169633  0.005356  -0.044433     -3.15 R
 36  0.217000  0.188287  0.005356   0.028713      2.03 R

R denotes an observation with a large standardized
residual.
```

Table A. 11. One-way ANOVA of texture analyzer measurements during refrigerated storage for 2 weeks period (Tukey's analysis)

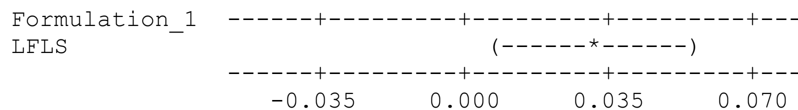
One-way ANOVA: Texture_1 versus Formulation_1 at time 0

```
Source         DF        SS        MS      F      P
Formulation_1   2  0.006003  0.003001  15.13  0.001
Error          12  0.002381  0.000198
Total          14  0.008384

S = 0.01409   R-Sq = 71.60%   R-Sq(adj) = 66.87%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ---+---------+---------+---------+-----
-
HFHS   5  0.10740  0.00829  (------*------)
HFN    5  0.12306  0.01309          (------*-----)
LFLS   5  0.15544  0.01885                          (------*------)
                            ---+---------+---------+---------+-----
-
                             0.100     0.120     0.140     0.160

Pooled StDev = 0.01409
```

```
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Formulation_1

Individual confidence level = 97.94%


Formulation_1 = HFHS subtracted from:

Formulation_1      Lower   Center    Upper
HFN             -0.00809  0.01566  0.03941
LFLS             0.02429  0.04804  0.07179
```



```
Formulation_1  ------+---------+---------+---------+---
HFN                          (-----*------)
LFLS                                   (------*------)
               ------+---------+---------+---------+---
                  -0.035     0.000     0.035     0.070


Formulation_1 = HFN subtracted from:

Formulation_1     Lower   Center    Upper
LFLS            0.00863  0.03238  0.05613
```



```
Formulation_1  ------+---------+---------+---------+---
LFLS                             (------*------)
               ------+---------+---------+---------+---
                  -0.035     0.000     0.035     0.070
```

Table A. 12. General linear model of ANOVA for viscosity of unbroken gel of selected formulations during storage at 4°C for 2 weeks periods.

General Linear Model: Viscosity unbroken gel versus Formulation, Replicate, ...

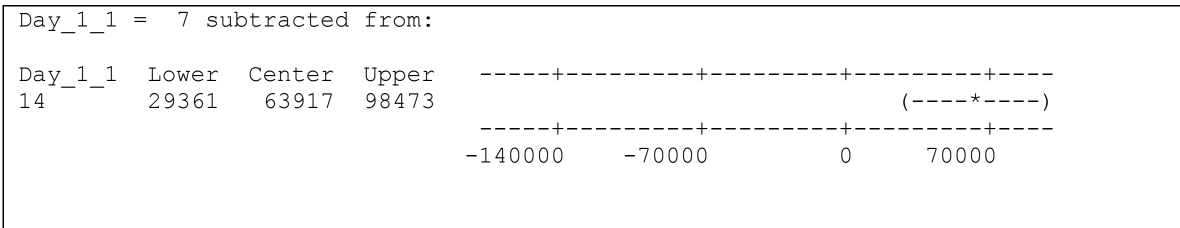
```
Factor        Type   Levels  Values
Formulation   fixed       3  HFHS, HFN, LFLS
Replicate     fixed       2  1, 2
Day           fixed       3  0, 7, 14
Time          fixed       4  35, 40, 50, 60


Analysis of Variance for Viscosity unbroken gel (m.Pa.s), 
using Adjusted SS for
     Tests

Source         DF       Seq SS       Adj SS       Adj MS
F      P
Formulation     2  7.66918E+11  7.71817E+11  3.85908E+11
106.38  0.000
Replicate       1  26837885131  29303816628  29303816628
8.08  0.005
Day             2  42284163737  42997081705  21498540852
5.93  0.003
Time            3  3.07901E+11  3.07901E+11  1.02634E+11
28.29  0.000
Error         170  6.16711E+11  6.16711E+11   3627712374
Total         178  1.76065E+12


S = 60230.5   R-Sq = 64.97%   R-Sq(adj) = 63.32%
```

```
Unusual Observations for Viscosity unbroken gel (m.Pa.s)

     Viscosity
      unbroken
           gel
Obs    (m.Pa.s)      Fit  SE Fit  Residual  St Resid
 18      422000  552913   15450   -130913     -2.25 R
 21      473000  598011   15575   -125011     -2.15 R
 24      442000  615068   15450   -173068     -2.97 R
 31      697000  509469   14741    187531      3.21 R
 34      695000  526527   14564    168473      2.88 R
 39      418000  548043   13119   -130043     -2.21 R
 76      663000  520872   13139    142128      2.42 R

R denotes an observation with a large standardized residual.
```

Table A. 13. Tukey's analysis of one-way ANOVA on viscosity of unbroken gel of #HFHS during storage for 14 days at 4°C

One-way ANOVA: Viscosity unbroken gel (m.Pa._2 versus Day_1_1 this is for HFHS which showed a decreased on day7 but increase on day 14

```
Source   DF           SS           MS      F      P
Day_1_1   2  1.50788E+11  75394050000  30.41  0.000
Error    57  1.41311E+11   2479137427
Total    59  2.92099E+11

S = 49791   R-Sq = 51.62%   R-Sq(adj) = 49.92%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ---------+---------+---------+---------+
 0     12  561500  90537                            (----*-----)
 7     24  426583  30019  (---*---)
14     24  490500  36366               (---*---)
                          ---------+---------+---------+---------+
                              450000    500000    550000    600000

Pooled StDev = 49791


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Day_1_1

Individual confidence level = 98.05%


Day_1_1 =  0 subtracted from:

Day_1_1    Lower   Center   Upper   -----+---------+---------+---------+----
 7       -177239  -134917  -92594   (-----*-----)
14       -113322   -71000  -28678            (-----*-----)
                                    -----+---------+---------+---------+----
                                   -140000    -70000         0     70000
```



```
Day_1_1 =  7 subtracted from:

Day_1_1  Lower  Center  Upper   -----+---------+---------+---------+----
14       29361   63917  98473                              (----*----)
                                -----+---------+---------+---------+----
                              -140000    -70000         0     70000
```

Table A. 14. Tukey's significance difference of one-way ANOVA of selected formulations at 1 day storage at 4°C.

One-way ANOVA: Viscosity unbroken gel (m.Pa._1 versus Formulation_1 on day 1 only

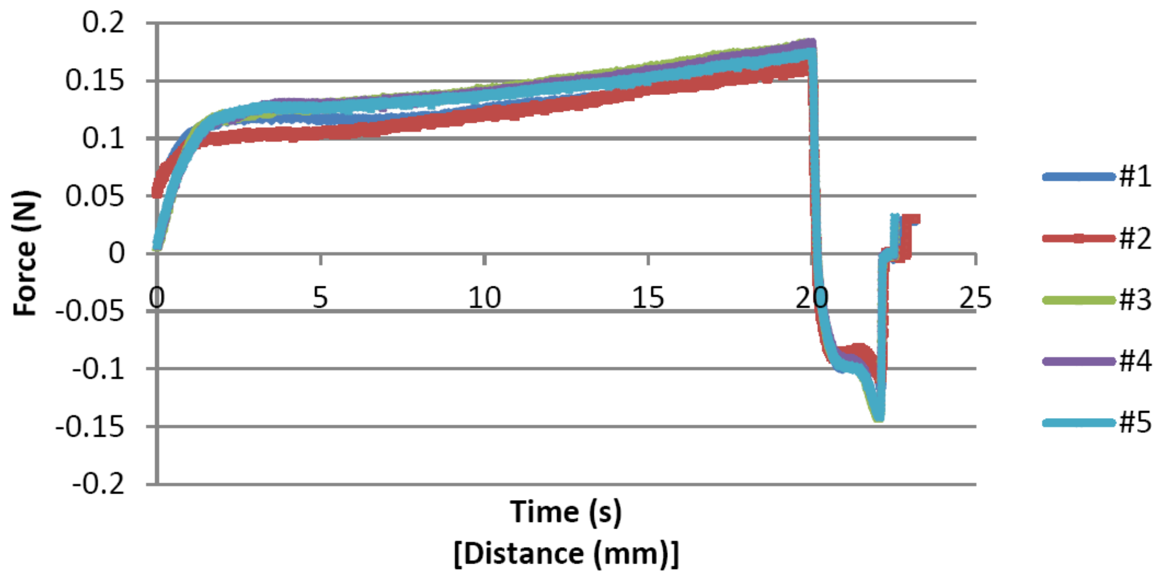
```
Source          DF           SS           MS     F      P
Formulation_1    2  97025917100  48512958550  9.57  0.001
Error           32  1.62205E+11   5068900805
Total           34  2.59231E+11

S = 71196   R-Sq = 37.43%   R-Sq(adj) = 33.52%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev   ----+---------+---------+---------+-----
HFHS   12  561500  90537             (------*------)
HFN    12  496917  53531   (------*------)
LFLS   11  626909  63653                       (------*-------)
                           ----+---------+---------+---------+-----
                          480000    540000    600000    660000

Pooled StDev = 71196


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Formulation_1

Individual confidence level = 98.06%


Formulation_1 = HFHS subtracted from:

Formulation_1    Lower  Center   Upper
HFN            -136106  -64583    6940
LFLS             -7721   65409  138540

Formulation_1  -------+---------+---------+---------+--
HFN                  (-----*-----)
LFLS                           (-----*------)
               -------+---------+---------+---------+--
                -120000         0    120000    240000


Formulation_1 = HFN subtracted from:

Formulation_1  Lower  Center   Upper  -------+---------+---------+---------+--
LFLS           56862  129992  203123                         (-----*-----)
                                      -------+---------+---------+---------+--
                                       -120000         0    120000    240000
```

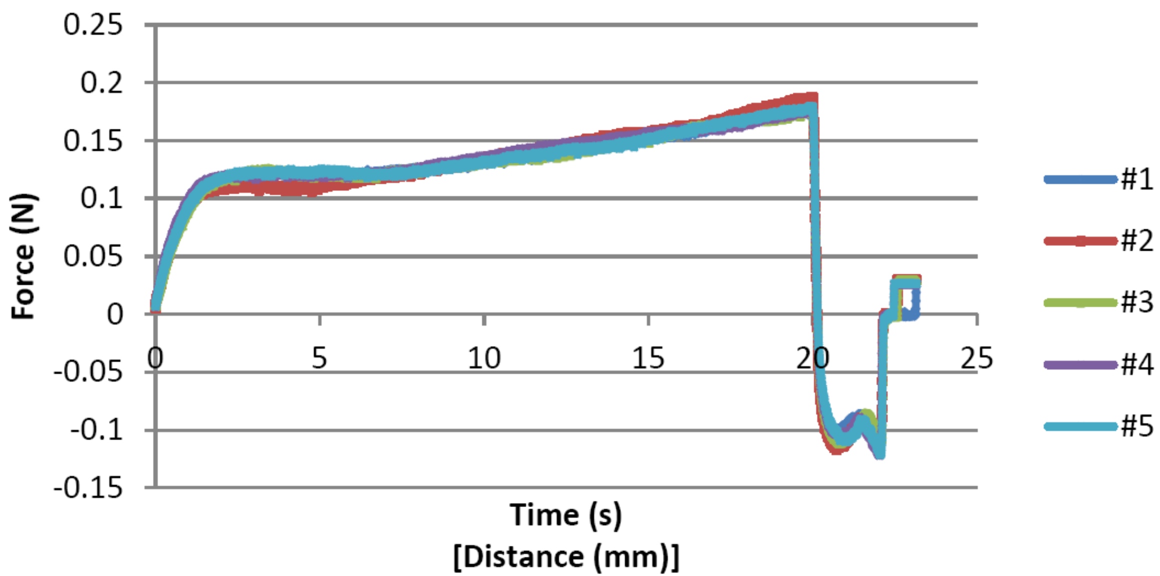
Table A. 15. General Linear Model of ANOVA analysis on viscosity of disturbed yogurt gel during storage at 4°C for 2 weeks.

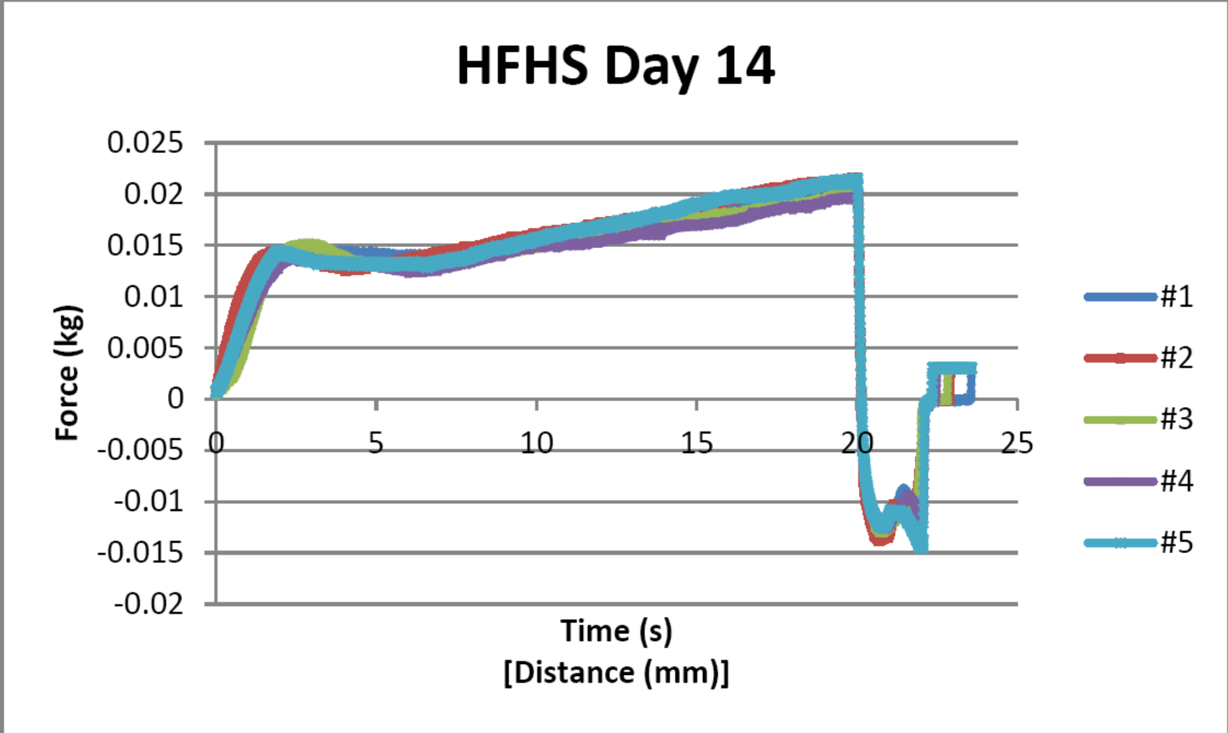
General Linear Model: Viscosity broken gel versus Formulation, Replicate, ...

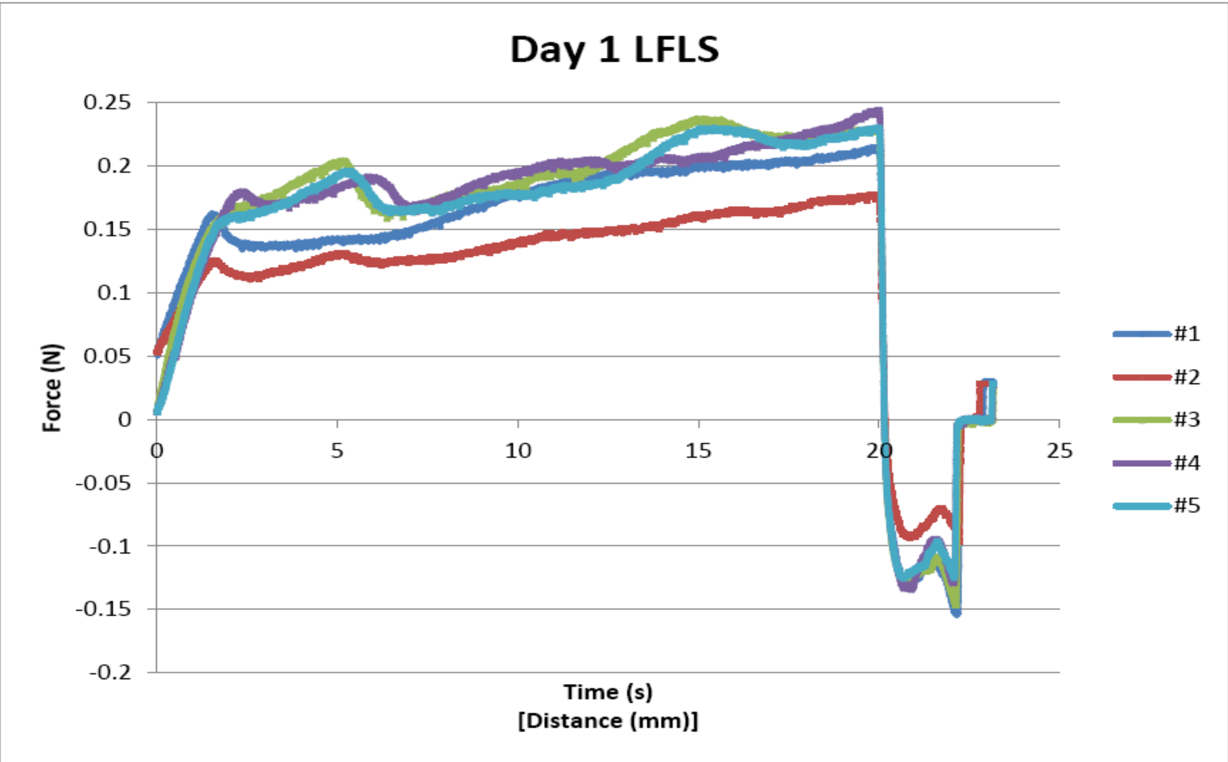
```
Factor        Type   Levels  Values
Formulation   fixed       3  HFHS, HFN, LFLS
Replicate     fixed       2  1, 2
Day           fixed       3  0, 7, 14
Time          fixed       4  35, 40, 50, 60


Analysis of Variance for Viscosity broken gel (mPa.s), using Adjusted SS
for
     Tests

Source         DF      Seq SS      Adj SS      Adj MS       F      P
Formulation     2  3160077972  3176005668  1588002834  106.66  0.000
Replicate       1   127898438   140207735   140207735    9.42  0.003
Day             2    93249766    92987213    46493607    3.12  0.047
Time            3   114198399   114198399    38066133    2.56  0.057
Error         170  2531041259  2531041259    14888478
Total         178  6026465834


S = 3858.56   R-Sq = 58.00%   R-Sq(adj) = 56.02%


Unusual Observations for Viscosity broken gel (mPa.s)

      Viscosity
     broken gel
Obs     (mPa.s)      Fit  SE Fit  Residual  St Resid
  3     11904.0  20991.3  1003.8   -9087.3     -2.44 R
 36     31025.0  14133.6   994.5   16891.4      4.53 R
 53     33368.0  20433.0   851.4   12935.0      3.44 R
 55     29900.0  20951.2   841.2    8948.8      2.38 R
 56     32712.0  20951.2   841.2   11760.8      3.12 R
 57     31306.0  20951.2   841.2   10354.8      2.75 R
 58     29994.0  21594.8   841.2    8399.2      2.23 R
 59     32712.0  21594.8   841.2   11117.2      2.95 R
 60     32431.0  21594.8   841.2   10836.2      2.88 R

R denotes an observation with a large standardized residual.
```

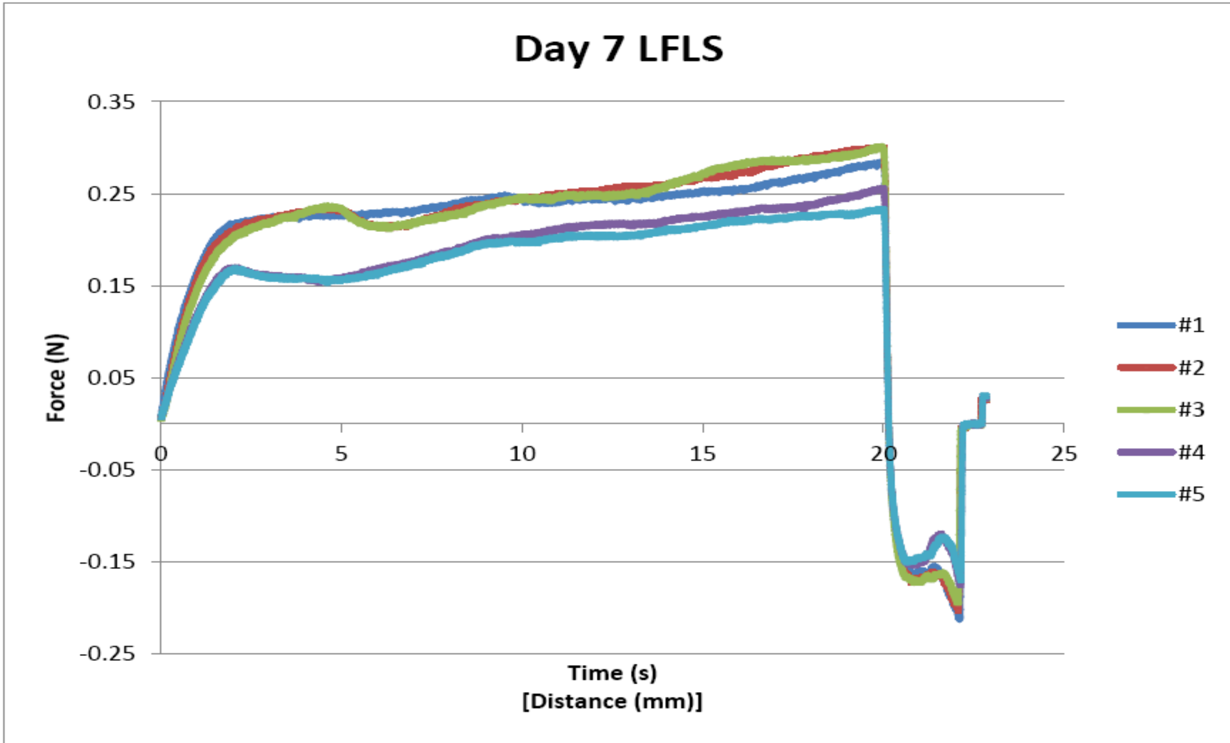
Texture Analyzer Profiles

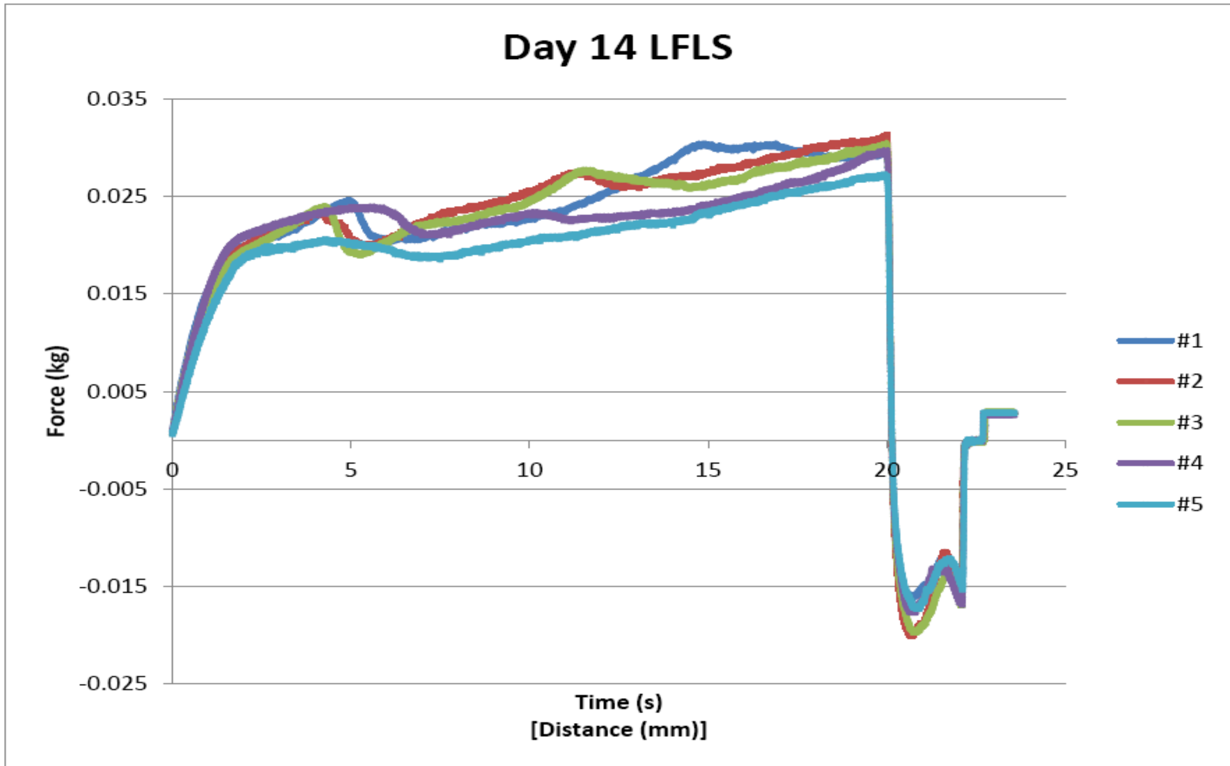

HFHS Day 1
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
Force (N)
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5

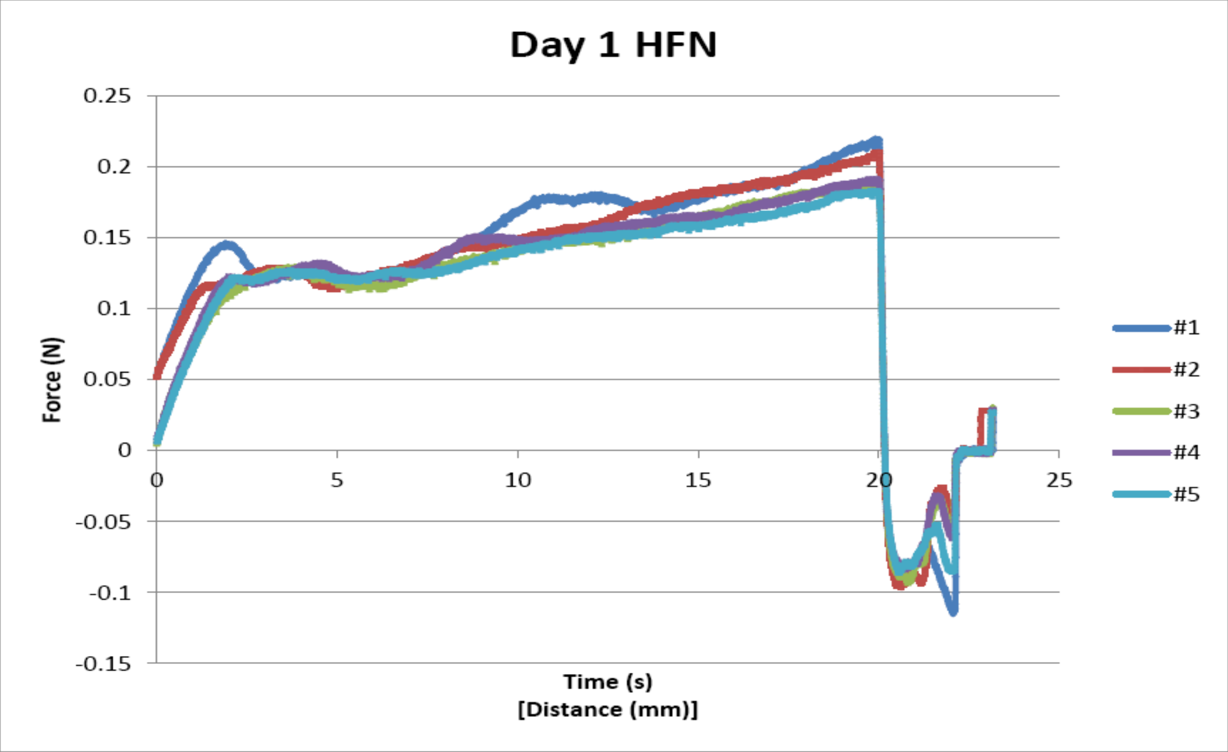

HFHS Day 7
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
Force (N)
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5

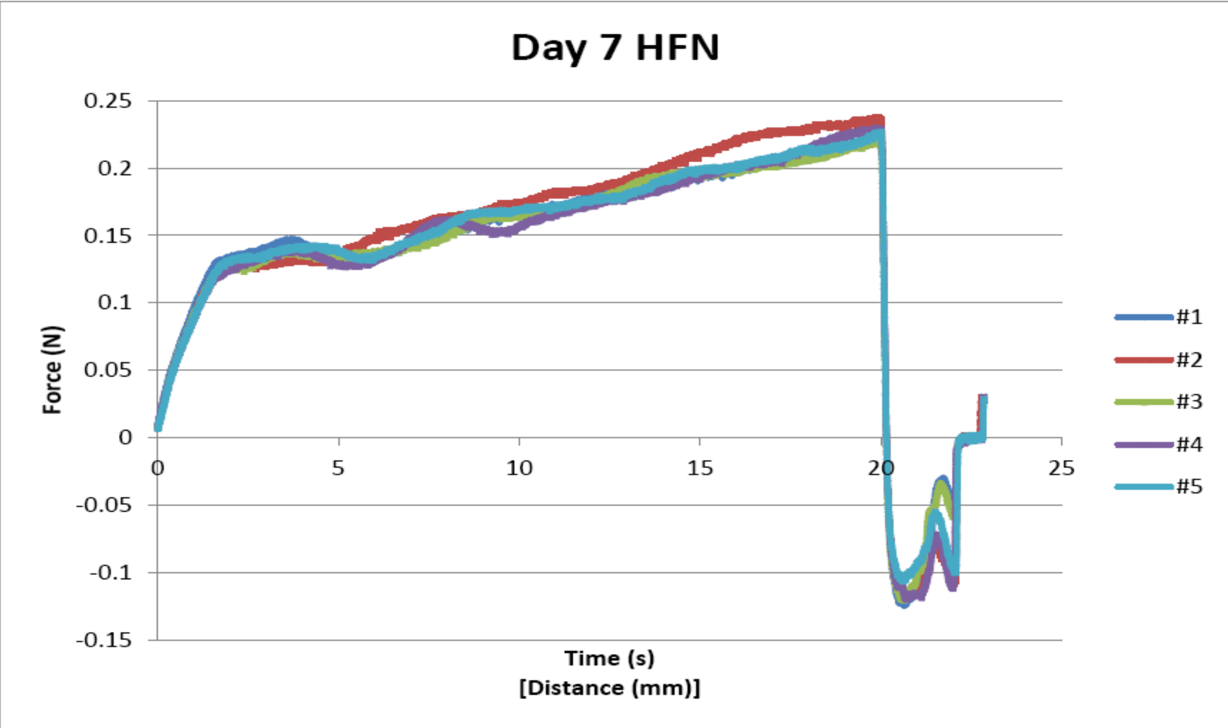

HFHS Day 14
Force (kg)
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5

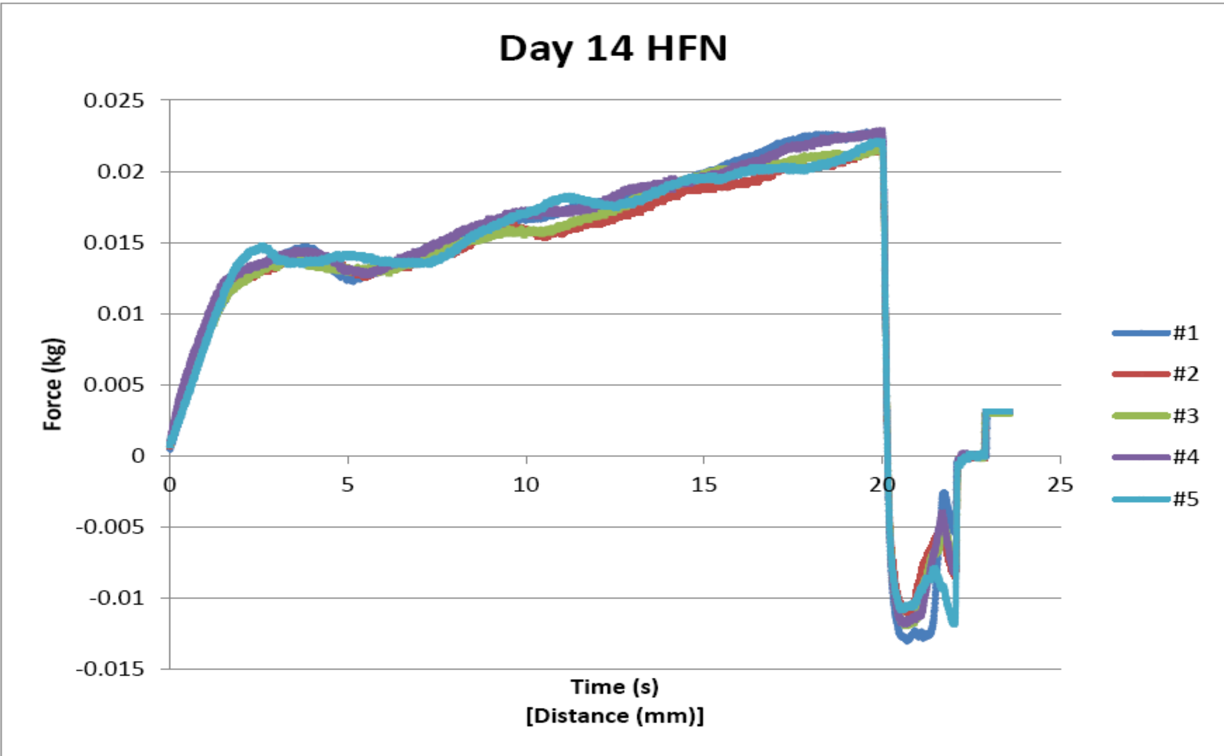

Day 1 LFLS
Force (N)
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5


Day 7 LFLS
0.35
0.25
0.15
0.05
-0.05
-0.15
-0.25
Force (N)
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5


Day 14 LFLS
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
Force (kg)
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5


Day 1 HFN
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
Force (N)
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5


Day 7 HFN
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
Force (N)
0
5
10
15
20
25
Time (s)
[Distance (mm)]
#1
#2
#3
#4
#5



G. Statistical Analysis of Sensory Results

Table A. 16. The effects of block design on panellists responses

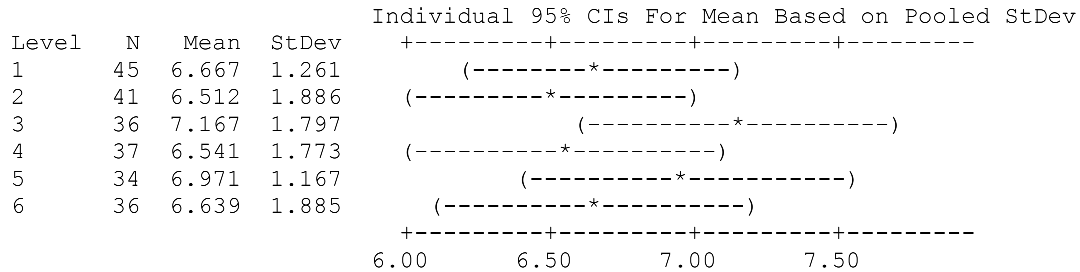
One-way ANOVA: Overall acceptability versus Block

```
Source    DF       SS    MS     F      P
Block      5    11.68  2.34  0.52  0.759
Error    223   997.51  4.47
Total    228  1009.19

S = 2.115   R-Sq = 1.16%   R-Sq(adj) = 0.00%

                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  -+---------+---------+---------+--------
1      45  5.767  2.147         (---------*---------)
2      41  6.073  2.054             (----------*----------)
3      36  6.028  2.420            (----------*-----------)
4      37  5.865  2.030         (-----------*----------)
5      34  6.059  1.808            (-----------*-----------)
6      36  5.417  2.170  (----------*-----------)
                         -+---------+---------+---------+--------
                        4.80      5.40      6.00      6.60

Pooled StDev = 2.115

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Block

Individual confidence level = 99.56%
```

One-way ANOVA: Appearance versus Block

Source	DF	SS	MS	F	P
Block	5	12.57	2.51	0.92	0.467
Error	223	607.71	2.73		
Total	228	620.28			

S = 1.651   R-Sq = 2.03%   R-Sq(adj) = 0.00%



```
                         Individual 95% CIs For Mean Based on Pooled StDev
Level   N   Mean  StDev    +---------+---------+---------+---------
1      45  6.667  1.261        (--------*---------)
2      41  6.512  1.886    (---------*---------)
3      36  7.167  1.797                (----------*----------)
4      37  6.541  1.773    (----------*----------)
5      34  6.971  1.167            (----------*-----------)
6      36  6.639  1.885      (----------*----------)
                           +---------+---------+---------+---------
                         6.00      6.50      7.00      7.50
```

Pooled StDev = 1.651

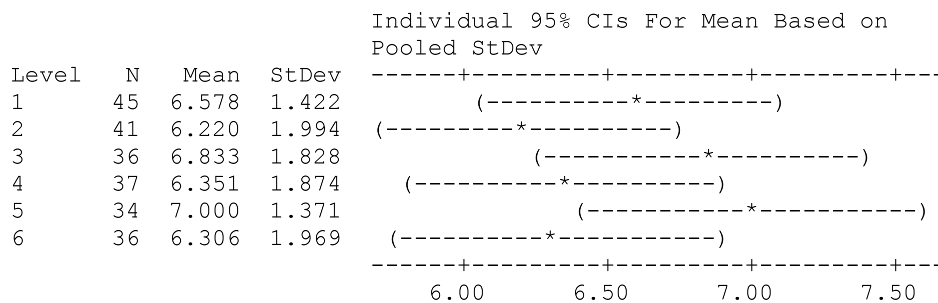
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Block

Individual confidence level = 99.56%

One-way ANOVA: Texture versus Block

Source	DF	SS	MS	F	P
Block	5	17.86	3.57	1.16	0.332
Error	223	689.07	3.09		
Total	228	706.93			

S = 1.758   R-Sq = 2.53%   R-Sq(adj) = 0.34%



```
                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  ------+---------+---------+---------+---
1      45  6.578  1.422         (----------*---------)
2      41  6.220  1.994  (---------*----------)
3      36  6.833  1.828             (-----------*----------)
4      37  6.351  1.874    (----------*----------)
5      34  7.000  1.371                (-----------*-----------)
6      36  6.306  1.969   (----------*-----------)
                         ------+---------+---------+---------+---
                             6.00      6.50      7.00      7.50
```

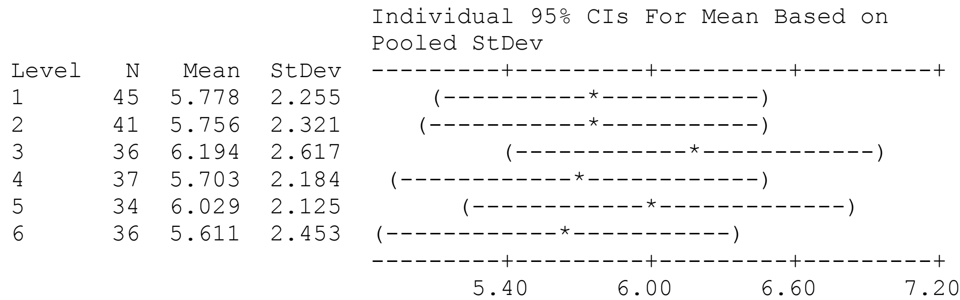
Pooled StDev = 1.758

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Block

Individual confidence level = 99.56%

One-way ANOVA: Sweetness versus Block

```
Source   DF       SS    MS     F      P
Block     5     8.79  1.76  0.32  0.898
Error   223  1210.23  5.43
Total   228  1219.02

S = 2.330   R-Sq = 0.72%   R-Sq(adj) = 0.00%
```

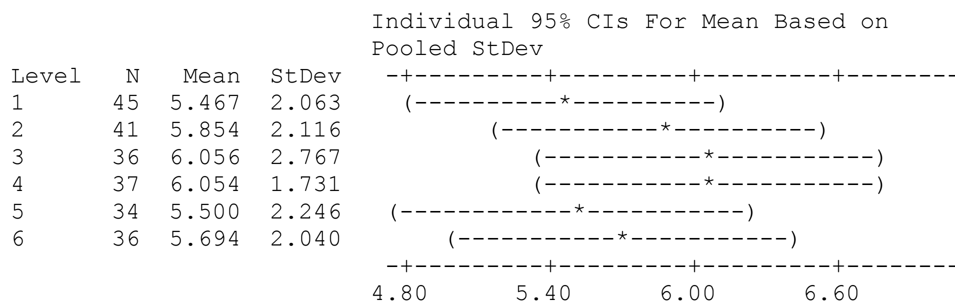


```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N   Mean  StDev  ---------+---------+---------+---------+
1      45  5.778  2.255      (----------*-----------)
2      41  5.756  2.321     (------------*-----------)
3      36  6.194  2.617           (------------*------------)
4      37  5.703  2.184   (------------*------------)
5      34  6.029  2.125        (------------*-------------)
6      36  5.611  2.453  (------------*-----------)
                         ---------+---------+---------+---------+
                              5.40      6.00      6.60      7.20

Pooled StDev = 2.330
```

```
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Block

Individual confidence level = 99.56%
```

One-way ANOVA: Sourness versus Block

```
Source   DF       SS    MS     F      P
Block     5    13.02  2.60  0.55  0.737
Error   223  1054.24  4.73
Total   228  1067.27

S = 2.174   R-Sq = 1.22%   R-Sq(adj) = 0.00%
```

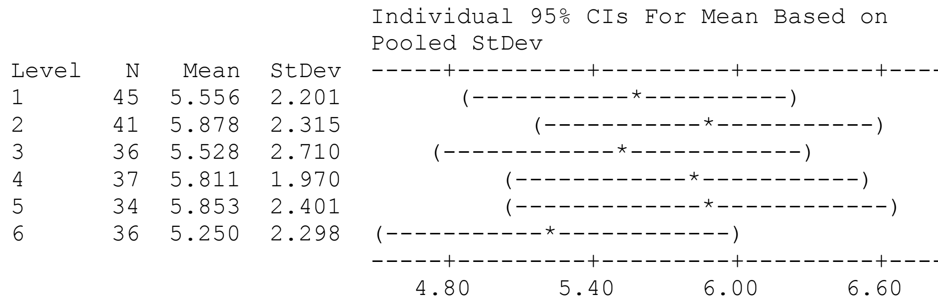


```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N   Mean  StDev   -+---------+---------+---------+--------
1      45  5.467  2.063    (----------*----------)
2      41  5.854  2.116          (-----------*----------)
3      36  6.056  2.767             (-----------*-----------)
4      37  6.054  1.731             (-----------*-----------)
5      34  5.500  2.246   (------------*-----------)
6      36  5.694  2.040       (-----------*-----------)
                          -+---------+---------+---------+--------
                         4.80      5.40      6.00      6.60

Pooled StDev = 2.174
```

```
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Block

Individual confidence level = 99.56%
```

One-way ANOVA: Flavour versus Block

```
Source   DF       SS    MS     F      P
Block     5    11.19  2.24  0.42  0.837
Error   223  1199.16  5.38
Total   228  1210.35

S = 2.319   R-Sq = 0.92%   R-Sq(adj) = 0.00%
```



```
                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  -----+---------+---------+---------+----
1      45  5.556  2.201        (-----------*----------)
2      41  5.878  2.315             (-----------*-----------)
3      36  5.528  2.710      (------------*------------)
4      37  5.811  1.970           (------------*-----------)
5      34  5.853  2.401           (-------------*------------)
6      36  5.250  2.298  (-----------*------------)
                         -----+---------+---------+---------+----
                            4.80      5.40      6.00      6.60

Pooled StDev = 2.319


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Block

Individual confidence level = 99.56%
```

Table A. 17. Sensory analysis of 77 panellists

Panellists	Block	Formulation	Appearance	Texture	Sweetness	Sourness	Flavour	Overall acceptability
1	1	1	7	7	7	5	7	7
3	3	1	8	6	8	7	7	7
5	3	1	7	8	7	8	7	8
7	2	1	8	6	6	6	6	7
8	5	1	8	8	8	8	8	7
10	6	1	8	8	7	5	6	7
11	5	1	9	8	9	9	9	9
12	4	1	8	8	8	8	8	8
13	5	1	5	7	6	5	6	6
15	6	1	8	8	8	7	9	8
16	3	1	5	8	8	8	7	6
17	2	1	6	8	7	6	8	7
18	2	1	8	8	8	8	8	8
19	6	1	7	4	8	8	7	7
20	3	1	8	7	7	5	6	7
21	5	1	7	7	7	8	8	8
22	6	1	7	7	8	8	7	7
23	5	1	8	7	8	7	6	8
24	6	1	8	8	7	7	8	8

25	2	1	7	8	6	7	7	7
27	5	1	7	7	8	5	8	8
28	1	1	8	8	9	9	9	9
29	1	1	8	9	8	9	9	9
30	4	1	8	7	7	7	9	8
32	4	1	8	8	9	8	8	8
33	5	1	7	7	8	5	8	8
34	4	1	8	8	4	7	7	7
35	2	1	7	8	8	8	8	8
36	4	1	8	8	7	7	6	7
37	3	1	7	6	9	7	8	8
38	1	1	6	6	8	5	5	7
39	3	1	9	9	9	9	9	9
40	3	1	9	7	8	9	9	8
41	6	1	7	6	8	6	6	7
42	1	1	7	6	7	6	7	7
43	4	1	9	9	9	9	9	9
44	6	1	9	8	9	5	7	8
45	1	1	8	8	8	8	9	9
46	4	1	7	5	8	8	6	7
47	3	1	7	4	3	8	7	7
48	3	1	8	6	6	4	8	7
49	6	1	7	8	8	7	4	6
50	6	1	8	8	7	8	8	8
51	1	1	7	6	8	5	7	7
52	1	1	6	5	8	7	8	8
55	1	1	8	8	8	8	8	9
56	3	1	8	8	8	8	7	8
57	5	1	7	7	9	9	9	8
57	5	1	8	8	6	4	5	6
58	4	1	7	7	7	6	7	8
59	5	1	8	7	9	9	9	8
60	2	1	9	9	9	9	9	9
62	1	1	7	7	7	7	7	7
63	2	1	9	9	9	9	9	9
64	2	1	7	7	7	7	7	8
66	3	1	9	9	9	9	9	9
70	2	1	5	6	8	7	6	6
71	4	1	4	4	4	5	5	6
73	6	1	9	7	9	9	9	9
74	4	1	5	7	7	4	8	7

75	5	1	7	4	8	8	8	8
76	3	1	8	7	9	9	9	8
77	2	1	7	7	8	6	8	8
79	1	1	8	6	4	5	8	7
80	5	1	7	8	7	8	8	7
1	1	2	7	7	4	6	4	6
5	3	2	8	6	6	7	6	6
9	1	2	8	7	8	9	6	8
15	6	2	8	8	7	7	7	7
18	2	2	8	7	7	6	6	7
22	6	2	7	6	7	7	6	6
26	4	2	7	8	8	7	6	7
27	5	2	5	7	5	6	7	6
29	1	2	9	8	9	8	8	8
30	4	2	8	7	3	6	5	7
32	4	2	6	7	5	8	6	6
33	5	2	8	8	2	3	7	7
37	3	2	8	7	9	8	7	8
40	3	2	9	8	7	8	7	8
43	4	2	8	8	6	6	4	6
57	5	2	7	7	5	5	5	6
60	2	2	9	8	6	6	7	7
62	1	2	7	7	6	4	3	6
75	5	2	7	7	6	6	4	7
77	2	2	7	8	4	5	5	6
78	6	2	9	9	9	8	8	8
1	1	3	7	7	7	6	6	6.5
2	2	3	6	6	7	6	6	6
3	3	3	8	7	7	8	6	7
4	4	3	6	6	7	7	6	7
5	3	3	7	8	8	8	7	8
6	1	3	7	6	7	7	6	6
7	2	3	7	6	6	7	6	7
8	5	3	8	8	8	8	6	6
9	1	3	5	7	7	7	6	6
10	6	3	8	7	9	9	8	8
11	5	3	8	8	6	4	6	6
15	6	3	6	7	5	6	7	6
17	2	3	7	7	7	6	7	7
18	2	3	8	7	8	9	9	8
20	3	3	7	7	7	5	6	6

21	5	3	5	7	8	7	7	7
23	5	3	7	7	7	3	4	6
25	2	3	7	5	7	7	8	7
26	4	3	7	7	8	6	5	7
27	5	3	7	6	6	6	7	6
28	1	3	8	8	7	7	7	7
29	1	3	8	8	7	8	7	7
30	4	3	4	8	4	6	5	8
32	4	3	7	7	8	8	7	7
33	5	3	8	8	7	6	8	8
34	4	3	7	7	8	7	8	7
35	2	3	6	7	7	6	7	7
37	3	3	9	9	9	9	9	9
38	1	3	6	8	9	6	8	9
39	3	3	6	6	6	6	6	6
40	3	3	8	8	9	9	7	8
42	1	3	7	6	8	7	7	7
43	4	3	9	9	8	7	9	9
46	4	3	7	8	7	8	7	7
48	3	3	9	8	8	8	4	8
50	6	3	7	7	7	7	7	7
52	1	3	7	7	6	4	6	6
54	1	3	7	8	8	8	6	7
55	1	3	7	8	7	5	6	7
59	5	3	7	9	6	7	5	7
60	2	3	9	9	9	9	9	9
61	2	3	7	7	7	6	6	7
63	2	3	9	9	9	9	9	9
64	2	3	6	6	6	5	7	7
66	3	3	9	9	9	9	9	9
67	2	3	6	5	4	3	5	6
70	2	3	6	6	6	6	6	6
71	4	3	6	6	6	6	6	6
73	6	3	9	9	7	7	7	7
74	4	3	5	8	6	4	8	7
77	2	3	7	8	7	7	7	8
78	6	3	7	6	7	7	7	7
79	1	3	8	7	6	7	6	7
81	1	3	7	5	7	8	7	7
2	2	1	7	6	6	7	4	5
4	4	1	7	6	4	5	5	5

6	1	1	7	8	4	3	8	4
9	1	1	6	6	6	6	6	5
26	4	1	7	6	3	5	7	5
54	1	1	7	7	3	6	4	4
61	2	1	6	6	7	6	1	4
67	2	1	5	4	2	3	5	5
72	6	1	6	6	5	5	5	5
81	1	1	5	4	4	3	3	3
2	2	2	6	7	3	3	6	4
3	3	2	8	6	5	4	5	5
4	4	2	6	4	4	5	4	4
6	1	2	7	8	2	3	5	3
7	2	2	3	3	3	3	2	3
8	5	2	7	8	4	3	2	4
10	6	2	7	8	7	8	4	5
11	5	2	8	7	4	2	7	3
13	5	2	4	7	3	3	2	3
17	2	2	7	6	4	5	3	5
20	3	2	5	5	3	2	2	3
21	5	2	7	7	4	6	6	4
23	5	2	7	4	3	2	1	3
24	6	2	5	7	4	4	4	4
25	2	2	3	3	4	4	4	3
35	2	2	4	4	5	4	4	4
36	4	2	3	3	3	4	4	4
38	1	2	5	5	5	5	6	5
39	3	2	5	7	5	4	3	4
42	1	2	8	3	2	4	2	4
44	6	2	8	7	4	4	4	4
45	1	2	6	7	4	4	3	3
48	3	2	4	8	5	2	1	3
49	6	2	5	5	4	6	5	5
50	6	2	3	3	3	3	3	3
51	1	2	4	4	3	2	2	3
52	1	2	4	4	3	3	2	3
54	1	2	6	6	7	3	4	5
55	1	2	5	7	4	3	4	4
56	3	2	8	8	8	4	5	5
59	5	2	7	4	2	5	3	4
61	2	2	8	7	6	5	7	5
64	2	2	5	6	3	3	3	3

67	2	2	7	5	1	9	5	5
71	4	2	5	5	5	5	5	5
72	6	2	3	3	3	3	3	3
73	6	2	9	9	1	1	1	3
74	4	2	4	3	2	7	4	3
80	5	2	4	3	3	2	2	3
12	4	3	9	3	8	8	8	3
13	5	3	6	7	4	4	4	4
16	3	3	6	6	4	4	3	3
19	6	3	7	5	4	6	3	4
22	6	3	7	7	6	5	6	5
24	6	3	7	7	5	5	4	4
36	4	3	2	3	4	4	3	4
41	6	3	4	4	2	3	3	3
44	6	3	7	3	4	5	3	3
45	1	3	7	8	4	4	4	4
47	3	3	7	2	4	7	3	5
49	6	3	6	6	3	3	4	4
51	1	3	7	4	6	4	6	5
56	3	3	8	8	6	8	4	5
58	4	3	7	7	4	4	4	5
62	1	3	3	7	2	2	2	3
72	6	3	6	6	5	5	5	5
75	5	3	7	7	4	4	3	4
80	5	3	7	8	6	8	2	4
78	6	1	5	6	3	5	1	1
12	4	2	8	2	8	8	3	2
16	3	2	5	7	2	2	1	2
19	6	2	4	3	1	5	2	2
28	1	2	7	7	2	2	2	2
34	4	2	4	5	2	2	2	2
41	6	2	1	1	1	1	1	1
46	4	2	5	6	2	3	2	2
47	3	2	4	4	1	1	1	2
58	4	2	7	7	3	3	3	2
63	2	2	2	1	1	1	1	1
66	3	2	1	1	1	1	1	1
70	2	2	1	1	1	1	1	1
76	3	2	8	8	2	2	2	2
79	1	2	6	7	2	5	1	2
81	1	2	5	4	2	3	3	1

76	3	3	8	8	1	1	1	2

H. Statistical Analysis for Consumer Acceptance

Non-parametric Data

Table A. 18. Kruskal-Wallis test of consumer acceptance of selected yogurt formulations

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Appearance is the same across categories of Formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
2	The distribution of Texture is the same across categories of Formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
3	The distribution of sweetness is the same across categories of Formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
4	The distribution of sourness is the same across categories of Formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
5	The distribution of flavour is the same across categories of Formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.
6	The distribution of overllacceptability is the same across categories of Formulation.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Ranks

	Formulation	N	Mean Rank
Appearance	1	77	134.74
	2	77	92.89
	3	75	117.43
	Total	229	
Texture	1	77	129.19
	2	77	91.71
	3	75	124.35

	Total	229	
sweetness	1	77	152.30
	2	77	67.49
	3	75	125.48
	Total	229	
sourness	1	77	143.78
	2	77	74.93
	3	75	126.59
	Total	229	
flavour	1	77	158.55
	2	77	66.43
	3	75	120.15
	Total	229	
overllacceptability	1	77	156.44
	2	77	66.12
	3	75	122.65
	Total	229	

Test Statistics[a,b]

	Appearance	Texture	sweetness	sourness	flavour	overllacceptability
Chi-square	16.443	15.295	67.245	45.819	76.539	74.922
df	2	2	2	2	2	2
Asymp. Sig.	.000	.000	.000	.000	.000	.000

a. Kruskal Wallis Test

b. Grouping Variable: Formulation

```
*Nonparametric Tests: Independent Samples.
NPTESTS
  /INDEPENDENT TEST (Appearance Texture sweetness sourness flavour
overllacceptability) GROUP (Formulation)
  /MISSING SCOPE=ANALYSIS USERMISSING=EXCLUDE
  /CRITERIA ALPHA=0.05  CILEVEL=95.
```

Table A. 19. Multiple comparisons using Mann-Whitney test of selected yogurt formulations

Mann-Whitney Test

Ranks

	Formulation	N	Mean Rank	Sum of Ranks
overllacceptability	HFHS	77	89.49	6890.50
	LFLS	75	63.17	4737.50
	Total	152		
Appearance	HFHS	77	82.77	6373.00
	LFLS	75	70.07	5255.00
	Total	152		
Texture	HFHS	77	78.03	6008.50
	LFLS	75	74.93	5619.50
	Total	152		
sweetness	HFHS	77	87.38	6728.00
	LFLS	75	65.33	4900.00
	Total	152		
sourness	HFHS	77	82.84	6379.00
	LFLS	75	69.99	5249.00
	Total	152		
flavour	HFHS	77	91.05	7011.00
	LFLS	75	61.56	4617.00
	Total	152		

Test Statistics[a]

	overllacceptability	Appearance	Texture	sweetness	sourness	flavour
Mann-Whitney U	1887.500	2405.000	2769.500	2050.000	2399.000	1767.000
Wilcoxon W	4737.500	5255.000	5619.500	4900.000	5249.000	4617.000
Z	-3.778	-1.858	-.448	-3.150	-1.825	-4.195
Asymp. Sig. (2-tailed)	.000	.063	.654	.002	.068	.000

a. Grouping Variable: Formulation

```
NPAR TESTS
  /M-W= overllacceptability Appearance Texture sweetness sourness flavour BY
Formulation(HFHS HFN)
  /MISSING ANALYSIS.
```

Ranks

	Formulation	N	Mean Rank	Sum of Ranks
overllacceptability	HFHS	77	105.95	8158.00
	HFN	77	49.05	3777.00

	Total	154		
Appearance	HFHS	77	90.97	7005.00
	HFN	77	64.03	4930.00
	Total	154		
Texture	HFHS	77	90.16	6942.00
	HFN	77	64.84	4993.00
	Total	154		
sweetness	HFHS	77	103.92	8002.00
	HFN	77	51.08	3933.00
	Total	154		
sourness	HFHS	77	99.94	7695.00
	HFN	77	55.06	4240.00
	Total	154		
flavour	HFHS	77	106.50	8200.50
	HFN	77	48.50	3734.50
	Total	154		

Test Statistics[a]

	overllacceptability	Appearance	Texture	sweetness	sourness	flavour
Mann-Whitney U	774.000	1927.000	1990.000	930.000	1237.000	731.500
Wilcoxon W	3777.000	4930.000	4993.000	3933.000	4240.000	3734.500
Z	-8.001	-3.844	-3.604	-7.417	-6.294	-8.131
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.000	.000

a. Grouping Variable: Formulation

```
NPAR TESTS
  /M-W= overllacceptability Appearance Texture sweetness sourness flavour BY
Formulation(HFN LFLS)
  /MISSING ANALYSIS.
```

Ranks

	Formulation	N	Mean Rank	Sum of Ranks
overllacceptability	HFN	77	56.06	4317.00
	LFLS	75	97.48	7311.00
	Total	152		
Appearance	HFN	77	67.86	5225.50
	LFLS	75	85.37	6402.50

		N	Mean Rank	Sum of Ranks
	Total	152		
Texture	HFN	77	65.86	5071.50
	LFLS	75	87.42	6556.50
	Total	152		
sweetness	HFN	77	55.42	4267.00
	LFLS	75	98.15	7361.00
	Total	152		
sourness	HFN	77	58.86	4532.50
	LFLS	75	94.61	7095.50
	Total	152		
flavour	HFN	77	56.93	4383.50
	LFLS	75	96.59	7244.50
	Total	152		

Test Statistics[a]

	overllacceptability	Appearance	Texture	sweetness	sourness	flavour
Mann-Whitney U	1314.000	2222.500	2068.500	1264.000	1529.500	1380.500
Wilcoxon W	4317.000	5225.500	5071.500	4267.000	4532.500	4383.500
Z	-5.862	-2.510	-3.092	-6.035	-5.048	-5.610
Asymp. Sig. (2-tailed)	.000	.012	.002	.000	.000	.000

a. Grouping Variable: Formulation

Parametric Data

Table A. 20. Consumer acceptance of parametric data

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Appearance	Between Groups	65.353	2	32.677	13.308	.000
	Within Groups	554.926	226	2.455		
	Total	620.279	228			
Texture	Between Groups	69.756	2	34.878	12.371	.000
	Within Groups	637.179	226	2.819		
	Total	706.934	228			
sweetness	Between Groups	376.823	2	188.411	50.559	.000
	Within Groups	842.199	226	3.727		
	Total	1219.022	228			
sourness	Between Groups	230.295	2	115.148	31.092	.000

		Sum of Squares	df	Mean Square	F	Sig.
	Within Groups	836.971	226	3.703		
	Total	1067.266	228			
flavour	Between Groups	402.150	2	201.075	56.227	.000
	Within Groups	808.199	226	3.576		
	Total	1210.349	228			
overllacceptability	Between Groups	335.008	2	167.504	56.151	.000
	Within Groups	674.180	226	2.983		
	Total	1009.188	228			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Formulation	(J) Formulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Appearance	1	2	1.260*	.253	.000	.66	1.86
		3	.339	.254	.377	-.26	.94
	2	1	-1.260*	.253	.000	-1.86	-.66
		3	-.920*	.254	.001	-1.52	-.32
	3	1	-.339	.254	.377	-.94	.26
		2	.920*	.254	.001	.32	1.52
Texture	1	2	1.234*	.271	.000	.60	1.87
		3	.147	.272	.852	-.50	.79
	2	1	-1.234*	.271	.000	-1.87	-.60
		3	-1.087*	.272	.000	-1.73	-.44
	3	1	-.147	.272	.852	-.79	.50
		2	1.087*	.272	.000	.44	1.73
sweetness	1	2	3.013*	.311	.000	2.28	3.75
		3	.771*	.313	.039	.03	1.51
	2	1	-3.013*	.311	.000	-3.75	-2.28
		3	-2.242*	.313	.000	-2.98	-1.50
	3	1	-.771*	.313	.039	-1.51	-.03
		2	2.242*	.313	.000	1.50	2.98
sourness	1	2	2.338*	.310	.000	1.61	3.07
		3	.541	.312	.196	-.20	1.28

	2	1	-2.338*	.310	.000	-3.07	-1.61
		3	-1.797*	.312	.000	-2.53	-1.06
	3	1	-.541	.312	.196	-1.28	.20
		2	1.797*	.312	.000	1.06	2.53
flavour	1	2	3.195*	.305	.000	2.48	3.91
		3	1.171*	.307	.001	.45	1.89
	2	1	-3.195*	.305	.000	-3.91	-2.48
		3	-2.024*	.307	.000	-2.75	-1.30
	3	1	-1.171*	.307	.001	-1.89	-.45
		2	2.024*	.307	.000	1.30	2.75
overllacceptability	1	2	2.883*	.278	.000	2.23	3.54
		3	.897*	.280	.004	.24	1.56
	2	1	-2.883*	.278	.000	-3.54	-2.23
		3	-1.987*	.280	.000	-2.65	-1.33
	3	1	-.897*	.280	.004	-1.56	-.24
		2	1.987*	.280	.000	1.33	2.65

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Appearance

Tukey HSD[a,b]

Formulation	N	Subset for alpha = 0.05	
		1	2
2	77	6.01	
3	75		6.93
1	77		7.27
Sig.		1.000	.376

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 76.322.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

I. Consumer Segmentation using Cluster Analysis

Cluster Analysis of Observations: Panellists, Overall acceptability

Euclidean Distance, Centroid Linkage
Amalgamation Steps

Step	Number of clusters	Similarity level	Distance level	Clusters joined		New cluster	Number of obs. in new cluster
1	224	100.000	0.0000	74	223	74	2
2	223	100.000	0.0000	72	221	72	2
3	222	100.000	0.0000	146	220	146	2
4	221	100.000	0.0000	69	218	69	2
5	220	100.000	0.0000	67	216	67	2
6	219	100.000	0.0000	66	215	66	2
7	218	100.000	0.0000	65	214	65	2
8	217	100.000	0.0000	63	212	63	2
9	216	100.000	0.0000	61	210	61	2
10	215	100.000	0.0000	58	207	58	2
11	214	100.000	0.0000	129	204	129	2
12	213	100.000	0.0000	44	195	44	2
13	212	100.000	0.0000	41	192	41	2
14	211	100.000	0.0000	40	191	40	2
15	210	100.000	0.0000	114	189	114	2
16	209	100.000	0.0000	110	185	110	2
17	208	100.000	0.0000	32	183	32	2
18	207	100.000	0.0000	31	182	31	2
19	206	100.000	0.0000	29	180	29	2
20	205	100.000	0.0000	102	177	102	2
21	204	100.000	0.0000	101	176	101	2
22	203	100.000	0.0000	24	175	24	2
23	202	100.000	0.0000	99	174	99	2
24	201	100.000	0.0000	17	168	17	2
25	200	100.000	0.0000	16	167	16	2
26	199	100.000	0.0000	7	158	7	2
27	198	100.000	0.0000	5	156	5	2
28	197	100.000	0.0000	3	154	3	2
29	196	100.000	0.0000	64	139	64	2
30	195	100.000	0.0000	55	130	55	2
31	194	100.000	0.0000	38	114	38	3
32	193	100.000	0.0000	35	111	35	2
33	192	99.377	0.5000	77	152	77	2
34	191	99.221	0.6250	1	77	1	3
35	190	98.809	0.9552	1	153	1	4
36	189	98.754	1.0000	75	225	75	2
37	188	98.754	1.0000	150	224	150	2
38	187	98.807	0.9571	76	150	76	3
39	186	98.754	1.0000	148	222	148	2
40	185	98.807	0.9571	74	148	74	4
41	184	98.754	1.0000	69	217	69	3
42	183	98.754	1.0000	64	213	64	3
43	182	98.754	1.0000	62	211	62	2
44	181	98.754	1.0000	133	208	133	2
45	180	98.754	1.0000	57	206	57	2
46	179	98.807	0.9571	56	57	56	3
47	178	98.754	1.0000	202	203	202	2
48	177	98.754	1.0000	49	199	49	2
49	176	98.807	0.9571	48	49	48	3
50	175	98.754	1.0000	123	198	123	2
51	174	98.754	1.0000	46	197	46	2
52	173	98.807	0.9571	45	46	45	3
53	172	98.754	1.0000	119	194	119	2
54	171	98.807	0.9571	119	193	119	3

55	170	99.054	0.7587	118	119	118	4
56	169	98.754	1.0000	186	187	186	2
57	168	98.807	0.9571	35	186	35	4
58	167	98.754	1.0000	34	184	34	2
59	166	98.807	0.9571	33	34	33	3
60	165	98.754	1.0000	107	181	107	2
61	164	98.807	0.9571	106	107	106	3
62	163	98.754	1.0000	178	179	178	2
63	162	98.807	0.9571	104	178	104	3
64	161	98.754	1.0000	97	173	97	2
65	160	98.807	0.9571	97	172	97	3
66	159	98.754	1.0000	21	171	21	2
67	158	98.807	0.9571	20	21	20	3
68	157	98.754	1.0000	19	170	19	2
69	156	98.807	0.9571	18	19	18	3
70	155	98.754	1.0000	91	166	91	2
71	154	98.754	1.0000	90	165	90	2
72	153	98.807	0.9571	15	90	15	3
73	152	98.754	1.0000	89	164	89	2
74	151	98.807	0.9571	89	163	89	3
75	150	98.754	1.0000	85	161	85	2
76	149	98.807	0.9571	10	85	10	3
77	148	98.754	1.0000	159	160	159	2
78	147	98.807	0.9571	9	159	9	3
79	146	98.754	1.0000	81	157	81	2
80	145	98.754	1.0000	3	155	3	3
81	144	98.754	1.0000	70	145	70	2
82	143	98.807	0.9571	70	71	70	3
83	142	98.754	1.0000	143	144	143	2
84	141	98.754	1.0000	67	141	67	3
85	140	98.754	1.0000	60	135	60	2
86	139	98.754	1.0000	59	134	59	2
87	138	98.754	1.0000	51	128	51	2
88	137	98.807	0.9571	51	127	51	3
89	136	98.754	1.0000	125	126	125	2
90	135	98.754	1.0000	120	121	120	2
91	134	98.754	1.0000	109	110	109	3
92	133	98.754	1.0000	29	105	29	3
93	132	98.850	0.9223	29	104	29	6
94	131	98.754	1.0000	24	101	24	4
95	130	98.754	1.0000	17	93	17	3
96	129	98.754	1.0000	82	83	82	2
97	128	98.807	0.9571	6	82	6	3
98	127	98.754	1.0000	4	80	4	2
99	126	98.807	0.9571	4	79	4	3
100	125	98.754	1.0000	2	78	2	2
101	124	98.754	1.0000	65	66	65	4
102	123	98.754	1.0000	53	54	53	2
103	122	98.754	1.0000	39	40	39	3
104	121	98.754	1.0000	30	31	30	3
105	120	98.850	0.9223	30	106	30	6
106	119	98.754	1.0000	27	28	27	2
107	118	98.754	1.0000	22	23	22	2
108	117	98.754	1.0000	7	8	7	3
109	116	98.686	1.0539	16	17	16	5
110	115	98.639	1.0920	32	33	32	5
111	114	98.570	1.1469	65	67	65	7
112	113	98.559	1.1563	27	29	27	8
113	112	98.551	1.1626	60	133	60	4
114	111	98.541	1.1707	88	89	88	4
115	110	98.556	1.1585	87	88	87	5
116	109	98.541	1.1707	9	86	9	4
117	108	98.523	1.1850	2	4	2	5

118	107	98.517	1.1898	18	20	18	6
119	106	98.442	1.2500	142	143	142	3
120	105	98.442	1.2500	124	125	124	3
121	104	98.442	1.2500	47	123	47	3
122	103	98.437	1.2539	69	70	69	6
123	102	98.308	1.3573	72	74	72	6
124	101	98.297	1.3660	44	45	44	5
125	100	98.297	1.3660	14	15	14	4
126	99	98.253	1.4018	35	36	35	5
127	98	98.237	1.4142	132	205	132	2
128	97	98.237	1.4142	200	201	200	2
129	96	98.237	1.4142	116	190	116	2
130	95	98.237	1.4142	112	188	112	2
131	94	98.313	1.3536	112	113	112	3
132	93	98.237	1.4142	95	169	95	2
133	92	98.313	1.3536	95	96	95	3
134	91	98.237	1.4142	73	149	73	2
135	90	98.237	1.4142	55	129	55	4
136	89	98.237	1.4142	25	102	25	3
137	88	98.307	1.3585	24	25	24	7
138	87	98.237	1.4142	99	100	99	3
139	86	98.386	1.2952	98	99	98	4
140	85	98.237	1.4142	42	43	42	2
141	84	98.313	1.3536	41	42	41	4
142	83	98.237	1.4142	37	38	37	4
143	82	98.237	1.4142	11	12	11	2
144	81	98.208	1.4372	18	97	18	9
145	80	98.199	1.4447	30	32	30	11
146	79	98.187	1.4542	10	11	10	5
147	78	98.185	1.4559	24	26	24	8
148	77	98.173	1.4659	39	117	39	4
149	76	98.171	1.4676	9	84	9	5
150	75	98.160	1.4759	72	147	72	7
151	74	98.125	1.5041	48	50	48	4
152	73	98.180	1.4598	48	200	48	6
153	72	98.075	1.5444	94	95	94	4
154	71	98.037	1.5751	59	209	59	3
155	70	98.037	1.5751	120	122	120	3
156	69	98.037	1.5751	61	62	61	4
157	68	98.037	1.5751	52	53	52	3
158	67	98.196	1.4474	52	202	52	5
159	66	98.027	1.5828	56	58	56	5
160	65	98.009	1.5976	56	60	56	9
161	64	98.000	1.6045	7	9	7	8
162	63	97.975	1.6249	10	162	10	6
163	62	97.979	1.6209	7	10	7	14
164	61	97.951	1.6441	55	132	55	6
165	60	97.924	1.6653	1	2	1	9
166	59	98.352	1.3220	1	3	1	12
167	58	97.882	1.6994	14	16	14	9
168	57	97.954	1.6416	14	92	14	10
169	56	97.865	1.7129	47	196	47	4
170	55	97.838	1.7348	44	47	44	9
171	54	97.819	1.7497	35	37	35	9
172	53	97.723	1.8264	142	219	142	4
173	52	97.713	1.8350	72	75	72	9
174	51	97.712	1.8356	68	69	68	7
175	50	97.671	1.8680	5	81	5	4
176	49	97.724	1.8256	1	5	1	16
177	48	97.642	1.8920	18	22	18	11
178	47	97.628	1.9033	39	41	39	8
179	46	97.605	1.9214	73	76	73	5
180	45	97.885	1.6967	73	151	73	6

181	44	97.558	1.9592	118	120	118	7
182	43	97.531	1.9804	44	48	44	15
183	42	97.530	1.9814	52	55	52	11
184	41	97.751	1.8044	51	52	51	14
185	40	97.369	2.1103	24	27	24	16
186	39	97.330	2.1417	35	112	35	12
187	38	97.223	2.2276	115	116	115	3
188	37	97.213	2.2361	136	137	136	2
189	36	97.377	2.1045	59	136	59	5
190	35	97.199	2.2473	61	63	61	6
191	34	97.178	2.2636	142	146	142	6
192	33	96.997	2.4088	108	109	108	4
193	32	96.843	2.5324	18	94	18	15
194	31	96.955	2.4430	18	98	18	19
195	30	96.774	2.5879	44	124	44	18
196	29	96.772	2.5896	13	87	13	6
197	28	96.702	2.6457	51	56	51	23
198	27	96.682	2.6621	35	39	35	20
199	26	96.669	2.6725	68	72	68	16
200	25	96.490	2.8161	7	13	7	20
201	24	96.486	2.8191	44	118	44	25
202	23	96.417	2.8746	1	6	1	19
203	22	96.314	2.9573	30	108	30	15
204	21	96.292	2.9750	65	142	65	13
205	20	96.278	2.9857	65	68	65	29
206	19	96.034	3.1815	14	18	14	29
207	18	96.034	3.1820	35	115	35	23
208	17	95.935	3.2608	61	64	61	9
209	16	95.761	3.4007	51	59	51	28
210	15	96.310	2.9607	51	131	51	29
211	14	95.551	3.5694	14	91	14	31
212	13	95.347	3.7326	30	35	30	38
213	12	95.280	3.7870	1	7	1	39
214	11	95.164	3.8800	65	73	65	35
215	10	95.014	4.0000	138	140	138	2
216	9	94.828	4.1495	51	61	51	38
217	8	94.605	4.3278	24	103	24	17
218	7	93.874	4.9143	14	24	14	48
219	6	92.752	5.8144	65	138	65	37
220	5	91.973	6.4396	30	44	30	63
221	4	87.319	10.1730	51	65	51	75
222	3	87.303	10.1860	1	14	1	87
223	2	79.376	16.5452	30	51	30	138
224	1	67.168	26.3395	1	30	1	225

Final Partition
Number of clusters: 3

	Number of observations	Within cluster sum of squares	Average distance from centroid	Maximum distance from centroid
Cluster1	87	7034.28	8.05444	14.5965
Cluster2	63	2603.94	5.85196	10.4495
Cluster3	75	5861.04	8.10088	14.2693

Cluster Centroids

Variable	Cluster1	Cluster2	Cluster3	Grand centroid
Panellists	15.5517	42.0000	67.52	40.2800

```
Overall acceptability    5.9253    6.0317      5.68    5.8733


Distances Between Cluster Centroids

           Cluster1  Cluster2  Cluster3
Cluster1     0.0000   26.4485   51.9689
Cluster2    26.4485    0.0000   25.5224
Cluster3    51.9689   25.5224    0.0000
```

J. Accelerated Shelf-life

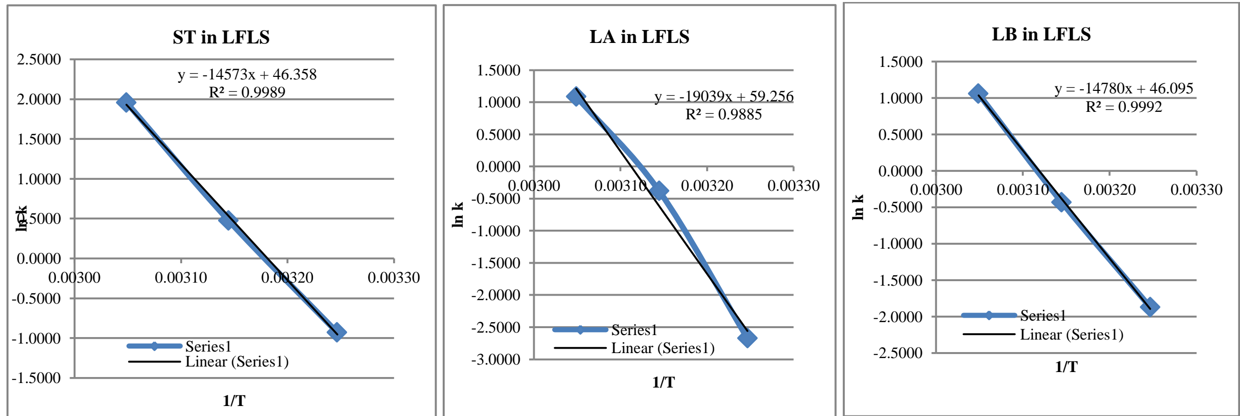


Figure A. 4. The Arrhenius relationship between bacteria thermal reduction and temperature of LFLS formulation.

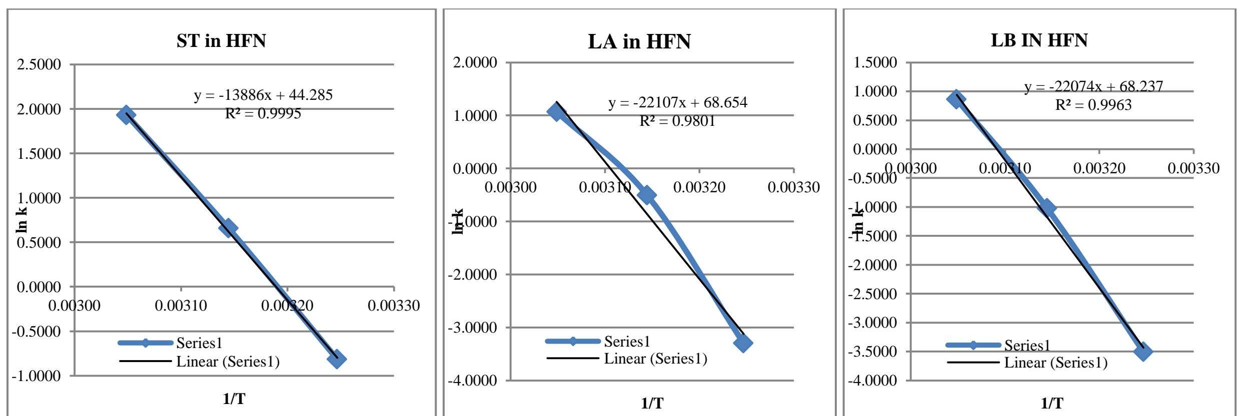


Figure A. 5. The Arrhenius relationship between bacteria thermal reduction and temperature of HFN formulation.

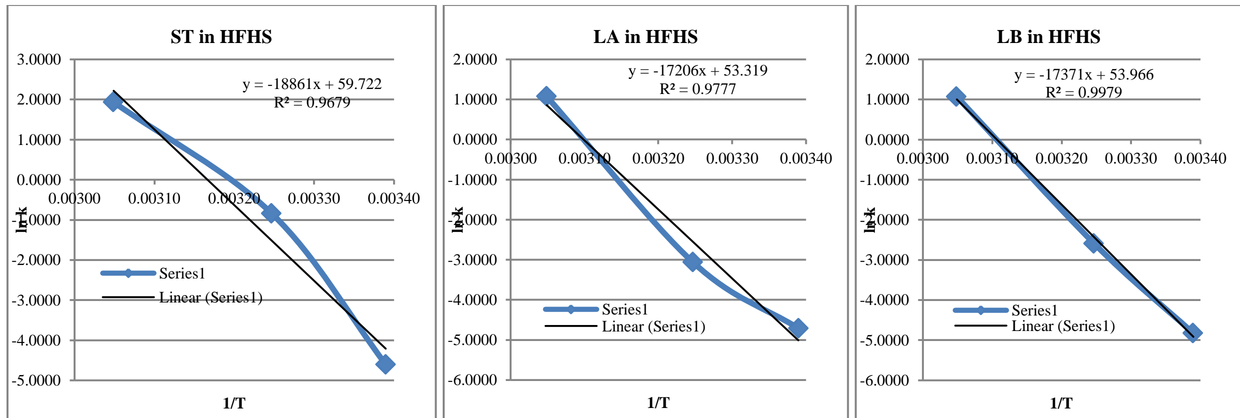


Figure A. 6. The Arrhenius relationship between bacteria thermal reduction and temperature of HFHS formulation.

Raw data of accelerated shelf life

Samples	Temperature	Day	ST average	LA average	LB average
LFLS	4	0	2150000	2.05E+08	25000000
LFLS	4	7	315000	2.25E+08	25000000
LFLS	4	13	3450000	1.9E+08	1.05E+08
LFLS	4	21	440000	4.05E+08	85000000
LFLS	4	34	935000	3.95E+08	22500000
LFLS	4	49	1657500	5.63E+08	1.03E+08
LFLS	4	56	775000	3.15E+08	7.58E+08
LFLS	4	83	600000	3.3E+08	26666667
LFLS	22	0	2150000	2.05E+08	25000000
LFLS	22	7	1700000	1.85E+08	30000000
LFLS	22	13	2050000	2.8E+08	1.05E+08
LFLS	22	21	875000	3.3E+08	5000000
LFLS	22	34	102500	3.55E+08	50000000
LFLS	22	49	727500	7.28E+08	45000000
LFLS	22	56	327500	2.35E+08	52500000
LFLS	35	0	2150000	4.8E+08	65000000
LFLS	35	2	625000	2.05E+08	25000000
LFLS	35	4	385000	1.85E+08	24000000
LFLS	35	6	500000	1.51E+08	20000000
LFLS	35	13	20000	1.28E+08	15500000
LFLS	35	15	3250	1.2E+08	2500000
LFLS	45	0	2150000	2.05E+08	23000000
LFLS	45	1	5000	1.4E+08	14500000
LFLS	45	2	4400	71500000	7500000
LFLS	45	3	1850	46000000	4750000
LFLS	45	4	600	10400000	1430000

LFLS	45	5	115	8910000	1066000
LFLS	55	0	400000	3.3E+08	35666667
LFLS	55	0.0625	106666.7	1.9E+08	21866667
LFLS	55	0.125	65000	1.68E+08	20100000
LFLS	55	0.1875	17000	1.25E+08	13650000
LFLS	55	0.25	14666.67	92066667	9440000
LFLS	55	1	133.3333	13680000	1504000
LFLS	55	1.0625	100	10200000	1362000
HFN	4	0	2150000	4.25E+08	25000000
HFN	4	7	1350000	4.05E+08	95000000
HFN	4	13	700000	2.5E+08	1E+08
HFN	4	21	1250000	8.15E+08	15000000
HFN	4	34	732500	9.43E+08	1.05E+08
HFN	4	49	1887500	1.8E+09	3.08E+08
HFN	4	56	1955000	5.13E+08	1.05E+08
HFN	4	83	600000	4.17E+08	1.13E+08
HFN	22	0	2150000	4.25E+08	25000000
HFN	22	7	6600000	3.7E+08	35000000
HFN	22	13	260000	4.75E+08	2E+08
HFN	22	21	245000	8.05E+08	2.28E+08
HFN	22	34	1265000	6.63E+08	52500000
HFN	22	49	917500	1.12E+09	47500000
HFN	23	56	420000	5.1E+08	72500000
HFN	35	0	2150000	4.25E+08	45000000
HFN	35	2	3420000	3.2E+08	28000000
HFN	35	4	450000	2.53E+08	26200000
HFN	35	6	280000	2.38E+08	26100000
HFN	35	13	35000	1.95E+08	25000000
HFN	35	15	1950	2.3E+08	22400000
HFN	45	0	2150000	4.25E+08	25000000
HFN	45	1	1300	2.8E+08	25000000
HFN	45	2	500	83000000	6000000
HFN	45	3	80	53000000	4000000
HFN	45	4	50	31900000	6200000
HFN	45	5	30	25080000	5040000
HFN	55	0	566666.7	4.17E+08	1.47E+08
HFN	55	0.0625	16666.67	3.69E+08	1.33E+08
HFN	55	0.125	15000	2.87E+08	50000000
HFN	55	0.1875	60000	2.64E+08	37600000
HFN	55	0.25	3000	2.29E+08	30000000
HFN	55	1	100	21960000	8760000
HFN	55	1.0625	55	21000000	8660000
HFHS	4	0	5550000	2.55E+08	28000000

HFHS	4	7	3100000	1.7E+08	25500000
HFHS	4	13	3050000	2.2E+08	23500000
HFHS	4	21	1750000	3.05E+08	30000000
HFHS	4	34	4800000	4.25E+08	32500000
HFHS	4	49	4925000	3.23E+08	1.06E+08
HFHS	4	56	7400000	3.95E+08	44250000
HFHS	4	83	2500000	2.83E+08	40000000
HFHS	22	0	5550000	2.75E+08	39666667
HFHS	22	7	4750000	2.7E+08	31333333
HFHS	22	13	5850000	3.15E+08	36500000
HFHS	22	21	2950000	4.3E+08	37666667
HFHS	22	34	2325000	2.88E+08	36000000
HFHS	22	49	4400000	4.84E+08	57666667
HFHS	35	0	5550000	2.55E+08	29000000
HFHS	35	2	4550000	2.3E+08	23500000
HFHS	35	4	1075000	92500000	23000000
HFHS	35	6	350000	2.24E+08	14500000
HFHS	35	13	10000	95000000	10200000
HFHS	35	15	20500	1.28E+08	9600000
HFHS	55	0	2500000	2.83E+08	1.17E+08
HFHS	55	0.0625	36666.67	2.06E+08	29333333
HFHS	55	0.125	15000	2.03E+08	8500000
HFHS	55	0.1875	13500	1.22E+08	8000000
HFHS	55	0.25	6000	1.08E+08	7400000
HFHS	55	1	133.3333	14360000	1800000
HFHS	55	1.0625	100	10980000	1420000

Example of Sorption Isotherms of yogurt powder at different temperatures

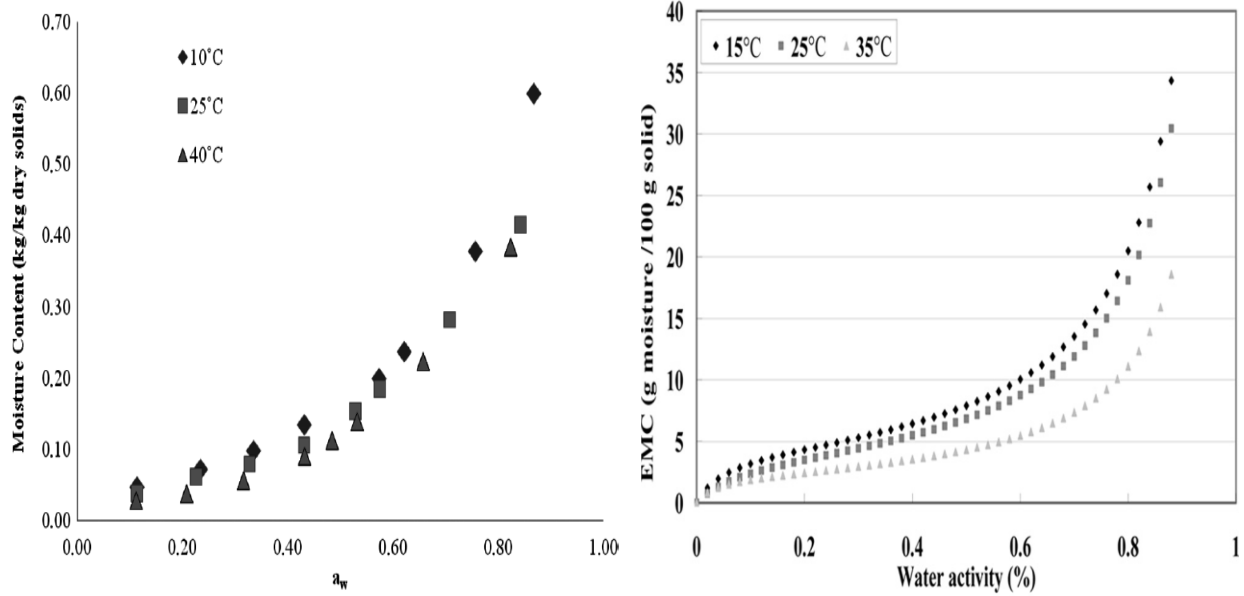


Figure A. 7. (Left) Adsorption isotherms of yogurt powder at different temperatures (Koç et al., 2010); (right) Adsorption isotherms of milk powder at different temperatures (Ko et al., 2008).

K. Sensory Questionnaire

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COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

INFORMATION SHEET

TITLE of Work: Development of Probiotics Rich Yogurt Dry Mix

Researcher(s) Introduction

Researchers Name:	Maureen F Kosasih	Supervisors Name:	Dr. Tony Mutukumira
Contact Details:	maureen_kosasih@hotmail.com	Contact Details:	a.n.mutukumira@massey.ac.nz

You are invited to take part in a consumer sensory evaluation to evaluate the probiotics rich yogurt.

The types of activities that this work involves includes *tasting* and *evaluating*

Your participation in this activity will take approximately *10 minutes*.

This work is sponsored by a commercial company that is likely to benefit from this work. Any money that Massey University receives for such work contributes to the teaching of students.

We are selecting people for this exercise who meet the following criteria:
Consume (any type) yogurt at least once a week

The foods you will be tasting contain the following components that can be harmful or cause allergic reactions with certain groups of people. You will be excluded from taking part if you are allergic, or may be adversely affected by any of the following:

- *Milk and milk derivatives*

The type of food that you will be testing is *yogurt* which contains following ingredients; milk, sugar, flavour, colour, and yogurt cultures (*Lactobacillus* and *S.thermophilus*).

The information collected in this study will be used to complete an assignment in partial fulfilment of the Master of Technology in Food Technology. No data linked to an individual's identity will be collected.

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- decline to answer any particular question;
- withdraw from the study (specify timeframe);
- ask any questions about the study at any time during participation;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be given access to a summary of the project findings when it is concluded.

If you have any questions about this work, please contact one of the people indicated above.

You are welcome to a summary of the results.

Please indicate if you wish to receive a summary of the results from this trial

YES ☐ NO ☐

- *"This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 09/39. If you have any concerns about the ethics of this research, please contact Professor Julie Boddy, Chair, Massey University Human Ethics Committee: Southern A telephone 06 350 5799 x 2541, email humanethicsoutha@massey.ac.nz."*

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CONSENT FORM

Development of Probiotics Rich Yogurt Dry Mix

CONSENT FORM

THIS CONSENT FORM WILL BE HELD FOR 3 YEARS

- I have read and understood the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.
- I agree to voluntarily participate in this study under the conditions set out in the Information Sheet.
- I understand I have the right to withdraw from the study at any time and to decline to answer any particular questions.
- I have advised and discussed the Researcher of any potentially relevant cultural, religious or ethical beliefs that may prevent me from consuming the Foods under consideration.

Participants Signature: .... **Date:** ....

Full Name - printed ....

Te Kunenga ki Pūrehuroa

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CONFIDENTIALITY AGREEMENT FOR PARTICIPANTS

Note: *This confidentiality agreement is to be applied when participants need to be provided information about the project that may need to be kept confidential for reasons of commercial sensitivity or to protect the views and opinions of individuals participating in focus groups from being disseminated to people not participating.*

Development of Probiotics Rich Yogurt Dry Mix

CONFIDENTIALITY AGREEMENT

I .... (Full Name - printed)

agree to keep confidential all information concerning the project ....

I will not retain or copy any information involving this project.

Signature: .... **Date:** ....

Full Name - printed ....

Initials: Product no. #

NOTE: EACH SAMPLE MUST BE EVALUATED ON A SEPARATE FORM

Please TICK THE BOX that best describes your feelings about this sample. *You are welcome to rinse your mouth with water before and between samples.*

1. How would you rate the **APPEARANCE** of this sample (visual)? You are welcome to stir the sample using the spoon provided.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Please comment: ....

2. How would you rate the **TEXTURE** of this sample?

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Please comment: ....

3. How would you rate the **SWEETNESS** of this sample?

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Please comment: ....

4. How would you rate the **SOURNESS** of this sample?

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Please comment: ....

5. How would you rate the **FLAVOUR** of this sample?

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Please comment: ....

6. How would you rate the **OVERALL ACCEPTABILITY** of this sample?

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

Please comment: ....

L. Phosphate Buffer for HPLC

A portion of 1.74g of K_2HPO_4, 12.37g KH_2PO_4, and 21.41g Na_2SO_4 were diluted with distilled water to 700 mL. A dropwise of phosphoric acid solution (85%) or potassium hydroxide solution (10 mol/L) was added to the solution to adjust the pH to 6.0. The solution was then diluted to 1000 mL and thoroughly mixed. Prior to use, the buffer solution was filtered through 0.45 μm membrane filter.