Improvement of a commercial probiotic product through metabolite profiling and quality assurance system development

---

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Applied Science

at

Lincoln University

by

Nagaiah Koneswaran

Lincoln University

2014

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Applied Science.

Improvement of a commercial probiotic product through metabolite profiling and quality assurance system development

by

Nagaiah Koneswaran

In recent years**,** use of probiotic formulation for animal health and nutrition has gained popularity to produce high quality animal based food products i.e. milk and meat. As a result, number of probiotic products in the market has increased. However, there is an increasing concern about the quality and safety of these products and the importance of ensuring quality control of the probiotics has widely been accepted. BioBrew Ltd produces liquid animal probiotics widely administered to the farm animals in New Zealand. In this study more than 300 samples at various stages of production were analysed. Sampling across New Zealand was carried out for a period of eight months (August 2013 to March 2014). BioBrew Animal Nutritional supplement (BBAN) is molasses fermented liquid formulation having multi-species of Lactic Acid Bacteria (LAB) and yeast cells.

Viability study was undertaken to enumerate LAB and yeast cell counts in BBAN using traditional plate count methods. Production involved three stages fermentation and viable LAB cell counts in stage 1, 2 and 3 differed significantly ($P < 0.05$). However, viable counts in stage 3 and final product with different storage days did not differ significantly ($P < 0.05$) and cell densities on average were found to be more than 10^9 CFU/ml in the product, which was higher than Minimum Suggested Level (MSL). On contrary, average yeast cell densities reached maximum in stage 1 and 2 (9.97 and 10.24 log CFU/ml respectively) dropped to 5.47 log CFU/ml at the end of storage period. On average, yeast cell counts in the product were just below the MSL, irrespective of product from different production sites.

Analysis confirmed that the product contained considerable amount of lactic acid and ethanol in BBAN. Headspace Solid-Phase Micro-Extraction Gas Chromatograph Mass Spectrometry (HS-SPME/GCMS) analysis was used to detect the semi-volatile and volatile metabolites in this probiotic formulation. Lactic acid, ethanol and butyric acid were primary metabolites detected in higher quantities in BBAN formulation. Lactic acid, acetic acid, ethanol, butyric acid, diacetyl were the important metabolites identified with antimicrobial properties.

Products were screened for potential foodborne pathogens and confirmed BBAN formulation were not detected positive for the presence of any bacterial pathogens over the period of study. However, contamination with yeast (*Rhodotorula rubra*) observed in 2 batches during the period of study. Favourable conditions for this detected yeast contamination were identified and were effectively controlled through measures suggested in this study.

Based on our study, model quality assurance (QA) system was proposed to produce consistent, high quality and safe BBAN. Model QA system includes products testing for pH, temperature, viable counts, safety assessment and presence of metabolites at different stages as well as during various storage days.

In summary, it was confirmed that BBAN met the quality control parameters. BBAN has lactic acid, butyric acid, ethanol, acetic acid and diacetyl as major bioactive compounds. BBAN batches tested were free from pathogenic contamination. This study suggested a model QA system to enhance quality and safety of the product.

Keywords: probiotics, BioBrew, BBAN, viable counts, colony forming unit (CFU), metabolite profiling, GCMS, SPME, metabolic compounds, chromatogram, internal standard, relative amount, pathogen, quality assurance (QA).

Acknowledgements

I wish to thank, first and foremost, my supervisor Dr. Malik Altaf Hussain for his guidance, encouragement, support and advice throughout the course of this study. I owe him a great debt of gratitude for allowing me to do research under him for this funded project with scholarship.

I am sincerely thankful to Dr. Craig Bunt for agreeing to co-supervise my project. Completing thesis writing would have been all the more difficult were it not for the critiques, valuable suggestions, and editing provided by him.

I cannot find words to express my gratitude to my associate supervisor, Don (Technical Director, BioBrew Ltd) and Andre Prassinos (Managing Director, BioBrew Ltd) for allowing me to work their product and getting funds for this project. Thanks to BioBrew Crew for always motivating, encouraging me and editing and giving positive feedback of my thesis.

My special thanks to Callaghan Innovation without their funding, this project would not have been possible.

Metabolite studies would not have been possible, hadn't I received the GCMS technical support from the Technical officer, Breitmeyer Jason. I share the credit of my GCMS work with Jason who taught me patiently all the techniques, guided and even edited my GCMS draft with his valuable feedback.

I owe my deepest gratitude to laboratory manager, Omega Amoafo who was very friendly and always approachable for any support. Also, I am thankful to Dr. Simon Hodge for his significant help with statistical analysis.

I am indebted to my colleagues and friends, for their moral support, who always wanted to see me in good position. Special thanks go to Arash Rafat who gave his valuable time for editing my final draft.

Last but not least, it gives me great pleasure in acknowledging the unconditional love and support from my little princesses (Thanoshika and Thaaranie), my wife (Sasikala) for being with me in all the ups and downs of my entire life and my family in Sri Lanka.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

°C	Degree Celsius
µl	micro litre
µm	micro metre
µg	microgram
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
AGP	Antibiotic growth promoters
Balc	Balclutha
BBAN	BioBrew Animal Nutrition
CAS	Chemical Abstracts Service
CFU	Colony Forming Unit
CE	Competitive exclusion
cm	centri metre
CO_2	Carbon dioxide
Ctr1	Control 1
Ctr2	Control 2
DGGE	Denaturing gradient gel electrophoresis
D-GPT D	Glutamate-pyruvate transaminase
D-LDH	D-Lactate dehydrogenase
EI	Electron impact mode
EFSA	European Food Safety Authority
FAO	Food and Agriculture
FDA	Food and Drug Administration
GC-MS	Gas Chromatography and mass spectrometry
GIT	Gastrointestinal tract
g	gram
GRAS	Generally regarded as safe
HS	Headspace
IBC	Intermediate bulk container
IBD	Irritable bowel syndrome
ID	Internal diameter
IS	Internal Standard
KI	Kovats index
LAB	Lactic acid bacteria
Lb.	Lactobacillus
l	litre
L-LDH	L-Lactate dehydrogenase
L3	Lot 3
m	metre
mm	milli metre
mg	milli gram
ml	milli litre
MSL	Minimum suggested level
MRS	De Man, Rogosa and Sharpes
NAD^+	Nicotinamide-adenine dinucleotide
NaCl	Sodium chloride
NIST	National Institute of Standards and Technology
PCR	polymerase chain reaction
PEA	phenyl ethyl alcohol
PDA	Potato dextrose Agar plates
QA	Quality assurance

QPS	Qualified presumption of safety
RT	Retention time
SCFA	Short-chain fatty acid
SEM	Standard error of mean
SPC	Solid phase cytometry
SOP	Standard operating procedure
SPME	Solid phase microextraction
S3 D3	Stage 3 day 3
ST	Storage day
TOC	Total ion current
VOC	Volatile organic compounds
v/v	volume per volume
Waik	Waikato
w/v	weight per volume
WHO	World health organization
VSFC	Volatile sulphur flavour compounds
YGC	Yeast glucose chloramphenicol

Chapter 1

Introduction

Demand for safe and high quality food of animal origin has increased, especially after recent outbreaks of foodborne diseases and food poisoning scandals (Hussain & Dawson, 2013). Zoonotic diseases are transmitted from animal-based food to humans. Antibiotics at low doses/sub-therapeutic levels have been used as growth promoters in animal feeds and to improve the quality of animal meat with a lower percentage of fat and higher protein content. The control of zoonotic pathogens such as *Salmonella, Campylobacter, Escherichia coli* and *Enterococci* is perceived to be another benefit of these antibiotic growth promoters (AGP) (Cogliani et al., 2011). However, the use of antibiotic as AGP in livestock was banned due to worldwide concern about the development of antibiotic resistance in humans (Castanon, 2007). The ban on use of AGP has been a challenge for animal nutrition and health increasing the need for alternative methods to improve animal production and control or/and prevent pathogenic bacteria.

In this context, the use of probiotics has been put forward to be used as a best practice alternative to AGP and much research has been done to substantiate the beneficial effects of probiotics such as improvement of gut microbial balance and acting against gut pathogens in animals. Probiotic use in agriculture started several decades ago (Fuller, 1999) and today hundreds of animal probiotic products are available in the global marketplace. However, there is an increasing concern about the quality and safety of these products and the importance of ensuring quality control of the probiotics has widely been accepted.

Quality-control properties must be continuously controlled and optimized and these properties can be evaluated in two steps. i.e., quality analysis before and after administration of these products. Properties before administration may include viability and survival throughout the manufacturing process, safety of product and metabolite profiling, etc. On the other hand quality parameters after administration may include bile and acid stability, colonization properties, immunogenicity, adhesive properties, etc. Most of these quality control properties are related to the selection criteria of probiotic strains, but probiotic properties may be influenced by long-term industrial processing and storage conditions. Thus, in addition to technological properties, functional properties should be considered in quality-control measures (Tuomola et al., 2001).

Viability is regarded as most important quality assurance parameter (Tuomola et al., 2001). Probiotic microorganisms have to encounter a series of stress conditions during processing and very low pH and detergent like bile salt when it travels through the gastrointestinal tract (GIT). These conditions seriously challenge the viability of probiotic microbes. Therefore, it is important to measure the viability of the probiotic products as claimed by the producers. It has been reported that the number of probiotic microbes found in certain products were below what was declared or they were altogether absent (Wannaprasat et al., 2009). Although health benefits from probiotic use depend on number of viable counts present in the product, it also varies with target animal and probiotic species used. MSL of probiotic viable counts in product is 10^6 CFU/ml (Adhikari et al., 2003; Vandenbergh, 1993). However, viable counts range 10^6-10^8 CFU/ml is expected to be efficacious, although the recommended dose in some countries is even higher than this level. For example the recommended viable counts of probiotics in Canada is 10^9 CFU/dose (CFIA, 2009).

A number of quality analysis studies of probiotics in various countries have been involved for the samples collected from the market shelf (Table 1.1).

Table 1.1 Quality testing of the probiotics in different countries.

Country	Quality testing	Reference
Belgium	213 products were studied and most of the products had wrong label information (mislabelling).	(Huys et al., 2006)
Canada	Two out of 25 tested animal probiotics were in good quality, rest of them had low viable numbers, misspelling of bacteria and lack of information about the contents.	(Weese & Martin, 2011)
China	28 probiotic product tested. Inconsistent Bacterial counts of specific species with the labelling, missing strain and wrong labelling.	(Chen et al., 2014)
Europe	Half of the tested products had fewer lactobacilli than claimed and mislabelling with respect to the bacterial species also reported.	(Coeuret et al., 2004)
New Zealand	Of 9 probiotics, all had at least the label viable cell numbers.	(Olivier et al., 2008)
New Zealand	Three out of four probiotic feed supplements had very poor viable cell numbers.	(Bennett et al., 2013)
Nigeria	Yoghurt tested for quality testing did not give adequate information about the product and product found to be contaminated with *Escherichia coli*, moulds and Bacillus.	(Ifeanyi, 2013)
Taiwan	Of tested solid and liquid probiotics, solid product did not have viable LAB and incorrect labelling on the package.	(Lin et al., 2006)
Thailand	Of 13 probiotics tested, all products had inaccurate labelling, misnaming and inaccurate viable cell numbers or species.	(Wannaprasat et al., 2009)
USA	58 commercial yoghurt were tested for quality testing most of the products contain viable strains below the level declared on the labels.	(Ibrahim & Carr, 2006)

Products are examined for viable probiotic counts, presence of pathogens or metabolites etc. Based on the information provided on the label of the product, test results are compared with content of the product. However, very few or none of the studies have been reported with a continuous quality analysis for the probiotic with each stage of production and with continuous evaluation of quality analysis until its expiry date.

Production of metabolic compounds by probiotics play a major role in controlling pathogenic and spoilage bacteria and some compounds exhibit anti-inflammatory effects. LAB produce a wide range of antibacterial such as organic acids (e.g., lactic acid and acetic acid, proteinaceous compounds (bacteriocins), antifungal peptides/proteins, low molecular mass peptides and other compounds such as reuterin and reutericyclin (Lefteris et al., 2006). Yeast acidifies the growth medium and tolerates high concentrations of ethanol. Production of antimicrobial compounds such as antifungal killer toxins or “mycocins” and antibacterial compounds demonstrate antagonistic activities toward undesirable bacteria and fungi in the probiotic products (Hatoum et al., 2012). Production of antimicrobial substances has been considered as one of several important probiotic mechanisms and a great deal of research focuses on the production of antimicrobial substances (Adhikari et al., 2003).

Probiotics are fermented products and usually considered safe from pathogens or food spoilers because of the low pH and production of antimicrobial substances (Vinderola et al., 2000). However, the use of probiotic products are associated with three types of hypothetical safety issues. Those include occurrence of diseases, antibiotic gene transfer in the GIT and toxic metabolites secreted by probiotic microbes (Snydman, 2008). Furthermore, the observations of reported post-production contamination in the probiotics (Ifeanyi, 2013) emphasizes the need for evaluating the product for potential pathogens in the product.

BioBrew Ltd produces fresh, living brews which are specialised microbial products for the agricultural sector. BBAN is a liquid probiotic widely administered to the farm animals in New Zealand. The aim of this MSc project is to develop scientific knowledge to produce a consistent quality commercial probiotic product for use in animal production. The ultimate goal is to design quality assurance system for production process. In general, sampling of probiotics at a given time from the market place has been used for quality control studies. However, we adopted a unique approach in this study and we consider quality control properties at many stages of production, storage stability through to the expiration of the product shelf-life.

This master's research has the following specific objectives:

- To identify the product characteristics which are responsible for its already demonstrated efficacy (metabolite profiling)
- To assess the viability of probiotic microbes throughout production and storage (microbiological quality testing)
- To develop a QA system to produce safe and high quality product
- Strategies to enhance product safety and stability (shelf- life and safety)

Chapter 2

Literature review

2.1 Probiotics and their characteristics

Probiotics are defined as "live microorganisms that are administered in order to provide a health benefit on the host" (FAO/WHO, 2001). The use of probiotics has gained interest as a natural way to improve health of both humans and animals. The global market for probiotic products was estimated at USD 24.23 billion in 2011 and expected to reach USD 44.9 billion in 2018. Application and demand for probiotic products have been increasing over the years and more than 500 probiotic products have been introduced in the past decade (Research and Markets, 2013).

Probiotic microbes help to prevent gastrointestinal disorders and improve and maintain a well-balanced intestinal flora (Lavermicocca, 2006). Well-investigated and documented health benefits include increasing natural resistance to infectious diseases in the GIT, improving lactose intolerance, suppressing traveller's diarrhoea and reducing bloating (Liong, 2007). Probiotic foods for human consumption have been marketed in Japan since 1920 (Svensson, 1999).

In order to achieve desired health effects the selection of suitable microbial strains plays a vital role. Selection criteria include bio-safety of the strains, origin of the strains, resistance to *in vivo*/*vitro* conditions, adherence and colonization ability to the intestinal epithelium, antimicrobial activity against enteric pathogens, viability or survival rate and resistance during processing and passage through the GIT (Kosin & Rakshit, 2006). Generally bacteria have been used in probiotic products. Fungi/yeast has also been used to a lesser extent. Bacteria used in the probiotics include *Lactobacillus, Bifidobacterium, Bacillus, Enterococcus, Pediococcus, Streptococcus and Clostridium* species whereas fungal or yeast probiotics include *Saccharomyces cerevisiae, Saccharomyces boulardii and Aspergillus oryzae* species (Canani et al., 2011; Cutting, 2011; Gueimonde et al., 2009). Depending on requirements, probiotic preparations may be mono-strain or multi-strain products. However, multi strain probiotic products, through synergistic adhesion effects, could act as broad spectrum protection against pathogenic infection in different host animals (Collado et al., 2007; Timmerman, 2004). Prebiotics are non-digestible oligosaccharides which act as carrier for probiotic microbes. Combined administration of probiotics and prebiotics are called synbiotics which provide definite health benefits through their synergistic action (Harish & Varghese, 2006).

2.2 Mechanism of action of probiotic microbes

In order to provide health benefits probiotic microbes exert different mechanisms of action in animals. The modes of actions are as follows:

(1) Alteration of intestinal microflora:

Food/feed supplements containing probiotics have been demonstrated to alter the existing intestinal microflora so as to give benefits to the host. Probiotics maintain the balance and multiplication of beneficial microbial populations in the GIT resulting in the prevention and combat of digestive disorders (Corcionivoschi et al., 2010).

(2) Immunomodulation of the host animal:

Probiotics have the ability to shape the immune system of the host animal through physiological action in the intestines (Isolauri et al., 2001). A study demonstrated the *Bacillus cereus var.toyoi* altered the immune status and functionalities of systemic immune cell populations in piglets (Schierack et al., 2007).

(3) Competitive exclusion (CE):

Nurmi and Rantala (1973) found that administration of gut contents prepared from healthy adult chickens protected newly hatched chicks from *Salmonella* colonization of the gut. Probiotic bacteria eliminate pathogenic bacteria by different CE techniques which include competition for physical attachment sites, direct and indirect competition for nutrients, enhancement of host immune system, and production of antimicrobial substances (Callaway et al., 2008).

(4) Production of inhibitory substances by probiotics against pathogenic microbes:

Probiotic microbes secrete bioactive compounds/metabolites which act against pathogenic organisms found in the gut. Metabolites produced by LAB include lactic acid, formic acid, acetic acid, propionic acid, benzoic, and phenyllactic acids. These metabolites play an important role in inhibiting pathogenic bacteria (Tharmaraj & Shah 2009).

2.3 Animal probiotics

The primary goal of livestock production is the delivery of safe, reliable and high-quality foods for human consumption. Recent outbreaks of food-borne diseases stress the need for reducing bacterial pathogens in foods of animal origin. Foodborne pathogens such as *Salmonella* and *Campylobacter*

are transmitted along the food chain and can be the source of human illness (Santini et al., 2010). In the past, antibiotics have been added to animal feeds at sub-therapeutic levels to act as growth promoters (Dibner & Richards, 2005). However, the European Union banned the use of antibiotics as growth promoters in 2006 due to the worldwide concern about development of antimicrobial resistance and transference of antibiotic resistance genes from animal to human microbiota (Castanon, 2007). This resulted in an increased demand for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the use of antibiotics (Verstegen & Williams, 2002).

In this context, the wider use of probiotic formulations has become popular using live microbial cultures to feed various animals as a substitute for the production-enhancing antibiotics. The use of probiotics to improve productivity in livestock is currently generating a great deal of interest.

Animal probiotics are live microbial feed supplements that enhance performance and/or improve intestinal microbial balance of host animals (Fuller, 1989). The strong demand by consumers for high quality and safe foods of animal origin have consolidated the use of probiotics in animal nutrition as a way to raise healthy animals without chemical interaction, and thus to produce foods of reliable quality and safety. Probiotics in animal nutrition can confer improved resistance to pathogenic bacterial colonization, enhancing host mucosa immunity which reduce pathogen load and improve health status of the animals (Choct, 2009).

2.4 Application of probiotics in animal farming

2.4.1 Animal production/nutrition

Application of probiotics in animal feed has dual purposes associated with animal production and animal health. Probiotic microbes have the ability to modulate gut microflora and probiotic strains separately and in combination can improve feed conversion rate, feed intake, daily weight gain and total body weight in farm animals (Torres-Rodriguez et al., 2007). The development of the probiotic approach to animal health started rapidly after the discovery of successful protection against *Salmonella* colonisation in newly hatched chicks by administering the gut suspension of healthy adult chickens (Nurmi & Rantala, 1973).

Bacterial probiotics have been effective in pigs, chickens and pre-ruminant calves whereas yeasts and fungal probiotics such as *S. cerevisiae*, *S. boulardii* and *A. oryzae* have delivered results in adult ruminants (Fuller, 1999). Several studies have shown the supplementation of animals feed with probiotics has significantly increased meat, milk and egg production. For example, probiotic products

containing *S. cerevisiae* and *A. oryzae* have increased the milk production and milk solids-non-fats and tended to increase milk protein percentage in dairy cows (Yu et al., 1997).

2.4.2 Animal health

The gut microbiota is a complex natural resource that can be manipulated to reduce the impact of pathogenic microbes that affect animal production and the safety of food products. Animal feeds that are classified as probiotics, prebiotics and competitive exclusion cultures have been utilized as pathogen reduction strategies in animals that are used for food production with varying degrees of success. The use of probiotic and prebiotics aims to eliminate the foodborne pathogens that affect animal production or food safety (Callaway et al., 2008).

Farm animals subjected to environmental and management stresses can experience disturbances in the intestinal microbiota, which may favour pathogenic infection. Administration of probiotic mixtures (*S. boulardii, B. subtilis* H4 and LAB) in weaned piglets reduced *E. coli* counts in the intestine, lowered the incidence of diarrhoea, and increased production of lactic acid and volatile fatty acid concentration (Giang et al., 2010). Probiotic microbes such as *Bifidobacterium lactis* and *Lb. rhamnosus* reduced adherence of *E. coli, Salmonella*, and *Clostridium* species to the intestinal mucosa in swine (Collado et al., 2007). Baum et al. (2002) showed that the administration of live yeast (*S. cerevisiae* spp. *boulardi*) to weaned pigs for 3-4 weeks improved growth performance, villus height, epithelial cell proliferation and the number of macrophages at various sites of the small intestine. A similar study conducted by Bontempo et al. (2006), demonstrated that dieteray supplementation with *S. boulardi* prevented gastrointestinal disorders and promoted intestinal health in weaned piglets.

Each year an estimated 1.4 million people had *Salmonella* contamination resulting in 400 deaths in USA (Voetsch et al., 2004). Probiotics are used in poultry to prevent and combat digestive disorders. For example, when oral *Lactobacillus* live culture (10^6 and 10^8 CFU/ml) administered to chicks infected with *S. enteritidis*, it resulted in a significant decrease of these pathogens (Higgins et al., 2008). Campylobacteriosis is a zoonotic disease caused by *Campylobacter jejuni*. Poultry meat is main source of this disease and occurrence of this disease in human has been increasing in recent years in the European Union (Hugas et al., 2009). Probiotic strain *Bifidobacterium longum* PCB 133 has shown an anti-campylobacter activity and campylobacteriosis in humans can be reduced by incorporating this probiotic strain as feed additive in poultry nutrition (Santini et al., 2010).

Digestive disorders (diarrhoea) are the leading cause of morbidity and mortality in new born calves. Faecal shedding of *E. coli* O157:H7 in cattle is directly relevant to levels of carcass contamination

(Elder et al., 2000). Administration of a *Lb. acidophilus* culture was effective in reducing *E. coli* O157:H7 shedding by more than 50% in finishing cattle and isolation from the hides of the cattle by up to 75% (Brashears et al., 2003; Younts-Dahl et al., 2004). Yeasts as probiotics have been used in cattle to improve health and as a dietary supplement to influence animal performance. *S. cerevisiae* as a probiotic feed supplement was found to be responsible for increased food intake, increased milk production and increased body weight in ruminants (Corcionivoschi et al., 2010).

2.5 Characteristics of animal probiotics products

2.5.1 Viability of probiotic organisms

Before a probiotic microorganism can start its biological role, it has to survive a series of stresses imposed during feed processing and passage through the GIT. Viability and stability of the probiotic products therefore have been both a technological and a marketing challenge for industry. Probiotic animal feeds should have probiotic strains and maintain the appropriate viable cell count during processing of the product and its entire shelf life. The present target of any probiotic food product in terms of viable cell numbers should be at least 10^6-10^7 CFU/ml at the end of its shelf life (Corcoran et al., 2006). Though different countries' specific requirements vary depending on products, many studies have indicated the viability claims by the producers have shown low populations of the probiotic bacteria in the probiotic product (Shah et al., 1995). Maintaining the viability of the probiotic cultures in functional foods during processing is the major challenge for the food/feed industry. Processes involving drying are often needed in order to keep the food/feed for prolonged storage. It is reported that the probiotic cultures exposed to spray drying show increased sensitivity to cell wall, cell membrane and increased DNA damage (Abee & Wouters, 1999).

Loss of viability of probiotic organisms in the probiotic products are due to several reasons such as level of oxygen in the products, acidity of products, acid produced during refrigerated storage (post acidification), sensitivity to antimicrobial substances produced by bacteria and oxygen permeation through the package (Dave & Shah, 1997). Selection of suitable probiotic strains plays a major role in improving the viability of probiotic organisms in probiotic products. There are many techniques that have been put forward to improve the viability of probiotics strains, including the selection of acid and bile resistant strains, microencapsulation, the use of oxygen-impermeable containers, two-step fermentation, stress adaptation, and the incorporation of micronutrients such as peptides and amino acids. Apart from manipulation of probiotics strains, it has been found some substances are known to improve the viability of the probiotic microbes. Babu et al. (1992) have reported that papaya pulp and tomato juice stimulated the growth of *Lb. acidophilus,* resulting in higher viable

counts, improved sugar utilisation and shorter generation times. This stimulation can be due to greater availability of simple sugars like glucose and fructose and minerals including magnesium and manganese which acts as growth promoters to *Lb. acidophilus.* Bifidobacteria, one of the most commonly used bacteria in probiotic products, show poor growth in milk. The viability of *B. longum* improved when the 0.01% of baker's yeast added to the milk (Shimamura, 1982).

Ross Crittenden et al. (2006) studied the effect of microencapsulation (film-forming protein-carbohydrate-lipid emulsion) on *B. infantis* survival rate. They found that the encapsulant protected *B. infantis* during non-refrigerated storage with exposure to oxygen and humidity.

Probiotic animal feed is manufactured in the form of liquids and pellets. Pelleting, extrusion and complementary processes require pressures and high temperature which may affect the viability of probiotics. For instance, a typical feed for broiler chickens is processed at about 75-85 ^{o}C for 15-20 min with a moisture content of 15%, before pelleting (Kosin & Rakshit, 2006). It is a significant challenge to maintain viability and survival of probiotic microbes during feed manufacturing. Thermophilic/thermotlolerant bacteria have the ability to withstand higher temperature during processing and storage. Selection of these microbes could enhance the viability of the probiotic products. Most of the thermophilic LAB has been studied for induced stress response, when exposed to heat shock. For example, a thermophilic probiotic strain (*Lb. paracasei*) showed 49% survival rate when subjected to spray drying at 80-85 ^{o} C (Gardiner et al., 2000).

Though most of the thermophilic lactobacilli originate from dairy products, some thermotolerant bacterial species have been successfully identified in chicken intestines, which includes *Lb. reuteri, Lb. thermotolerans, Lb. aminata sp. nov.,* and some varieties of *Lb. paraplantarum* (Niamsup et al., 2003; Sow et al., 2005). Probiotic cultures exposed to sub-lethal treatment demonstrate better adaptability and viability during lethal treatments of food/feed processing. For an instance, *Lb. paracasei* subjected to pressure pre-treatment showed higher survival rate than untreated cells when exposed to high temperature at 60 ^{o}C (Ananta & Knorr, 2004). Maintaining the viability of the probiotic microbes during spray drying and freeze-drying in feed processing is a major challenge for the industry. Pascual et al. (1999) showed that the glycerol and skim milk that act as cryoprotective agents could be used to preserve the probiotic strain in the freeze-drying. Probiotic microbes can be protected from the stress and spray-drying by composite carrier matrix systems which include different food matrices.

Dehydrated glucose syrups microencapsulated with the probiotic strains showed greatest survival rate compared to other carbohydrates used during spray- drying (Boza et al., 2004).

Prebiotics in combination with probiotics is called synbiotics. Use of prebiotics in probiotic culture may increase the growth and survival of the probiotic microbes. A study of the *in vitro* fermentation properties of a commercial prebiotic demonstrated Xylooligosaccharides and lactoluse were responsible for increased cell numbers in *Bifidobacterium* whereas fructooligosaccharides were found to be responsible for increased lactobacilli population (Rycroft et al., 2001). Resistant starches (prebiotics) used in the feed industry offer a surface for adherence to the probiotic microbes during processing, storage and transit through the upper regions of the gastrointestinal tract improve the viability of the probiotic microbes (Crittenden et al., 2001).

2.5.2 Bioactive compounds/metabolites in animal probiotic products

The production of metabolites by different probiotic microorganisms is considered to be a primary mode of action against pathogenic organisms present in the GIT as well as in food. These metabolites such as organic acids (lactic acid and acetic acid) carbon dioxide, ethanol hydrogen peroxide, diacetyl, acetaldehyde, acetoine, reuterin, reutericyclin are low mass molecules. Bacteriocin, one of the important metabolites with high molecular mass has extensively been used in the food industry as biopreservative (Suskovic et al., 2010). Utilization of antimicrobial metabolites in probiotic products in animal and human health applications has increased. These antimicrobial substances are used in the probiotic industry to control pathogenic organisms in the GIT and urogenital tract. The most important and best characterized antimicrobials produced by LAB are lactic acid and acetic acid. Organic acids such as formic acid, acetic acid, lactic acid, propionic acid, benzoic acid and free fatty acids are produced from sugars, amino acid and/or lipid metabolism in bacterial cells (Ray et al., 2000).

Tharmaraj and Shah (2009) studied the effect of probiotics on pathogenic and spoilage bacteria. They found that the varying quantities of organic acids (acetic, lactic, formic, propionic, butyric, benzoic, and phenyllactic) secreted by probiotic microbes (*Lb. acidophilus, Lb. casei*, *Lb. paracasei* subsp. *paracasei, B. animalis*, and *Propionibacterium freudenreichii* subsp. *shermanii*) were responsible for inhibiting the growth of pathogenic and spoilage microbes such as *E. coli, S. typhimurium, S. aureus,* and *B.cereus.* Secretion of antimicrobial substances is one of the most studied components in the anti-pathogenic activity of probiotic bacteria. *Lb. fermentum* strain SRJ-23 showed significant antimicrobial activity against human pathogenic bacteria such as *B. subtilis, E. coli,* and *S. aureus* by producing proteinaceous substance like bacteriocin which is a characteristic feature of many lactobacilli (Purohit et al., 2012). BIF is a novel protein secreted by *Bacillus longum* strain BL2928 inhibited the interaction between *E. coli* and human epithelial cells (Fujiwara et al., 1997).

Production of short-chain fatty acid (SCFA) metabolites by probiotic bacteria have an important protective function in the intestine and exert anti-inflammatory effects in different animals (Licciardi et al., 2010). SCFA includes butyrate, acetate and propionate. The butyric acid-producing anaerobic bacterium, *Faecalibacterium prausnitzii* is a novel probiotic used for the treatment of inflammatory bowel disease (Neish, 2004). Lipophilic compounds secreted by *Bifidobacterium* strains (CA1 and F9) have strong antimicrobial activity against *S. typhimurium* SL1344 and *E. coli* 1845 (Lievin et al., 2000).

Though different probiotics exhibit different mechanisms of action to confer health benefits to the host animal, they are host-specific and strain-specific in their action. Selection of probiotic organisms with a combination of different modes of action may amplify the protective range of bio-therapeutic preparations (Filho-Lima et al., 2000).

2.5.3 Quality control of animal probiotics products

Testing for an adequate number of viable microbes present in a probiotic product throughout its production cycle and storage has generally been used as the only way to measure the quality of commercial probiotic products over the years. Though the viability of probiotic microbes in probiotic products plays an important role in quality issue, it is not the only criterion for quality assurance. Probiotic strains should be able to deliver the functional health characteristics for which they were originally selected. Strain resistance against gastric acid and bile salt can be determined by *in vitro* testing. Advancement in molecular techniques can also be used to examine strain stability. To develop an effective quality control system other functional properties of probiotics such as adhesion characterization, immunogenic effects and competitive exclusion have to be studied. However, probiotic microbes exhibit host-specific and strain-specific differences in their actions. Maintaining strain characteristics of the probiotic microbes is considered to be an important factor to deliver desired health effects. Some studies reveal that dried probiotic preparations may have contaminants (Arthur et al., 2002). Therefore, it is necessary for careful identification of strains via hygienic practices to avoid contamination.

In order to measure the functionality and safety of the probiotic products used in the animal feeding, preliminary *in vitro* screening could be done to measure the following parameters: adhesion studies, survival in the GIT, antimicrobial activity and antibiotic susceptibility. Identification and characterization by advanced molecular methods such as microarrays improve the detection of multiple characteristics of probiotic strains. It has been reported that the organisms cited in the labels of certain probiotic products have not been found within the product and often the products contain species other than those claimed on the label (Huff, 2004; Wannaprasat et al., 2009).

Therefore, correct labelling of the probiotic product and the name of the exact taxonomic species with internationally accepted nomenclature is indispensable to avoid confusion and misidentification. It is recommended that the claim made by the producer about the preparation should reflect the actual composition of the product until the expiry date maintained in the recommended storage conditions with a decrease of one or two logarithmic units maximum (Czinn et al., 2009).

Efficacy of the probiotics may be affected by the dose, timing and duration of the administration of probiotics and the age of the animal. Higher dose of probiotics given for a short time seemed to be more effective in controlling acute infectious diarrhoea than lower doses of administration (Sazawal et al., 2006). New-born animals are more susceptible to environmental pathogens. Administration of probiotics for new-born animals could help in colonization of the bacteria which can modulate expression of genes in epithelia cells thus creating a favourable habitat for themselves (Siggers et al., 2007).

Adhesion characterization of probiotic microbes is an important quality control method for assessing the effect on the gut barrier. Tuomola et al. (2001) studied the adhesion ability of some common probiotic strains by using a human colon carcinoma cell line (Caco-2) and human ileostomy glycoproteins as an *in vitro* model for intestinal epithelium and mucus respectively. *Lb. casei* showed higher adhesive characteristics amongst tested microbes.

2.5.4 Safety of Animal Probiotics Products

Most of the probiotic microbes used in animal probiotic products are considered to be safe, while some of the microbes such as Enterococci species and *B. cereus* have exhibited some problems when used as probiotic microbes. *B. cereus* is associated with the production of an emetic toxin and enteric toxin and the Enterococci species may be responsible for harbouring transmissible antibiotic resistance (Arturo et al., 2006). Microorganisms used in probiotics such as *Lactobacilli, Lactococci, Bifidobacterium* and yeast are classified as Generally Regarded As Safe (GRAS). Though some microbes including *Bacillus, Enterococcus, Streptococcus* and other spore-forming bacteria have not been categorized under the GRAS status, they have been used in the probiotics industry (Snydman, 2008). Therefore safety and quality of the probiotic products has become important and the safety evaluation of probiotics use has already been implemented in different countries.

In the USA, specific utilization of microorganisms for human consumption should possess the GRAS status regulated by the Food and Drug Administration. A similar approach has been introduced in Europe where European Food Safety Authority (EFSA) has introduced the concept of Qualified

Presumption of Safety (QPS) similar in purpose to the GRAS approach. The QPS system is considered to be more flexible as it takes into account number of additional criteria to evaluate the safety of bacterial probiotics. Those additional criteria include history of safe use in the food industry and the development of antibiotic resistance or virulence determinants (Wassenaar & Klein, 2008).

In its recent updates EFSA has listed microorganisms that are safe to use in animal feeds (Table 2.1). The status of some microbes has been updated and some microbes have even been excluded from the list. For example, bacterial species such *as Lb. lactis* and *Bacillus amyloliquefaciens* which already held QPS status have been reviewed for their safety usage and extended their QPS status with some conditions. Furthermore*, A. oryzae* has been excluded from the QPS list because of its potential for mycotoxin production (EFSA, 2012).

Table 2.1 Microbes that are claimed as safe to use as probiotics in animal feeds certified by EFSA EFSA (2012).

Genus	Species
Lactobacillus	*Lb. acidophilus* *Lb. amylolyticus* *Lb. amylovorans* *Lb. brevis* *Lb. buchneri* *Lb. casei* *Lb. bulgaricus (L.delbrueckii* subsp*. bulgaricus)* *Lb. casei rhamnosus (Lb. rhamnosus)* *Lb. delbrueckii* subsp. *lactis* *Lb. farciminis* *Lb. fermentum* *Lb. helveticus* *Lb. mucosae* *Lb. paracasei* *Lb. pentosus* *Lb. plantarum* *Lb. reuteri* *Lb. sakei* *Lb. salivarius*
Bifidobacterium	*B. animalis* *B. longum*
Streptococcus	*S. cremoris (L. lactis subsp. cremoris)* *S. thermophiles*
Bacillus	*B. subtilis*
Pediococcus	*P. acidilactici* *P. pentosaceus*
Propionibacterium	*P. freudenreichii shermanii*
Saccharomyces	*S. cerevisiae*
Kluyveromyces	*K. marxianus var. lactisK1* *K.marxianus-fragilis*

Generally probiotic microbes are considered to be responsible for four types of potentially negative side effects; gene transfer, systemic infections, deleterious metabolic activities and excessive immune stimulation in susceptible individuals (Marteau & Boutron-Ruault, 2002). One hypothetical risk associated with probiotic use is antibiotic-resistance gene transfer taking place between probiotic and pathogenic bacteria and this is considered to be a major area of concern. Plasmids containing antibiotic resistance genes have been discovered in LAB. Although antibiotic resistant genes have been found in *Lb. acidophilus, Lb. reuteri, Lb. plantarum and Lb. fermentum* isolated from feces of animals, raw meat, and silage (Gevers et al., 2003), the transfer of the Lactobacillus plasmids to pathogenic microbes is quite rare (Dessart & Steenson, 1991; Mathur & Singh, 2005). However, transfer of lactose fermentation plasmids to *Lb. casei,* and transfer of bacteriocin production to *Lb. johnsonii* have been reported (Ahn et al., 1992).

Hypothetical risk of bacteremia and endocarditis by the use of probiotics have been reported and species of LAB, *Enterococcus* species and *Bifidobacteria* have been identified as cause of bacteremia and Endocarditis (Cannon et al., 2005). However there have been no studies to prove that these microbes are actual cause of bacteriamia and endocarditis. Sullivan and Nord (2006) studied *Lactobacillus* bacteremia with the probiotic microbes such as *Lb. paracasei* subsp. *paracasei, Lb. acidophilus* NCFB 1478 and *Lb. rhamnosus* strain GG. They concluded there was no evidence for *Lactobacillus* bacteremia and it could instead be due to the polymicrobial action.

In addition to these risks associated with probiotic use, some theoretical risks have been raised with the use of probiotics include the potential for transmigration, production of undesirable metabolites in GIT and adverse immunologic effects. Therefore, it is required to have universally accepted regulatory systems to monitor the probiotic safety.

2.6 BBAN

BBAN is fresh, living and liquid animal probiotics produced by BioBrew Ltd which claims the use of BBAN improves health and overall wellbeing of animals. BBAN contains multi species and multi strain of LAB and yeast (4 LAB species and 3 yeast species). This animal probiotic product is available in 5, 10 and 20 litre plastic containers for consumers. BBAN does not need any special storage requirement and shed conditions are appropriate for storage. Containers have to be tightly capped and stored out of direct sunlight. BBAN is suitable for oral application in all domesticated species at all stages of development and may be administered via stock water, feed, or direct drenching.

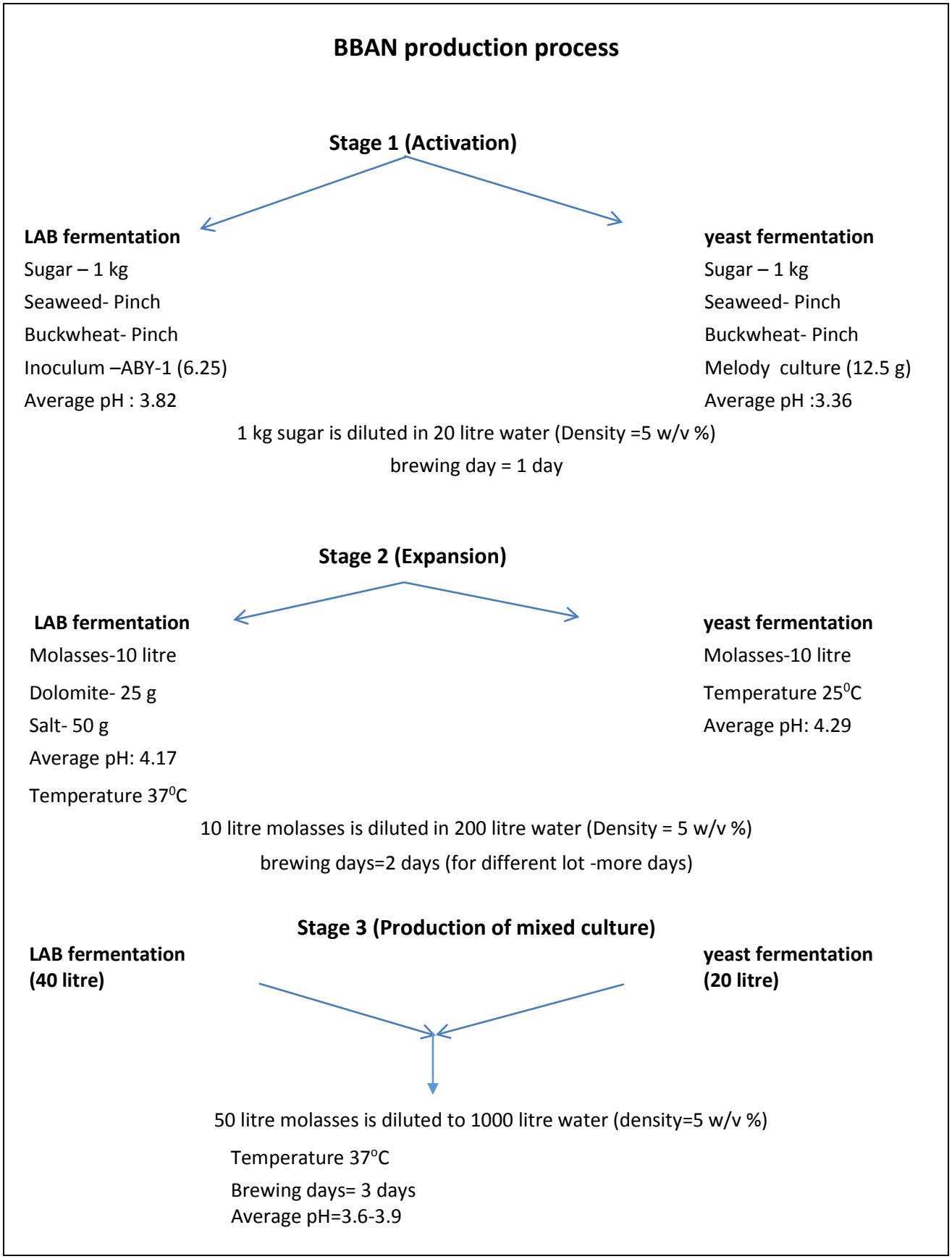

Figure 2.1 Steps involved in standard commercial BBAN production.

Production of BBAN involved several steps. Each step is different from other steps in terms of fermentation temperature, number of fermentation days and ingredients(Figure 2.1).

Stage one/initial fermentation - one day

Stage two - 2-3 days

Stage three/Mixed fermentation - 3-4 days

Storage/shelf-life - one month

Production of BBAN is a three stage fermentation process. Commercial LAB(ABY-1) and wine yeast (Melody) are used as starter cultures for BBAN production. In the first stage, using sugar based medium,starter cultures are normally grown in individual containers at different temperatures for one day. stage 2 production is expansion of stage 1, LAB and yeast are fermented in parallel in the molasses media for 2-3 days. temperature in both stage 1 and 2 is maintained at $37^{o}C$ and $25^{0}C$ for LAB and yeast fermentation respectively.however, $37^{o}C$ is maintained in stage 3 in which fermented products of stage 2 are combined together and allowed to ferment for 3 days. At the end of mixed fermentaion final product is ready to use and BBAN is decanted in the plastic containers.

Chapter 3

Microbiological quality and safety of the probiotic product

3.1 Introduction

The probiotic market is expanding year after year with an increasing variety of products available for different host species. Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). In order to attain therapeutic health benefits from probiotics there should be sufficient number of viable microbes present during production and storage (Ljungh & Wadström, 2006). However the determination of the number of viable microbes required to be present in a product to ensure efficacy is subject to debate and some countries have their own national standard to set the number of viable microbes in a "probiotic" product. For example, the recommended level of viable microbes in commercially available probiotics in Canada is 10^9 CFU per serving (CFIA, 2009) whereas the minimum bacterial count of the Chinese national standard for probiotic products is 10^6 CFU/g (Chen et al., 2014). In general, a viable cell population of microbes with probiotic characteristics between 10^6-10^8 CFU/ml is considered sufficient to be efficacious.

The determination of viable cell concentration in probiotic products is critical in quality evaluation (Champagne et al., 2011). However, many probiotic products in the marketplace with low microbial counts, i.e., poor quality, have been reported when products are independently examined through quality testing (Coeuret et al., 2004). Deficiencies included inaccurate labelling or fewer viable microbes than the amount declared by the manufacturer and no information whether the viable counts were present until expiry date or at the time manufacture (Chen et al., 2014; Wannaprasat et al., 2009).

Very few studies have been conducted on quality of animal probiotic formulation when compared with human probiotics. However, quality studies of animal probiotics are equally as important as human probiotic studies. Poor quality products not only reduce the beneficial effects from probiotic use, they also challenge the safety of the target organisms. Animal probiotics should be of especially high quality because of administration of poor quality probiotics in farm animal may not yield desired outcome.

The primary objective of this project was to test the microbiological quality for the commercial animal probiotics (BBAN) in terms of viability assessment at many stages of production for each batch until its shelf-life expiry.

3.2 Materials and methods

3.2.1 Growth media

3.2.1 1 MRS agar plates

De Man, Rogosa and Sharpes (MRS) agar (De Man et al., 1960) was used to detect and enumerate *Lactobacillus* colonies. MRS agar media was prepared either by dissolving 62 g of MRS agar in 1 litre of dH_20 or by adding 15 g of bacteriological agar with 52 g of MRS broth in 1 litre of distilled water. All media were supplied by Oxoid Ltd. (Hampshire, England) and sterilised by autoclaving at 121 ^oC for 15 min.

3.2.1.2 YGC plates

Yeast Glucose Chloramphenicol (YGC) agar was used to enumerate yeast colonies. YGC plates were prepared by combining 5 g/l of yeast extract, 20 g/l glucose and 15 g/l agar in dH_20. pH was adjusted to 6.6 before autoclaving and just before pouring into plates, 50 mg/l of chloramphenicol was added to YGC to inhibit bacterial growth.

3.2.1.3 PDA plates

In addition to YGC plates, Potato Dextrose Agar (PDA) plates were used to enumerate yeast counts in the product. Also PDA plates were used to detect *Rhodotourula rubra* presence in the product.

3.2.1.4 MacConkey agar

MacConkey agar was used to detect gram negative bacteria (especially, *E.coli*) in the sample/product

3.2.1.5 Baird-Parker agar

Baird-Parker agar is a selective agar media to detect the *Staphylococci* species. Especially, Baird-Parker agar was used to find out if *Staphylococcus aureus* was in the sample

3.2.1.6 3M Petrifilm

In addition to these selective agar media, 3M petrifilm plates were used to check whether the product contained any foodborne pathogens.

3.2.2 Production schedule

BioBrew Ltd. is a Commercial animal probiotic company which has four manufacturing sites in New Zealand located in Canterbury, Balclutha, Waikato and Taranaki (www.biobrew.net.nz) Manufacturing plant in Canterbury and Waikato are the chief production sites and production of BBAN involved several steps. Each step is different from other steps in terms of fermentation temperature, number of fermentation days and ingredients.

Stage one/initial fermentation - one day

Stage two - 2-3 days

Stage three/Mixed fermentation - 3-4 days

Storage/shelf-life - one month

3.2.3 Sampling

Sampling during different stages of production and over the duration of storage was undertaken to enumerate viable counts of probiotic microbes present in the product. Sample collection began from August 2013 to March 2014 for eight months at the Canterbury manufacturing site. Samples at many stages of production were collected in the plastic test tubes and tested immediately after brought to the laboratory. Samples for shelf life were stored in the laboratory environment in the plastic container.

Three consecutive batches of BBAN were sampled for quality testing from day one of first stage of formulation to 30th day of shelf-life. Subsequently, sampling and testing was made at the end of each stage and weekly during storage for the different batches of probiotic products produced for eight months.

Sampling across other manufacturing sites was made for comparative quality testing for the probiotic formulation with same production stage. This was done by collecting samples from all other manufacturing sites every month for three months.

3.2.4 pH analyses

The pH of the samples was measured using Orion 3-Star pH meter (Thermo Fisher Scientific Inc., USA) after calibrating with commercial pH 4.0 and 7.0 buffers (Thermo Fisher Scientific, MA, USA). pH of the product must of monitored to make sure the viability of the microbial counts is not affected by the low pH which put the cells in stressful conditions resulting loss of viability.

3.2.5 Enumeration of LAB and yeast

For plate counting, 100 µl of BBAN sample was mixed with 900 µl of peptone water (0.1%). The samples were then serially diluted and appropriate dilutions (up to 10^{-8} cells/ml) were spread onto different agar media. Spreader (hockey stick) was used to spread sample evenly onto the agar plate. MRS agar plates were incubated under anaerobic conditions at 37 ^{o}C for 48 hours whereas YGC plates were incubated under aerobic condition at 25 ^{o}C for 48 hours. Anaerobic conditions for MRS plates were maintained using click-clack jars and AnaeroGen™ sachets (Oxoid Ltd, Hampshire, UK). Subsequently, colonies were manually enumerated and yeast and bacterial concentrations in the original sample was estimated.

3.2.6 Safety tests

BBAN sample (1000 µl) was pipetted onto the MacConkey agar, Baird-Parker Agar and 3M petrifilm. Spreader was used to spread the sample evenly on agar plates and petrifilm. After incubation at 37 ^{o}C for 48 hours, plates were inspected for any possible pathogens present in the sample.

3.2.7 Statistical analysis

Statistical analysis of data was carried out using Minitab (Minitab version 17, Minitab Inc.). The comparisons of differences between the means of the treatments were tested by analysis of variance by the General Linear Model (ANOVA-GLM) at the significance level of $P < 0.05$. Grouping was done using Tukey's Method at 95.0 % confidence level.

3.3 Results

3.3.1 Changes in pH during production and over the storage period of time

Average pH value during different stages of production and storage period varied between lactic acid and yeast fermentation. At the end of stage 1 of lactic acid fermentation, pH of the product dropped from 6.1 to 3.82 and went up to 4.17 in stage 2 (Figure 3.1).

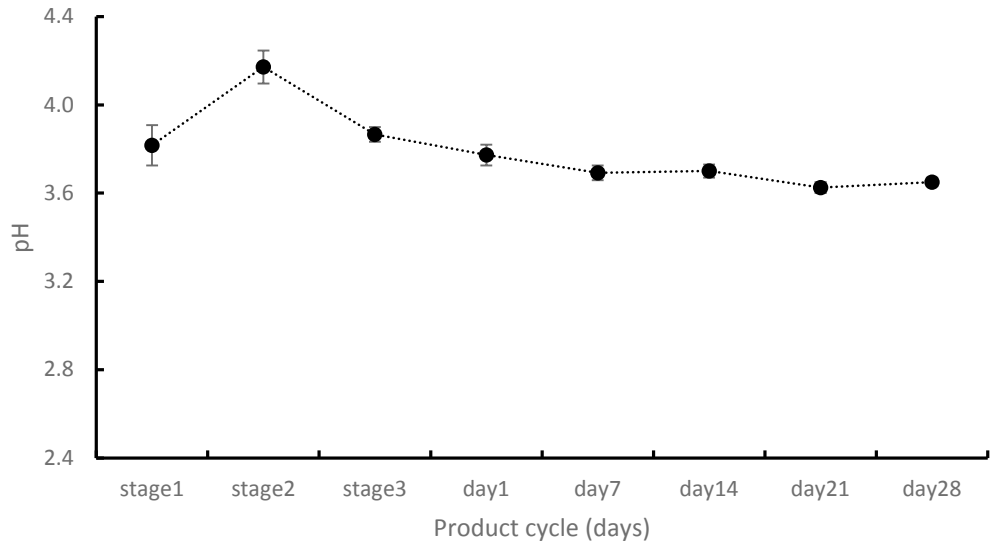

Figure 3.1 Changes in pH value in lactic acid fermentation during production and over the storage period of time.

pH changes in yeast fermentation also showed similar pattern where pH range was 3.36 - 4.29 between stage 1 and stage 2, respectively (Figure 3.2). However, there were no significant differences in pH value between mixed fermentation and different storage days. Low pH value is a good indicator of how acidic the product is. Shelf-life of the product has been designated one month. Both LAB and yeast acidified the product in combined cultures reaching a final pH of between 3.6 - 3.9 when the product was available for customers.

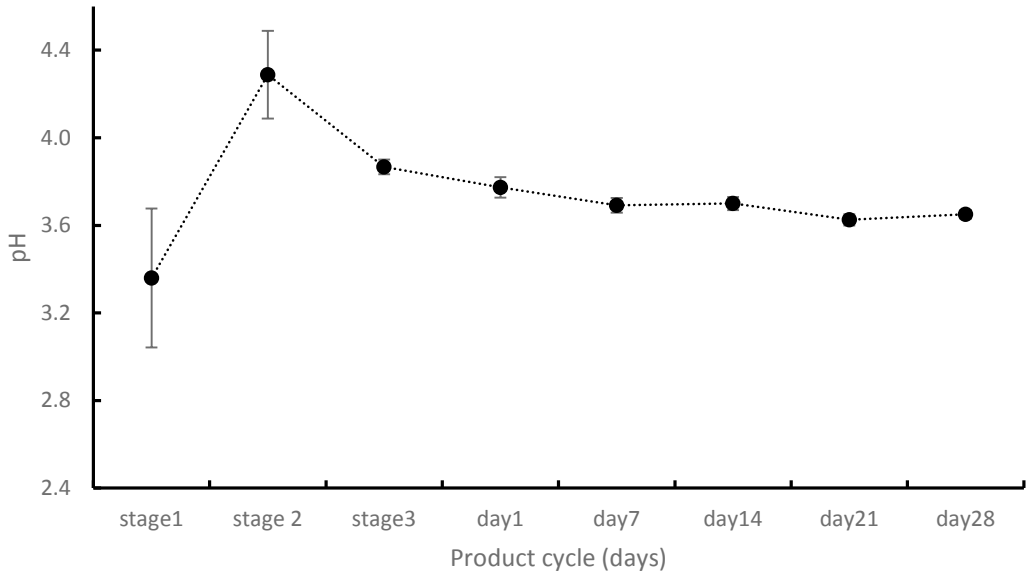

Figure 3.2 Changes in pH values in yeast fermentation during production and over the storage period of time.

3.3.2 The influence of storage on the survival of LAB in BBAN

The data collected at the end of each stage and during storage at weekly interval was subjected to analysis. Average log number of bacterial and yeast cell numbers were obtained from several batches (Appendix A.1 and A.2).

Generally lactobacilli fermentation as a single culture is undertaken using sugar media as a substrate and allowed to ferment overnight during stage 1. Most of the samples collected after several hours of fermentation at stage 1, estimated the number of viable count of 7.67 log CFU/ml. Molasses is used as substrate in step 2 fermentation and the fermentation lasts for 2-3 days. The average bacterial colonies (9.3 log CFU/ml) in step 2 was found to be higher than that of initial fermentation and difference in cell counts between stage 1 and 2 were significantly different ($P < 0.05$) (Figure 3.3).

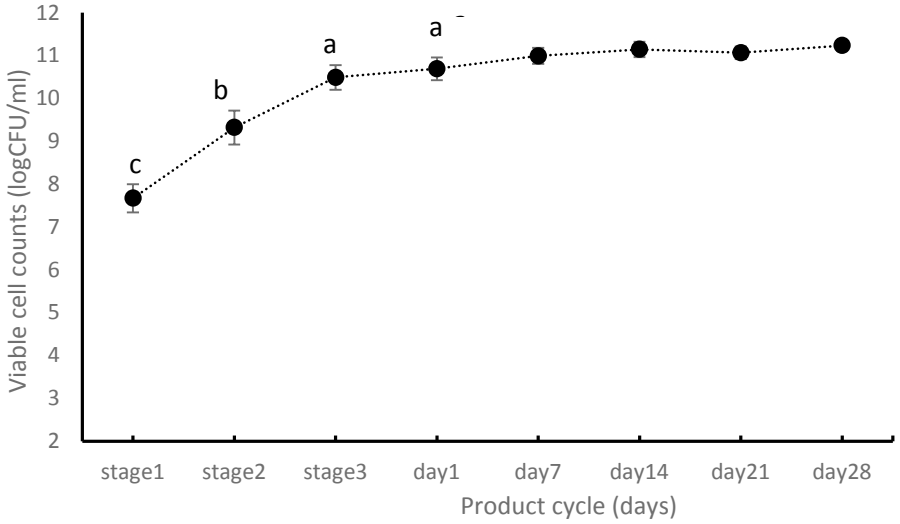

Figure 3.3 Mean values of several batches. Bacterial cell concentration at many stages and over the storage period; [a, b, c] superscript letters differ significantly (P < 0.05).

Step 3 fermentation (mixed fermentation) is similar to stage 2 in terms of substrate and at the end of mixed fermentation BBAN is ready for marketing. There was a significant difference between the results obtained from stage 2 and mixed fermentation (P < 0.05). In contrast, cell numbers in mixed fermentation did not differ statistically with different storage days and the average bacterial viable counts obtained were between 10.49 - 11.24 log CFU/ml (Figure 3.4).

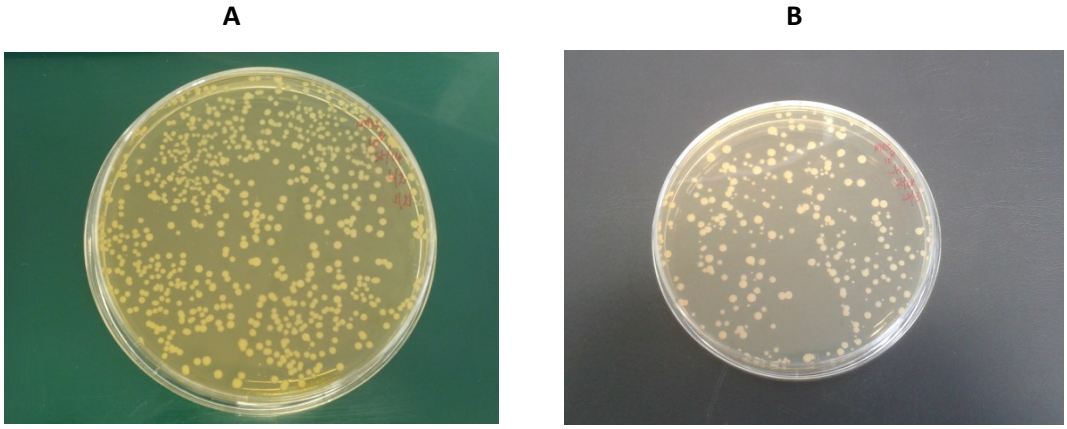

Figure 3.4 Typical LAB counts with 10^{-8} dilution of the BBAN samples at 14th (A) and 28th (B) day of storage days.

Changes in cell densities in stage 1 and 2 fermentation seemed to be not affecting the cell numbers at the end of mixed fermentation. Almost all the batches had sufficient lactobacilli numbers ranging from 10.49 ± 0.29 log CFU/ml of at the end of stage 3.

To assess the stability of the BBAN, viability of the product tested until 30th day of storage. Log number of viable lactobacilli counts did not have significant differences between various storage periods until its expiry day and cell counts were observed 11.24 ± 0.12 log CFU/ml at the end of shelf-life. Therefore, it is certain that this BBAN contained viable lactobacilli counts higher than minimum recommended dosage until its expiry day. The results indicated that the influence of storage did not affect the viability of the LAB in BBAN. There was no reduction in bacterial counts; rather product had consistent bacterial counts towards the end of shelf-life.

3.3.3 The influence of storage on the survival of yeast culture in BBAN

The BBAN produced by BioBrew Ltd consists of multi-species and multi strains to diversify the health benefits. It contains LAB and yeast to deliver health benefits to the farm animals.

The total viable yeast colonies contained in BBAN in stage 1 and stage 2 ranged between 9.97-10.24 log CFU/ml. There was no significant difference was detected between initial fermentation and stage 2 as well as between mixed fermentation and day 1 of storage (Figure 3.5).

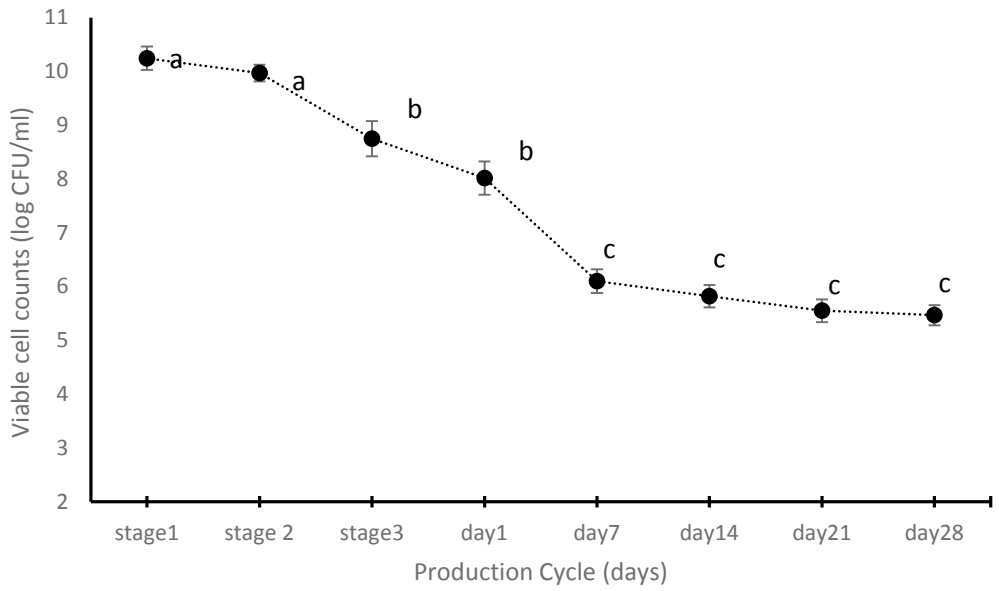

Figure 3.5 Mean values of several batches. Yeast cell concentration at many stages and over the storage period of time. $^{a, b, c}$ superscript letters differ significantly (P < 0.05).

In general, population of yeast tended to decrease when the storage period of the BBAN increased. The total yeast counts found in BBAN in first and 28th of storage day were 8.02 ± 0.31 and 5.47 ± 0.19 log CFU/ml respectively (Figure 3.6). Therefore, it is clear, that the growth of yeast colonies has been significantly affected in co-culture.

Statistically significant difference in cell numbers was obtained between stage 2 and mixed fermentation and day one storage and other storage days ($P < 0.05$). Influence of storage on viability of yeast was found to be negative and yeast cell numbers from first week of storage until its shelf-life did not show statistically significant difference.

A **B**

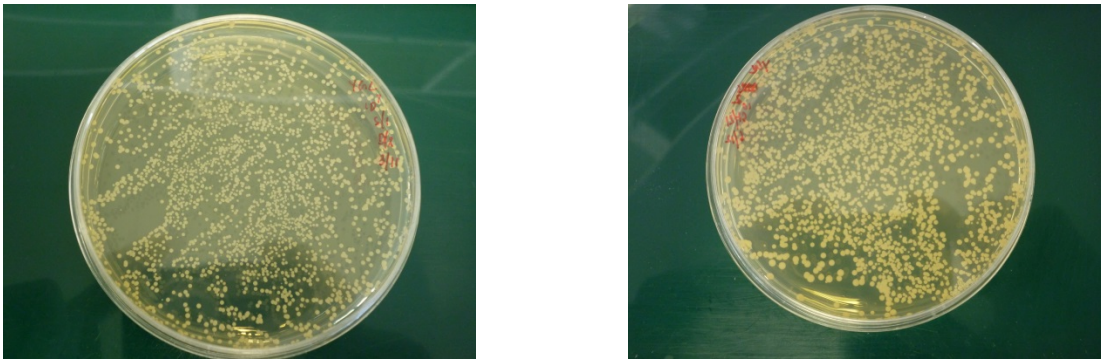

Figure 3.6 Overview of viable yeast cell colonies at stage 1 (A) and 21st (B) day of storage with 10^{-7} and 10^{-2} dilution, respectively.

3.3.4 Viability at many stages of production and at the end of shelf-life

The total LAB and yeast counts in BBAN samples found at the end of expiry day were 11.24 ± 0.12 and 5.47 ± 0.19 log CFU/ml respectively. It was noted there was significant differences in LAB and yeast counts at the expiry date (Figure 3.7).

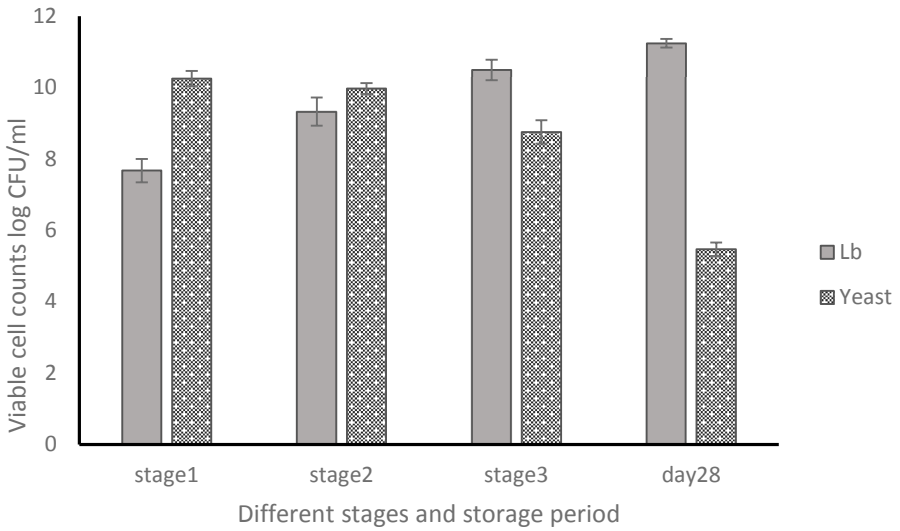

Figure 3.7 Lactobacilli and yeast cell concentrations at different stages of production and at the end of shelf-life.

3.3.5 The influence of extended storage on the viability of LAB and yeast

Viability of the bacterial strains in the product did not change with storage day until its expiry of the product. The study conducted to determine the viable counts of LAB and yeast for the extended shelf-life. Total viable counts of LAB and yeast in the product with two weeks extended storage remained same as it was found on the eve of its expiry date (Figure 3.8). There were no reductions in LAB and yeast cell counts when the product with 2 weeks extended shelf-life.

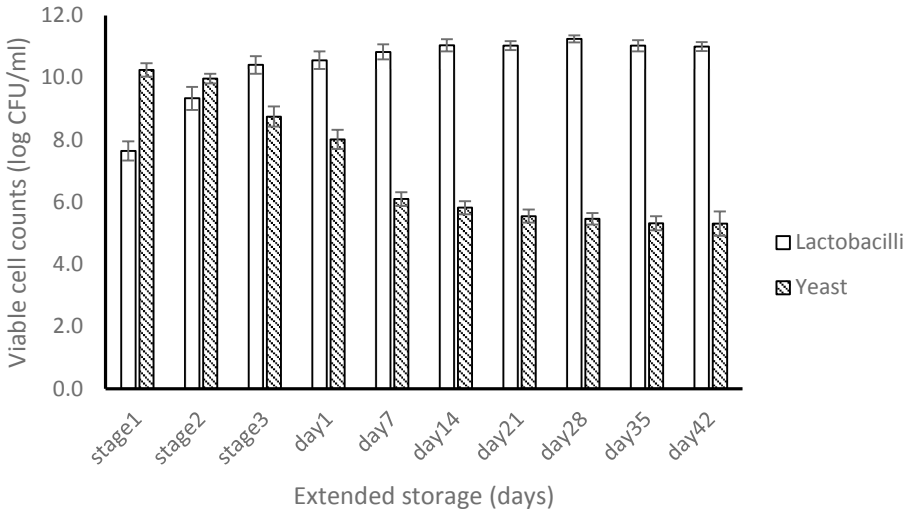

Figure 3.8 Average viable Lactobacilli and yeast cell concentration (log CFU/ml) of several batches with extended storage.

3.3.6 Comparison of different production sites for viability assessment

Viability of the product amongst different production sites were consistent and cell counts of LAB and yeast ranged between 9.43-11.23 and 5.29 -5.69 log CFU/ml, respectively. Yeast cell counts did not differ significantly amongst different production sites. However, LAB cell densities in Canterbury production sites were found to be slightly higher than that of other productions sites (Figure 3.9). Number of samples analysed in Canterbury site was much higher than that of other sites. Production of BBAN in Canterbury site and other sites had similarity in the viability analysis and there were no significant differences between various production sites.

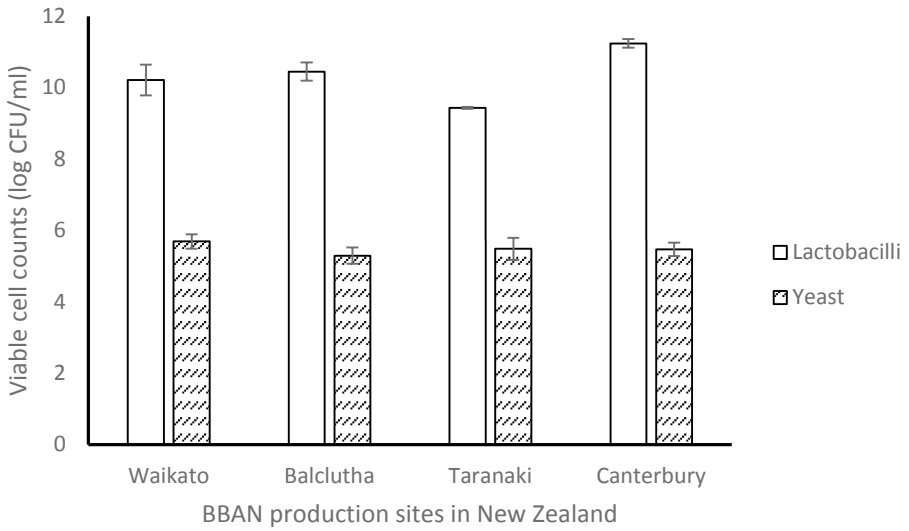

Figure 3.9 Overview of the log number of viable LAB and yeast cell numbers in the animal formulation obtained from different production sites.

3.3.7 Viability assessment on monthly basis

Investigating the microbial quality analysis on a monthly basis and evaluation of microbial cell counts on 7th and 28th day of storage were analysed. LAB cell numbers in the study period consisted > 10^{10} log CFU/ml at 7th and 28th days of storage except month of August where cell counts at 7th day of storage was 10^{9}- 10^{10} log CFU/ml (Figure 3.10). Bacterial cell numbers between first and last week of storage did not show significant difference whereas yeast cell concentrations between 7th and 28th storage days, differed significantly. In most cases, yeast cell numbers ranged between 10^{4} to 10^{6} CFU/ml until its expiry day in all months except months of November and December 2013 in which cell concentrations were >10^{6} at 7th day of storage.

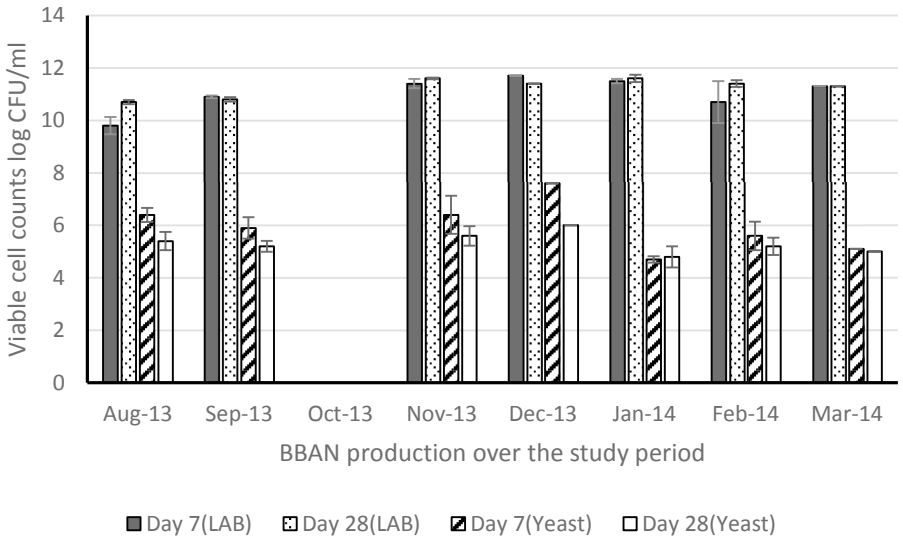

Figure 3.10 Overview of the log number of LAB and yeast cell numbers for the BBAN produced in different months (study period) in Canterbury production site.

3.3.8 Safety assessment

Assessment of safety in the BBAN was measured for the product collected from all four production sites at various stages of production. Product tested for general foodborne pathogens such as gram negative bacteria (especially *E. coli* and total Coliform*)* and *Staphylococci* species showed negative and none of bacterial foodborne pathogens detected in the product. However, unusual pink colonies were observed in two batches of BBAN produced in the month of October 2013. In order to identify these unknown pink colonies, they were isolated, stained and looked under the microscope and the digital images of these colonies were taken (Figure 3.11 and 3.12). Morphological appearance, characteristics of these suspected organism and literature review suggested that contamination of the product could be due to *Rhodotorula species* (Banzatto, de Freita, & Mutton, 2013; Wirth & Goldani, 2012).

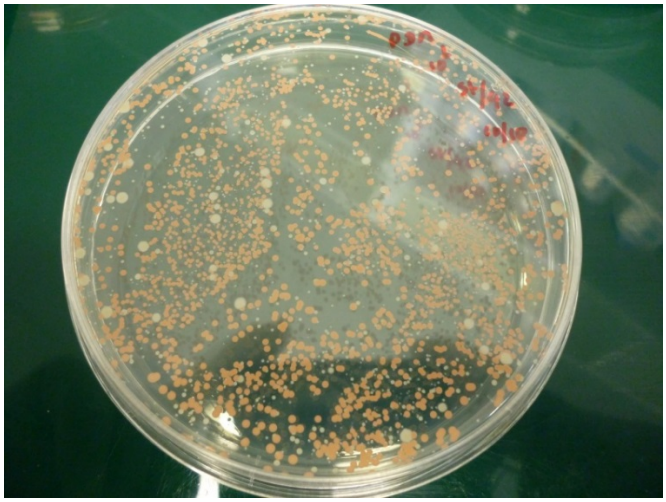

Figure 3.11 Overview of pink colonies found in the BBAN produced in the month of October 2013.

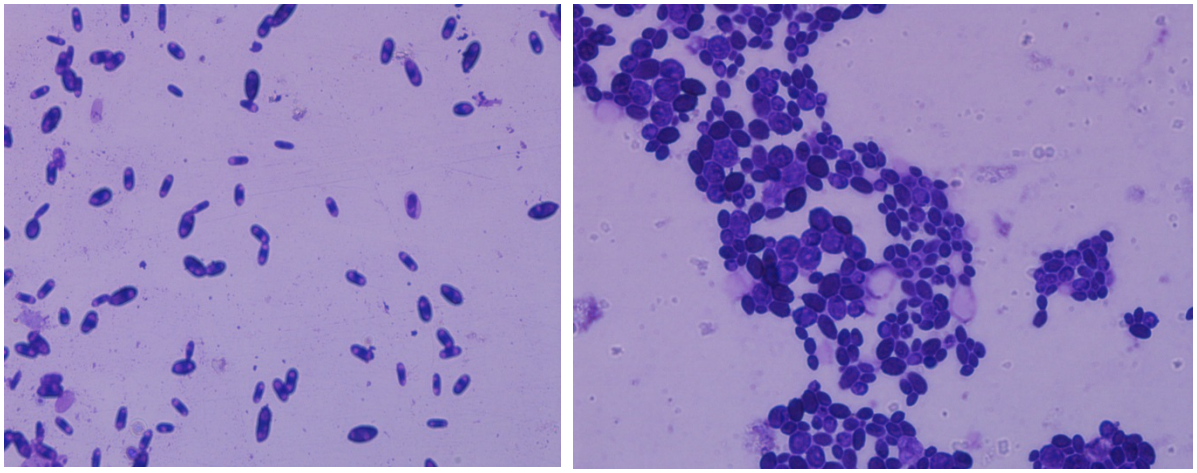

Figure 3.12 Digital images of suspected pink colonies tested under a microscope (×100)

Further literature review showed that BBAN might have been contaminated by *R. rubra (R. musigelaneous).* This would have been confirmed by using molecular technique and sequencing technique. This problem was observed 2 months after the study started. Limited time period forced to focus on how to manage this problem in the future.

3.3.8.1 Management of contamination

According to the literature, *R. rubra* has strong affinity with plastic containers and is prevalent everywhere (Wirth & Goldani, 2012). BBAN is decanted in different size of plastic bottles. Following steps were taken to manage the contamination in production sites.

Rescheduling the production technique

- All the production was temporarily suspended
- All the containers including IBC containers were soaked and washed by bleaching

- Once the production resumed, switched to two step fermentation in contrast to usual three step fermentation. This lasted at least for one month
- Unloading used containers were separated from production site and washed by bleaching before reuse.
- To avoid cross contamination in the laboratory
    - Fresh culture media was prepared
    - Fresh 0.1% peptone water was prepared
    - As usual all the tips, micro tubes, pipettes were autoclaved
    - Routine good lab practice was undertaken

3.4 Background of *R. rubra*

Rhodotorula is ubiquitous yeast species commonly found in water, air, soil, ocean and other environmental sources. *R. rubra* which produces characteristic pink to red colonies have long been considered as non-pathogenic and used in the carotenoid production (Wirth & Goldani, 2012).
Carotenoid which has anti-carcinogenic and pro vitamin properties have been successfully produced by *R. rubra* with different culture media such as molasses, sugarcane juice, and syrup as substrates (Banzatto et al., 2013). It should be noted that BBAN uses molasses as a substrate for microbial fermentation. Recent studies described *Rhodotorula* species have been identified as an emerging pathogen in human and animals. It is reported that these species are responsible for skin and lung infections in animals and fungemia disease in immunocompromised patients (Wirth & Goldani, 2012). Among farm animals there are reports of skin infection in chicken; lung infection and otitis in sheep and cattle (Table 3.1).

Table 3.1 Infection caused by *Rhodotorula* species in human and animals.

Species	Target organism	Disease	Reference
R. mucilaginosa	Human	Fungemia	(Wirth & Goldani, 2012)
R. glutinis		Endocarditis	
R.mucilaginosa (*R. rubra*)	Chicken	Skin infection	(Aruo, 1980)
	Sheep	Lung Infection	(Garg, 1980)
	Sea lion	Epididymitis	(Alvarez-Perez et al., 2010)
	Cat	Dermatitis	

3.5 Discussion

In recent years, application of fermented liquid feed in animal nutrition has been increased dramatically due to number of reasons. Using cheaper ingredients, aiming to reduce the use of antibiotics in animal feeds and use of environmental friendly ingredients are some of the reasons. BBAN is a fermented liquid probiotic widely used as a functional food in domesticated animal nutrition. Amongst different quality control parameters, viability of the probiotic strains is an important microbiological quality parameter of the probiotics (Tuomola et al., 2001).

Quality study for the samples collected continuously and periodically would ensure a consistently high-quality product. In our study we evaluated quality analysis of the BBAN with each stage of production until and beyond its expiry date. Samples collected routinely to determine the viability assessment.

In this study, we evaluated 23 batches of BBAN (from initial fermentation until its shelf-life) produced between August 2013 and March 2014 in Canterbury production site and several random samples from other production sites. More than 300 samples were analysed and Plate counting on Selective agar media were used to enumerate the colonies of LAB and yeast found in the formulation. LAB were cultivated on lactic acid medium and were enumerated based on colony morphology.

Although modern methods are rapid and able to produce higher number of cell counts (Vanhee et al., 2010) plate count method is used traditionally to quantify the viable cell numbers especially, it is preferable when higher number of samples for the routine determination of colony counting.

Exposure of LAB starter culture to the different substrates and fermentation involving 3 steps ensures viability of the LAB. Major end products of the bacterial fermentation such as lactic acid lower the pH and before the media reaches its minimum level of pH, the next stage of fermentation is undertaken. The volume and density of the culture media varied between different stages of production. Molasses was diluted with water to a final density (5% w/v) before mixed fermentations started. Gradual adaptation to the low pH environment and supply of enough substrate for the growth kept the LAB viable until its expiry date. Consistent pH throughout the storage period was recorded and pH range varied between 3.6-3.9.

Health benefits from probiotic use depend on how the viability of a product is maintained until its shelf-life. Determination of minimum dose of probiotics is critical to attain intended health benefit from probiotic use. Although a number of following factors determine minimum dosage, there is no internationally recognized regulatory body to determine the minimum dosage.

1. Target organism (probiotic for human use / animal use) dosage is host dependent as well as strain specific.

2. Target site of body part (for an example probiotics intended to use in stomach would need higher number of viable counts than the target site of mouth)

3. Strain used in probiotics - In order to get intended/given functional health benefits from probiotic use, dosage determination may be influenced by what strains used in the probiotic formulation and minimum dosage likely to vary with individual probiotics (Sanders et al., 2007).

4. Country of origin- expectation of minimum viable counts for the probiotic products vary with manufacturing country. Recommended level of viable counts of microbes in commercially available probiotics in Canada is 10^9 CFU per serving (CFIA, 2009) whereas minimum (bacterial counts) quantitative requirement of the Chinese national standard for the probiotic product remain 10^6 CFU/g (Chen et al., 2014).

Although the viable cell count level required to guarantee the health benefits from probiotic use has not been conclusively established (Reid, 2008), in general, probiotic formulations have been demonstrated to be efficacious, when the product contained total viable cell counts greater than

10^6-10^8 CFU/ml at the end of its shelf-life (Champagne et al., 2011).The average total number of cultivable LAB was found to be 10^{10} - 10^{11} CFU/ml for all the BBAN analysed between several batches. Log number of viable lactobacilli counts did not significantly differ between various storage periods until its expiry day and average cell counts were observed 11.24 ± 0.12 log CFU/ml at the end of shelf-life. Viability of LAB strains contained in all BBAN samples measured by traditional methods remained above 10^{10} cells per ml until their expiry dates. Hence, this microbial cell counts are substantially greater than the suggested minimum dose for probiotic effect.

Selection of multi-strain and multi-species for probiotic product development enhances the protective range and acts as a broad spectrum product. In order to diversify the beneficial effects using multi-strain and multi-species, BBAN consist of Lactobacilli and yeast strains in the product. Yeast as a single culture is used in Initial and step 2 fermentation and average cell concentration reaches to 9.5 - 10 log CFU/ml. However, population of yeast started to decrease when it was used as co-culture in mixed fermentation. There have been reports of positive and negative influence between LAB and yeast on their growth by each other. Positive interaction by yeast on LAB includes production of amino acid and vitamin synthesis (Roostita & Fleet, 1996) as well as production of CO_2, pyruvate, succinate and propionate which have stimulating effect on LAB growth (Leroy et al., 1993). On the other hand, galactose released by some LAB species may favour the growth of lactose-negative yeasts (Marshall & Tamine, 1997). Though their mechanism of interaction is not well understood, it is obvious, the growth of yeasts was significantly affected in co-culture and the yeast species reached between 5.47 log CFU/ml and 8.02 log CFU/ml over the period of storage.

In our study, stability assessment of bacterial counts over the storage period did not statistically differ and plate counts remained above 10^{10} cell counts/ml until their expiry days. Hence, the LAB growth seems to be not affected by the yeast in the co-culture and rather, positive interaction overwhelms negative influence of yeast. Results obtained from other production sites are in conformity with the results obtained from main production site. Mutual inhibition of growth has been reported as negative interaction between these two cultures. Compounds such as phenyl-lactic acid, 4-hydroxy-phenyl-lactic and cyclic peptides secreted by LAB have been reported to have inhibitive effect on yeast growth (Nielsen et al., 1998); while fatty acids produced by the metabolism of lipolytic yeasts have been affected the growth of LAB (Broome et al., 1979).

Assessing BBAN for the stability of the yeast counts reduced by half amount at the end of its shelf life. It should be noted that the MSL of viable counts in the probiotics at the time of consumption should be approximately 10^6 CFU/ml (Adhikari et al., 2003; Vinderola et al., 2000). Average viable counts of yeast colonies were just below the MSL after one week of storage period towards the end

of shelf-life. An average log reduction in the number of cells 5.82 and 5.47 were obtained during 2nd and 4th week of storage. Although yeast counts in BBAN slightly lower than the MSL, combination of LAB and yeast acts synergistically to provide health benefits. Combination of low pH produced by the LAB plus the alcohol and CO_2 produced by the yeasts collectively has inhibitory effect against many undesirable microorganisms (Ferreira & Viljoen, 2003). Plate count method enabled the enumeration of only viable yeast counts however, detection of viable non-culturable microbes by Solid Phase Cytometry (SPC) have also shown probiotic effects when the probiotics containing *S. boulardii* tested for quality analysis (Vanhee et al., 2010). Secretion of protein involved in inhibition of *Vibrio cholerae* toxin (Czerucka et al., 1989) and increased production of the brush border disaccharidases (Buts & de Keyser, 2006) are some of the examples of probiotic effects by the viable non-culturable cells.

Viability results obtained from the other production sites showed conformity with the Canterbury production site. All of the production sites had similar pattern of survival rate of LAB and yeast in the product. Assessment of stability of the product with extended storage period (up to 6 weeks) had interesting findings. While LAB counts were similar to 4th week of storage, yeast counts were found below the MSL at the end of 6th week.

The safety assessment results of this study demonstrated the BBAN have not been contaminated by general foodborne bacterial pathogens. However, contamination by *R. rubra* was successfully identified over the period of study. Conditions favourable for this contamination was effectively identified and eliminated. Similar contamination issue was not reported again during the period of study

3.6 Conclusions

Final product of the BBAN had more than recommended number of LAB viable counts irrespective of the storage days. However, yeast cell densities was higher in the first week of storage and started to decline towards the end of expiry date. Overall yeast counts in the product were just below the MSL. Viability results obtained from the other production sites showed conformity with the Canterbury production site. Viable LAB counts in the BBAN with extended storage (6 week) were similar to 4th week of storage and yeast counts were found below the MSL at the end of 6th week.

The safety assessment results of this study demonstrated the BBAN have not been contaminated by general foodborne bacterial pathogens. However, contamination by *R. rubra* was successfully identified over the period of study. Conditions favourable for this contamination was effectively identified and eliminated.

In conclusion, the quality of the BBAN in terms of viability and safety satisfied the requirements for quality parameters.

Chapter 4

Metabolite profiling

4.1 Introduction

The production of metabolites by different probiotic microorganisms is considered to be the main mode of action against pathogenic organisms present in the GIT as well as in food. Probiotics exert health benefits to the host organism through the production of metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetoine, carbon dioxide, reuterin, reutericyclin, bacteriocins and short- chain fatty acid (SCFA) (De Jong & Badings, 1990; Lindgren & Dobrogosz, 1990). Metabolites such as organic acids (lactic acid and acetic acid) carbon dioxide, ethanol hydrogen peroxide, diacetyl, acetaldehyde, acetoine, reuterin, and reutericyclin are categorized as low mass molecules whereas bacteriocin, one of the important metabolites and has extensively been used in the food industry as biopreservative, has been categorized as a metabolite with high molecular mass (Suskovic et al., 2010).

The most important and best characterized antimicrobial metabolites produced by LAB are lactic acid and acetic acid. The effect of lactobacilli on *C. jejuni* growth demonstrated that the concentration of lactic acid produced by lactobacilli was sufficient to kill the foodborne pathogen in broiler chicken (Neal-McKinney et al., 2012). Furthermore, the combination of metabolites produced by *Lb. plantarum* was effective in reduction of *Enterobacteriaceae* in the GIT of piglets (Thu et al., 2011). Yeasts have shown to exhibit antagonistic activities against undesirable bacteria and fungi by producing antimicrobial compounds such as antifungal killer toxins or “mycocins” and antibacterial such as ethanol and CO_2 (Hatoum et al., 2012). The BBAN contains both LAB and yeast culture. Low pH from LAB fermentation and the alcohol and CO_2 produced by the yeasts in combination have shown to be responsible for inhibition of many undesirable microorganisms (Ferreira & Viljoen, 2003).

SPME/GC-MS represents a novel method for studying metabolic profiles of biological samples. The aim of this investigation was to determine the volatile and non-volatile organic compounds found in the BBAN and to profile the diversity and nature of these compounds.

4.2 Materials and method

4.2.1 Materials

4.2.1.1 Non-volatile metabolites

a) Lactic acid assay

1. Buffer (25 ml, pH 10.0) + D-glutamate and sodium azide (0.02% w/v)

2. NAD^+ (contents was dissolved in 5.5 ml distilled water)

3. D-Glutamate-pyruvate transaminase suspension (D-GPT)

4. L-Lactate dehydrogenase suspension (L-LDH)

5. D-Lactate dehydrogenase suspension (D-LDH)

6. D-/L-Lactic acid standard solution (5 ml, 0.15 mg/ml of each) in 0.02% (w/v) sodium azide

b) Ethanol assay

1. Buffer (15 ml, pH 9.0) + sodium azide (0.02% w/v)

2. NAD^+ -Dissolve the contents in 12.4 ml of distilled water.

3 Aldehyde dehydrogenase solution

4. Alcohol dehydrogenase suspension

5. Ethanol standard solution (5 ml, 5 mg/ml) - 0.5 ml standard solution was diluted with 50 ml distilled water.

4.2.1.2 Volatile metabolites

a) Internal Standard (IS) - Cis-3-hexenyl acetate

Cis-3-hexenyl acetate (0.1122 g) 98% (Sigma Aldrich, New Zealand) was dissolved in 100 ml of 10% acetone in deionised water to form a stock solution. This was further diluted 20 times in deionised water to obtain a final concentration of 0.055 g/l.

b) Buffer -Tartaric acid

2.5 g of L-Tartaric acid ACS grade 99.5% (Sigma Aldrich, New Zealand) was dissolved in 500 ml of distilled water and adjusted to pH 3.5 with 4M sodium hydroxide.

c) Solvent

Diethyl ether high purity grade 99.5% (BDH Prolabo, Belgium).

d) Sulphuric acid (50%)

Analytical grade H_2SO4 (50 ml) was added to distilled water (50 ml) and stored at room temperature.

e) Sodium chloride

Crystalline sodium chloride AR grade (Merck, New Zealand).

4.2.2 Methods

4.2.2.1 Sampling

Samples that were collected for microbiological quality testing was preserved in the freezer were used for metabolite studies. Samples at many stages of production, different storage period and samples from other manufacturing sites were used for this study. In addition, samples from control batches at many stages of production and different storage periods were also used for metabolite profiling studies. Stage 1 of control batch was prepared at Lincoln university laboratory and rest of the process made at Canterbury production site. Thus, two control batches were produced to compare the test results with the usual BBAN products.

4.2.2.2 Non-volatile organic compounds

a) Sequential D- and L-lactic acid assay

Sequential D-/L-lactic acid (rapid) assay method (Megazyme International, Ireland, 2012) was used to determine the D- and L-lactic acid concentration in the selected BBAN samples according to the manufacturer's procedure. Samples collected from different sites with various stages of production were diluted 50 times. 1.5 ml distilled water, 0.5 ml buffer solution, 100 µl NAD^+ and 20 µl D-GPT were added to the cuvette containing 100 µl sample. Instead of sample, 0.1 ml distilled water and 0.1 ml standard solution was used as blank and control respectively. The absorbance at wavelength of 340 nm was recorded after 3 min of reaction (UVmini-1240 Spectrophotometer, Shimadzu). Subsequently, 20 µl D-LDH suspension and 20 µl L-LDH suspension were added and left for 5 and 10 min interval, respectively. The absorbance was recorded and concentration of D- and L-lactic acid was determined from the calculation made according to the manufacturer' instructions.

b) Ethanol assay

Ethanol assay procedure (Megazyme International, Ireland, 2012) was used to determine the concentration of ethanol present in the BBAN samples. Distilled water (2 ml), 200 µl of buffer solution, 200 µl of NAD^+ and 50 µl of aldehyde dehydrogenase solution were added to the cuvette

containing 100 µl sample which was diluted 100 times. Blank and control were prepared by adding 100 µl of distilled water and 100 µl of ethanol standard solution, respectively. Cuvette was allowed for 2 min and the absorbance at wavelength of 340 nm was recorded (UVmini-1240 Spectrophotometer, Shimadzu). Subsequently, 20 µl of alcohol dehydrogenase suspension was added and spectrophotometer reading was taken after 5 min. Concentration of ethanol was calculated according to the manufacturer manual instructions.

4.2.2.3 Volatile Organic Compounds (VOC)

Two GC–MS extraction methods were trialled to determine which method was most effective in profiling the volatile metabolites present in the BBAN samples. Of the two extraction methods headspace solid phase micro-extraction (HS-SPME) was found to be more sensitive and efficient in metabolite profiling compared to the solvent extraction methodology proposed.

a) Extraction of volatile metabolites with organic solvents

In order to extract volatile compounds from the sample, 5 ml of BBAN sample was centrifuged and the supernatant was taken into a 10 ml glass tube. NaCl (4 g) and 2 ml of 50% H_2SO_4 was added and mixed well. Volatile compounds were extracted in 5 ml of diethyl ether high purity grade 99.5% (BDH Prolabo, Belgium) added to the glass tube and inverted 20 times. Air was then used to dry the ether layer concentrating this from 5 ml to 2 ml.

The concentrated 2 ml ether layer was then transferred to a 2 ml gas chromatography vial and kept in a range hood for air drying until the final volume was 0.5 ml. It was then immediately capped and sealed with para film for analysis.

b) Headspace Chromatography /SPME

Samples collected at many stages of production, different storage periods and from different manufacturing sites were kept in the freezer at – 20 ^{o}C. Samples kept in the freezer were allowed to thaw in the fridge overnight at 4 ^{o}C. The following day samples from the fridge were centrifuged for 5 min and 10 ml of supernatant were transferred to 15 ml centrifuge tubes ready for HS-SPME GC-MS analysis. Cis-3-hexenyl acetate (0.055 g/l) was used as an internal standard (IS).

Sample preparation significantly influences the HS-SPME extraction sensitivity and subsequent peak shape of volatiles on the GCMS. A reliable and repeatable analysis was required. To optimise the technique different parameters such as sample volume, concentration of IS, headspace volume, extraction temperature and extraction time were examined.

Comparison of initial sample extractions showed improved chromatographic performance with reduced sample volume minimising peak overloading. As such the IS amount added was reduced to ensure its peak area did not dominate. It was decided that 1 ml of sample in a total volume of 9 ml was the best extraction sample volume to use. Further tests also showed that increased extraction temperature and time resulted in improved sensitivity (Kopsahelis et al., 2009). Hence 60 ^{o}C for 45 min was used instead of 30 ^{o}C for 30 min (initial experiments) in all subsequent sample analyses.

4.2.2.4 GCMS parameters for headspace analysis

The analysis of volatile compounds was determined using an automated HS-SPME GCMS technique, based on the work of Kopsahelis et al. (2009). This adapted method utilised the addition of cis-hexenyl acetate as an IS and involved sample dilution in a tartaric acid buffer to achieve consistent HS-SPME extraction between sample treatments and adequate chromatographic conditions for the VOC's detected.

Sample preparation involved pipetting 1ml of BBAN into 20 ml SPME sample vials followed by 7.98 ml of tartaric acid buffer (5 g/l, pH 3.5), 20 μl of internal standard solution (55 mg/l) and 3 g of crystalline sodium chloride immediately prior to capping. Samples were then placed in a sample tray held at 8 °C awaiting extraction. Samples were incubated for 45 min at 60 ^{o}C with their enclosed headspace exposed to a 2 cm long DVB/CAR/PDMS combination SPME fibre (p/n 57348-U, 50/30 μm thickness, 24 gauge, Supelco Bellefonte, PA, USA, through Sigma- Aldrich, Australia). During this exposure period the headspace volatiles, were adsorbed onto the fibre. Desorption of these volatiles occurred when the SPME fibre was inserted into the GC injection port for 10 min at 250 ^{o}C.

GCMS analysis was carried out on a Shimadzu GCMS-QP2010 gas chromatograph–mass spectrometer (Shimadzu Scientific Instruments Inc, Japan) equipped with a Combi-Pal autosampler (CTC analytics AG, Switzerland) ready for automated SPME. GCMS solutions version 2.7 was used as the data acquisition software. The chromatography was performed using an Rtx-Wax 60.0 m x 0.25 mm ID x 0.25 μm film thickness (Polyethylene Glycol - Restek, Bellefonte, PA, USA) GC column. Helium was used as the carrier gas with the GCMS set to a constant linear velocity of 29.1 cm/sec. The injector was operated in splitless mode for 5 min then switched to a 10:1 split ratio.

The column oven was held at 40 ^{o}C for 3 min (during desorption of the SPME fibre), then heated to 220 ^{o}C at 4 ^{o}C /min followed by a final ramp to 250 ^{o}C at 30 ^{o}C / min and held at this temperature for 11 min, total run time was 60 min. The interface and MS source temperatures were set at 250 ^{o}C and 230 ^{o}C respectively. The MS was operated in electron impact mode (EI) at an ionization energy of 70eV.

All analytes were analysed in full scan mode with selected VOC peaks (metabolites) integrated for semi-quantitative analysis utilising the IS present. Identification of VOC compounds was made using the NIST 11 (National Institute of Standards and Technology) and Wiley 10 (John Wiley & Sons Inc.) mass spectral libraries. Peak identifications were confirmed using published retention indices (Umano et al., 1999), (Lee & Shibamoto, 2000), (Acree & Arn, 2004), (El-Sayed, 2014) for wax (polyethylene glycol) GC columns.

4.3 Results

4.3.1 Non-volatile metabolites

Colorimetric enzymatic assays were used to evaluate lactic acid and ethanol concentrations in the BBAN samples. Lactic acid and ethanol assays were studied for samples of control batches 1 and 2, Canterbury, Waikato and Balclutha. Ethanol concentration was higher (400 mg/100 ml) when the product was 1 day old (ST1) and decreased when storage days increased in control 1 (Table 4.1). The ethanol concentration was 205 mg/100 ml when the product was 4 weeks old (ST28). Similarly ethanol concentration in control 2 was found to be 646 mg /100 ml and 205 mg/100 ml in ST1 and ST28 respectively. Total lactic acid concentration in control 1 and 2 varied with different storage days. While total lactic acid concentrations were 230 mg/100 ml (ST1) and 373 mg/100ml (ST28) in control 1, it was evaluated 346 mg/100 ml (ST1) and 230 mg/100 ml (ST28) in control 2. Total lactic acid and ethanol content were higher in the Lot 2 products of control batches. For example, total lactic acid and ethanol concentrations were 635 mg/100 ml and 482 mg/100 ml in lot2 ST28 of control 2.

The amount of lactic acid and ethanol were found to be higher in all other tested samples than control samples. Of tested samples, results obtained from control batches 1 and 2 showed better conformity than others. Ethanol, a primary end product of yeast fermentation, was higher in fresh products and decreased when storage days increased. Overall ethanol content in control samples were below 1% v/v. Samples collected from Canterbury and other production sites with different storage days contained more than 1% of ethanol which did not decrease with increasing storage days (Table 4.2). Samples that contained more than 1000 mg/100 ml ethanol concentration included Canterbury ST7 (1133 mg/100 ml), Canterbury L3 ST28 (1436 mg/100 ml), Canterbury ST28 (1077 mg/100 ml) and Waikato ST28 (1048 mg/100 ml).

Most the samples tested for lactic acid concentration showed that BBAN products contained higher percentage of L-lactic acid than D-lactic acid. However, samples of control batches such as Control 1 ST07, Control ST07 and ST28 showed higher amount of D-lactic acid than L-lactic acid. Higher lactic

acid concentration was obtained from the Canterbury L1 ST21 (573 mg/100 ml) while lowest lactic acid content was measured from control ST07. It seemed storage days and different manufacturing sites did not have any influence on lactic acid content. Control 1 ST21 (541 mg/100 ml), Canterbury ST21 (573 mg/100 ml) and Canterbury ST28 (501 mg/100 ml) were the samples measured with highest amount of lactic acid content.

Control batches produced under the laboratory condition. Production of Lactic acid and ethanol in the BBAN were higher in usual products than that of products produced in the laboratory environment. The mechanism of interaction between both yeast and LAB is not well understood.

Table 4.1 Lactic acid and ethanol concentrations in the final product (Control batch 1 and 2) at different sampling times during storage.

Control 1	Ethanol mg/100 ml	Ethanol % (v/v)	D-lactic acid mg/100 ml	L-lactic acid mg/100 ml	Total lactic acid mg/100 ml
ST 1	400	0.51	70	160	230
ST07	329	0.42	88	44	132
ST14	289	0.37	253	160	413
ST28	205	0.26	255	118	373
Control 2					
ST 1	646	0.82	120	226	346
ST7	421	0.53	163	102	265
ST28	378	0.26	151	79	230
Lot2 ST 1	304	0.39	96	260	356
Lot2 ST 21	581	0.74	173	368	541
Lot 2 ST28	635	0.80	170	312	482

ST: Storage; L: lot.

Table 4.2 Lactic acid and ethanol concentrations in BBAN samples collected at various production sites.

Canterbury	Ethanol mg/100 ml	Ethanol % (v/v)	D-lactic acid mg/100 ml	L-Lactic acid mg/100 ml	Total lactic acid mg/100 ml
140123 L1 ST7	1133	1.43	208	220	428
140123 L1 ST21	985	1.25	244	330	573
140123 L3 ST28	1436	1.82	168	170	338
140107 L 2 ST14	613	0.78	127	184	311
140107 L 1 ST28	808	1.02	130	208	338
140306 ST7	956	1.21	26	229	255
140306 ST28	1077	1.36	157	344	501

Different sites					
Waikato ST07	876	1.11	98	207	305
Waikato ST28	1048	1.33	187	213	401
Balclutha ST07	802	1.02	106	168	274
Balclutha ST14	815	1.03	107	186	293
Balclutha ST28	823	1.04	173	234	407

4.3.2 Volatile Metabolites

Analysis of metabolites in animal probiotics containing LAB and yeast is important since some metabolites produced by these organisms can have a positive effect in stimulating bacterial growth in the digestive tract and optimizing feed intake. The detection of metabolites in molasses fermented biological samples from mixed cultures of yeast and LAB has not been previously reported. The metabolite profiling study of BBAN was initially conducted using a liquid solvent extraction method (De Jong & Badings, 1990) as described in 4.2.2. The GCMS Chromatograms obtained showed that the number of peaks observed was very low, only 5 peaks were detected. Since this method was not considered effective, headspace analysis (HS-SPME-GC/MS) was investigated as an alternative using a low temperature extraction of 30 °C and an arbitrary incubation time of 30 min. Figure 4.1 shows a HS-SPME chromatogram for a stage 3 BBAN sample with many more peaks detected compared with the liquid solvent extraction trialled.

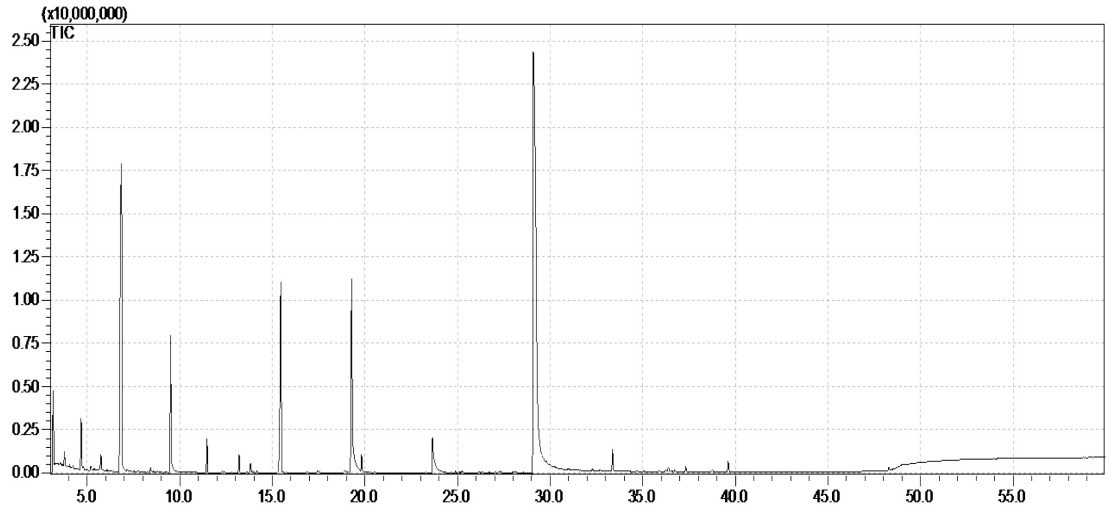

Figure 4.1 Typical HS-SPME/GC-MS chromatograms of a stage 3 BBAN sample.

Peak overloading occurred for two compounds, namely ethanol and butyric acid, when sample volumes of 2 ml or more were used. Hence a sample volume of 1 ml was adopted to improve the quality of these two peak shapes by reducing the column overloading. The amount of IS added was also investigated with the amount added set at 20 µl or a concentration of 122.2 µl in the 9 ml vial solution. These initial HS-SPME extraction conditions were used as BBAN products were considered biologically active, meaning that higher extraction temperatures would likely produce volatiles that were not originally in the samples. In the resulting chromatograms detected peaks whose similarity match was more than 90% were considered, identified according to the NIST 2011 and Wiley 10 mass spectral databases. These compound identities were then confirmed using published kovats indices for polyethylene glycol (wax) GC columns (www.pherobase.com), (www.chemspider.com), (www.flavornet.org), (Lee & Shibamoto, 2000). Although chromatograms obtained, showed vastly more peaks were detected, 34 detected in sample (Control 1 Stage 3 Day 3) and only a subset of these were identified and subsequently confirmed using published kovats indices. In this example, 31 compounds were identified with only 19 identities confirmed and about 18 compounds were identified as metabolites (Figure 4.2).

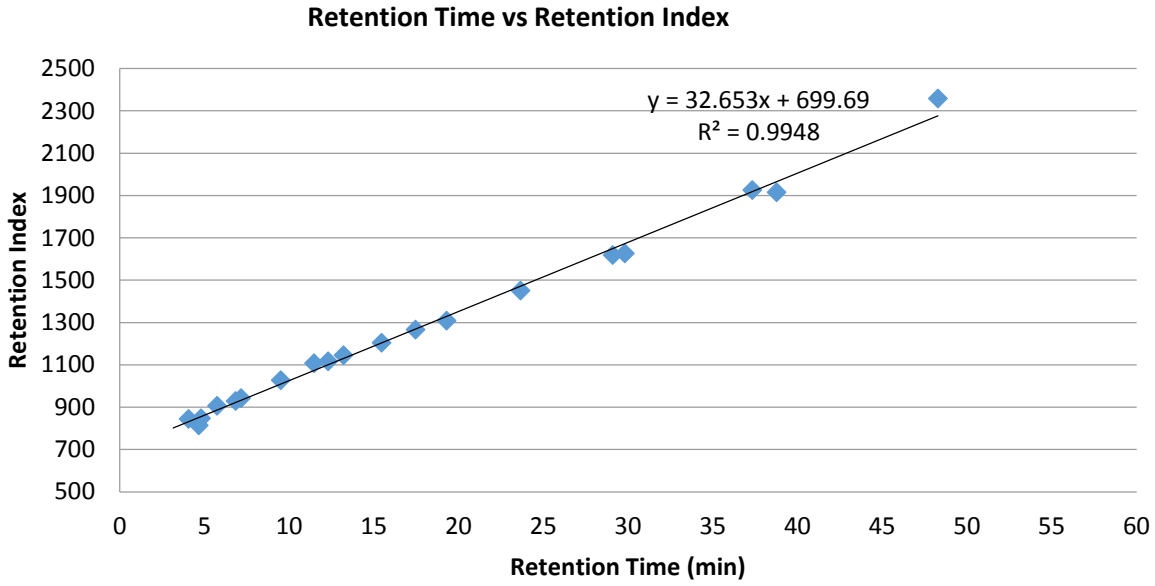

Figure 4.2 Confirmation of metabolites using Kovats indices for sample (Control 1 S3 D3) when vial temperature and extraction time were 30 °C and 30 min respectively.

Not satisfied that earlier assumptions concerning extraction temperatures were valid, other HS-SPME extraction conditions were investigated to see whether more metabolites would be detected. The HS-SPME extraction method used by Kopsahelis et al., (2009) was trialled with some

modifications, 1 ml of sample was used for extractions rather than the 10 ml as described by these authors. Keeping all other sample parameters the same as the initial method employed but increasing the extraction temperature and time, (60 °C for 45 min) resulted in more volatile and semi-volatile metabolites being detected. This method was found to be very effective in detecting metabolites. For example when the BBAN sample from Canterbury (ST10) was analysed, 140 peaks were detected, 100 of these were identified with 72 compound identities confirmed using published kovats indices (Figure 4.3).

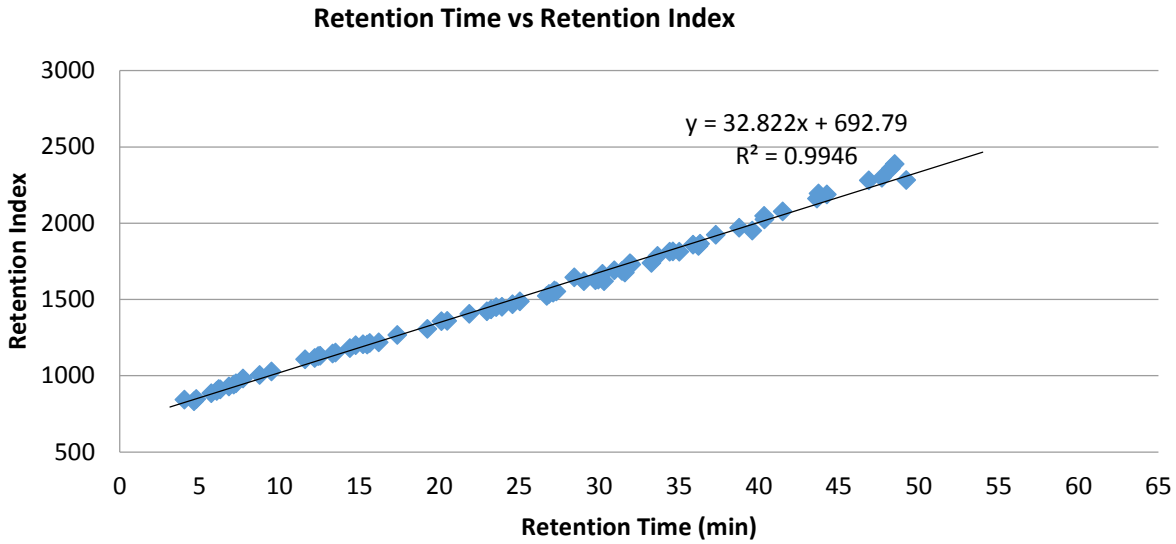

Figure 4.3 Confirmation of metabolites using Kovats indices when vial temperature and extraction time were, 60 °C and 45 min (1 ml of sample).

Of these confirmed, identified volatile compounds only a subset of these could be considered metabolites. In order to identify the metabolites derived solely from the yeast and LAB fermentation, samples containing unfermented molasses (stage 3 blank) were ran through the HS-SPME-GC/MS method. Metabolites exclusively from the BBAN products were identified by comparing the unfermented molasses samples with the fermented analogues. A total of 40 different metabolites belonging to the chemical class of organic acids, alcohols, esters, carbonyl compounds, sulphur compounds and miscellaneous compound were detected (Table 4.3).

In the current study BBAN samples were collected from different production sites (Canterbury, Waikato and Balclutha) at many stages of production (Stage 1, 2 and 3 and storage days). An initial study looking at the stages of production of the Canterbury site and utilised the initial HS-SPME/GC-MS extraction method of 30 °C for 30 min. From this study a smaller list of 32 metabolites was obtained (Appendix A and B). A second study then looked at a total 19 BBAN samples of final product collected at Canterbury (13 samples), Waikato (3 samples) and Balclutha (3 samples)

production sites with different storage days, were analysed using the HS-SPME/GC-MS methods described in 4.2.2.2 as this was found to be the most effective in the extraction of volatile compounds from these biological samples. Metabolites were profiled in these samples using the 40 identified metabolites listed in Table 4.3.

Table 4.3 Volatile metabolites from BBAN isolated by the SPME/GC-MS, categorized according to chemical class.

Chemical Class	Metabolite	KI published	Chemical Class	Metabolite	KI published
Ester	Ethyl formate	848	***Alcohols***	Ethanol	929
	Ethyl acetate	886		1-propanol	1040
	Ethyl propanoate	950		1-Butanol	1145
	Ethyl butyrate	1028		Isoamyl alcohol	1205
	Ethyl hexanoate	1220		1-hexanol	1360
	Butyl acetate	1105		3-octanol	1406
	Isoamyl acetate	1117		1-Octanol	1553
	Isobutyl butyrate	1152		2-pentanol	1130
	Isoamyl butyrate	1267		Isobutanol	1108
	Phenyl butyrate	-		Eugenol	2161
	Methyl salicylate	1786		(Z)-isoeugenol	2298
	Phenyethylbutyrate	1930		Menthol	1626
				Benzyl alcohol	1865
Organic acid	Acetic acid	1450		Phenyl ethanol	1925
	Butyric acid	1619		Phenol	1951
	Octanoic acid	2083		p-Ethylguaiacol	2048
	Hexanoic acid	1829		p-Cresol	2077
				4-ethyl-phenol	2195
Carbonyl compounds	Diacetyl	977			
	(3E)-3-penten-2one	1128	***Sulphur compounds***	Dimethyl sulfide	844
	gamma-nonalactone	2024		Methionol	1738
			Miscellaneous compound	2,5-dimethyl- furan	943

(http://www.chemspider.com, http://www.flavornet.org, http://www.pherobase.org, (Lee & Shibamoto, 2000))

The volatile compounds from both studies were tentatively quantified using the technique described by Kopsahelis et al., (2009) where the peak areas of the compounds of interest were divided by the peak area of the IS then multiplying this ratio by the initial concentration of the IS (expressed as µg/l). Concentrations reported in Appendix B, C and Table 4.4 are pseudo concentrations based solely on their comparison to the concentration of the IS. In SPME analyses such concentrations are indicative as the only way to determine an accurate concentration using this extraction technique is

to use internal standards that are chemically similar to each of the analytes of interest such as deuterated analogues. From the 42 metabolite compounds detected in the second study (Appendix D), 10 were selected as the major metabolites and these were used to compare BBAN samples from different sites (Table 4.4). All peak areas were obtained from the full scan chromatograph using the total ion current (TIC).

Table 4.4 Relative amounts (µg/l) of major volatile compounds identified by HS-SPME/GC-MS from different BBAN samples

Metabolite Compounds	KI published	RT (min)	Bal ST7	Waik ST7	Ctrl1 ST7	140123 ST7	140306 L2 ST7	Ctrl 2 ST7	Ctrl 1 ST 14	Balc ST14	Waik ST14	140107 L2 ST14
Ethyl acetate	886	5.72	51.4	96.3	26.6	135.9	75.7	56.5	43.7	100.6	82.1	146.5
Ethanol	929	6.84	1073.7	1212.0	969.4	1530.7	966.3	1215.5	1137.1	1429.6	1294.3	1031.7
Ethyl butyrate	1028	9.49	382.7	551.0	375.5	496.0	428.1	417.3	501.0	469.7	656.2	571.9
Isobutanol	1108	11.60	40.3	50.2	23.0	40.4	33.3	30.8	28.6	32.3	36.5	38.4
Isoamyl acetate	1117	12.20	16.8	38.1	9.8	31.2	20.5	20.6	14.0	14.7	43.4	30.3
Isoamyl alcohol	1205	15.48	372.7	530.2	266.3	485.8	317.6	396.6	324.4	421.0	591.2	376.4
Acetic acid	1450	23.56	285.1	230.7	251.5	406.2	320.2	315.0	336.4	369.2	109.6	458.6
Butyric acid	1619	29.03	3132.6	2179.5	3195.2	2781.4	2797.6	3304.2	3527.0	3327.7	2517.3	2610.5
Phenylethyl Alcohol	1925	37.29	184.9	268.4	104.5	340.5	152.8	194.3	142.9	215.0	325.7	168.8
Phenol	1951	39.57	114.6	6.4	124.3	122.5	90.4	115.6	167.1	127.5	6.1	68.1

Metabolite Compounds	KI published	RT (min)	Ctrl 2 ST 28	140107 ST28	140306 ST28	Ctrl1 ST28	Ctrl 2 L2 ST28	Waik ST28	140123 L3 ST28	Balc ST28	140107 ST42
Ethyl acetate	886	5.72	92.4	192.3	134.1	64.7	96.3	219.5	433.6	115.7	228.6
Ethanol	929	6.84	949.1	1084.0	1069.3	913.5	850.7	1657.1	1230.0	1177.8	975.2
Ethyl butyrate	1028	9.49	506.1	612.7	629.1	476.9	464.2	798.9	73.4	555.4	682.3
Isobutanol	1108	11.60	32.8	33.4	39.5	31.7	12.5	47.8	92.7	40.7	29.3
Isoamyl acetate	1117	12.20	21.9	32.8	32.4	13.6	17.8	75.4	120.7	25.6	38.2
Isoamyl alcohol	1205	15.48	312.7	390.5	357.0	267.6	170.7	677.0	781.7	380.3	341.1
Acetic acid	1450	23.56	289.1	445.5	392.5	293.5	466.4	361.5	361.0	401.6	458.1
Butyric acid	1619	29.03	2738.0	2568.1	3017.4	3074.5	2812.0	2617.6	229.2	3221.1	2331.3
Phenylethyl Alcohol	1925	37.29	156.9	174.5	183.6	115.3	58.6	364.5	408.4	197.5	157.8
Phenol	1951	39.57	93.5	72.5	112.5	137.2	111.1	8.7	9.6	117.3	66.7

RT: Retention Time; Waik: Waikato; Balc: Balclutha; Ctr1: Control 1; Ctr2: Control 2; L1, L2 and L3: Lots; µg/l Rel IS: Is a comparison to the concentration of the internal standard in the vial (122.2 µg/l) corrected for the dilution factor (X9); Amounts stated are indicative only and are not the true concentration (http://www.flavornet.org, http://www.pherobase.org, http://www.chemspider.com, (Umano et al., 1999),(Lee & Shibamoto, 2000)).

4.3.2.1 1st Study results

An initial study mainly focused on looking at the stages of production of the Canterbury site. As we discussed in 3.2.2 production cycle consisted three steps. Sugar was used as a substrate for yeast and LAB fermentation in stage 1 of production and very few volatile metabolites were detected. Ethanol (1987.5 µg/l) and isoamyl alcohol (1288.3 µg/l) were major end products in the yeast fermentation of stage 1 (S1 D1 Y) followed by isobutanol (176.2 µg/l), ethyl acetate (87 µg/l), diacetyl and phenyl ethanol (Appendix C).

However, the metabolite profile was different in the LAB fermentation (S1 D1 L) where acetic acid and diacetyl were the major metabolites detected. The use of molasses as a substrate and an increasing number of fermentation days may have been attributable in the detection of a larger number of volatile metabolites in the stage 2 and 3 fermented products (S2 D3 Y, S2 D3 L and S3 D3 - Appendix C). The number of metabolites and their relative amounts increased in stage 2 where molasses was used as a substrate. This was seen in the yeast samples from the stage 2 fermentation where butyric acid, acetic acid, dimethyl sulphide, ethyl butyrate, isoamyl butyrate, and phenol were found in addition to those identified in stage 1. Moreover, metabolites already identified in stage 1 were detected in higher quantities i.e. ethanol (3364.9 µg/l), isoamyl alcohol (3121.1 µg/l), ethyl acetate (615.3 µg/l) and isobutanol (327.7 µg/l).

Butyric acid (4154.6 µg/l) was detected in higher quantity in the stage 2 LAB fermented product (S2 D3 L) followed by acetic acid (103.8 µg/l), 1-butanol (185 µg/l), ethanol (60.4 µg/l) and phenol (53.2 µg/l). The largest number of metabolites were detected in the stage 3 mixed fermentation samples however quantities seen varied compared with samples from yeast or LAB alone. This showed that the metabolites produced in stage 3 mixed fermentation were not simply a summation of the quantities found in the single yeast and LAB stage 2 ferments. Here only acetic acid and ethyl butyrate exhibited quantities higher in stage 3 than stage 2 (Appendix C). Thus, it appears that the number of brewing days, the formulation and culture types influence metabolite production in the BBAN products.

4.3.2.2 Second Study results

A second study was undertaken to determine what differences if any, there were in the volatiles metabolites found in BBAN samples manufactured in Canterbury, Waikato and Balcultha at the various stages of production. To provide focus the 10 most abundant volatile metabolite compounds were selected for examination in this second study (Table 4.4). These were organic acids (acetic acid and butyric acid), alcohols (ethanol, isobutanol, isoamyl alcohol and phenylethyl alcohol), esters

(ethyl acetate, ethyl butyrate and isoamyl acetate) and phenol. It is important to note here that although these metabolites were selected due to their abundance, the degree to which they were detected was influenced by the SPME fibre type and the extraction conditions used. The abundance of a compound in the headspace of a vial is due in part to the boiling point/vapour pressure and polarity of that compound at the extraction temperature. The solid phases of the SPME fibre preferentially adsorb compounds present in the headspace based on the selectivity of their solid phase chemistry, with the DVB/CAR/PDMS fibre used best suited to the detection of a wide variety of compounds.

Two control batches along with production stage 3 samples taken from Canterbury, Waikato and Balclutha (varying storage times, 7-28 days) were analysed for volatile metabolites (Appendix D). Also included in this 19 sample study were two BBAN variant products from Canterbury, namely batch 140123 L3 ST28 (lot 3, 4 weeks production) produced using a starter culture which was more than 10 days old, and sample batch 140107 ST42 with an extended storage time of 6 weeks. These were analysed according to the HS-SPME extraction method described in section 4.2.2.2.

Butyric acid was found to be the major volatile component detected in all the BBAN analysed followed by ethanol, ethyl butyrate, isoamyl alcohol and acetic acid. At the end of 4 weeks of storage (ST28) more than 3000 µg/l of butyric acid was detected in Control 1, Balclutha and Canterbury (batch no: 140306). However the overall butyric acid content in all tested samples, irrespective of storage day, ranged between 2331-3527 µg/l except for 140123 L3 ST28 in which only 229.2 µg/l was detected. The highest concentration of butyric acid (3527.0 µg/l) was detected in Control 1 ST14 at 2 weeks storage.

The highest ethanol concentration (1657.1 µg/l) was detected in the Waikato sample (ST28) with 4 weeks of storage while the lowest ethanol concentration (850.7 µg/l) was measured in Lot 2 of control 2 at 4 weeks of storage. In addition, 798.9 µg/l of ethyl butyrate (Waikato ST28, 4 weeks storage) and 73.4 µg/l (Canterbury batch 140123 L3 ST28) were found to be the highest and lowest concentrations respectively. Isoamyl alcohol was found in its largest concentration (781.7 µg/l) in Canterbury sample 140123 L3 ST28 while the minimum concentration (170.7 µg/l) was found in Canterbury control 2 L2 ST28 sample at 28 days of storage. A maximum concentration of 408.4 µg/l phenyl ethanol was found in Canterbury sample 140123 L3 ST28 while the lowest concentration (58.6 µg/l) was detected in lot 2 of control 2 ST28. While lowest phenol concentrations (6.1-8.7 µg/l) were found in all three Waikato samples analysed, highest phenol concentration (167.1 µg/L) was measured in control 1 ST14 (2 weeks of storage). Notably, when compared with the other samples in

this study, those from Waikato exhibited high concentrations of isoamyl alcohol and phenyl ethanol and showed the lowest concentration of phenol.

More than 400 µg/l of Acetic acid were found in samples analysed from the Canterbury site such as batch: 140123 ST7, batch: 140107 Lot 2 ST14, batch: 140107 ST28, Control 2 lot 2 ST28 and batch: 140107 ST42 with 6 weeks storage.

It is worth noting that metabolites produced from BBAN under extended storage conditions (6 weeks storage) did not significantly differ from those produced under shorter storage conditions. The ethanol 975.2 µg/l, butyric acid 2331.3 µg/l and acetic acid 458.1 µg/l found in sample 140107 ST42 is consistent with the number of viable microbial counts present in the product after 6 weeks of production.

However, higher amount of alcohols, esters and lower amount of organic acids were detected in the product prepared from the several days old starter culture. Lower concentration of butyric acid (229.2 µg/L) and ethyl butyrate (73.4 µg/L); higher concentration of isoamyl alcohol (781.7 µg/L), phenyl ethyl alcohol (408.4 µg/L), isobutanol (92.7 µg/L), isoamyl acetate (120.7 µg/L) and ethyl acetate (433.6 µg/L) was detected in L3. Moreover, 1-propanol was detected only in L3 and which was not detected in any other samples. Concentration of major metabolites such as ethanol and butyric acid detected in single culture (stage 2) were higher than compounds detected in mixed culture.

4.4 Discussion

Metabolite profiling studies between dairy yeasts and LAB strains during milk fermentation (Álvarez-Martín et al., 2008) and molasses fermentation by combination of LAB and yeast strains at different temperature have been previously reported (Kopsahelis et al., 2009). However, to the best of our knowledge this is the first case to report the study of detecting metabolic compounds in molasses fermented product having mixed culture of yeast and LAB. The metabolic composition of BBAN may provide a window for elucidating the health benefits using probiotics. Viability results showed that LAB population were higher than MSL in BBAN products with different storage days. Yeast population found to be higher at the first week of storage and decreased towards the end of expiry day. Lactic acid is a major end product of sugar fermentation by LAB whereas ethanol being the major end product of yeast fermentation.

Growth of LAB and yeast can be considered as coupled process with lactic acid and ethanol production. Therefore it can be inferred that the lactic acid can be higher and maintained with different storage days, while ethanol decreased when storage increased. However, results obtained from lactic acid and ethanol assays did not behave according to cell densities. Only control batches 1 and 2 showed better conformity for ethanol concentration than other tested batches. Ethanol, a primary end product of yeast, was higher in the single culture and decreased when storage days increased in the final product. However, various amounts of lactic acid concentration was measured in tested samples. Lactic acid was ranged 132-573 mg/100ml amongst different batches. Though the exact mechanism is not understood for this variation, interaction between LAB and yeast may influence the amounts of metabolites production. For example, positive interaction by yeast have been reported to stimulate LAB through the production of CO_2, pyruvate, propionate and succinate (Leroy & Pidoux, 1993). Composition of growth media may influence the end products of fermentation. For an example, lactose is main energy source for LAB in the milk fermentation. Molasses, contains 75% sucrose and 25% glucose & fructoseis utilized as energy source for microbial fermentation (Olbrich, 2006). Therefore, organic acid profile of BBAN is influenced by molasses and combination of LAB and yeast. It should be noted that though growth of LAB and lactic acid production are closely associated processes, sometimes lactic acid production can be maintained by non-growing cells under undefined stress conditions (van de Guchte et al., 2002).

Both volatile and non-volatile study indicated that important antimicrobial compounds such as lactic acid, ethanol and other alcoholic compounds, acetic acid, butyric acid, diacetyl, and etc. were identified in the BBAN. It has been demonstrated that metabolites such as organic acids (lactic acid and acetic acid), ethanol, diacetyl, H_20_2, acetaldehyde and bacteriocin have been responsible for antimicrobial activities by probiotic action (Brki et al., 1995; Vandenbergh, 1993).

Beneficial effects by LAB and their metabolites included antimicrobial action against pathogenic organisms, prevention of food spoilage and the extension of the shelf-life of food. Additionally, these metabolites have a number of roles including providing nutritional substances, enhancing immunity response by removing toxins and stimulating activity of bile salt hydrolase to transfer cholesterol for therapeutics absorption (Fuller, 1989; Heyman, 2000; Holzapfel et al., 1998). For example, liquid metabolite combinations produced by *L. plantarum* improved growth performance, reduced the incidence of diarrhoea and increased the population of gut LAB and faecal SCFA when fed to post-weaned piglets (Thu et al., 2011).

Organic acids detected in the BBAN included mainly lactic acid, acetic acid, butyric acid, octanoic acid and hexanoic acid. Lactic acid and acetic acid are the most important and best characterized

antimicrobial against pathogenic and spoilage organisms. Lactic acid, produced by probiotic LAB functions as a natural antimicrobial and has been demonstrated to inhibit the growth of gram negative species of the bacterial families such as *Enterobacteriaceae* and *Pseudomonadaceae* (Doores, 1993). Furthermore, lactic acid has been recognized as a biopreservative in natural fermented products (Ray & Sandine, 1992). *In vitro* studies indicated that production of lactic acid by *Lactobacillus* species inhibited growth of *C. jejuni* and reduced colonization by disrupting membrane of *C. jejuni* in broiler chickens (Neal-McKinney et al., 2012). Acetic acid exhibits antagonistic effect against yeast species and the inhibitory effects by lactic acid and acetic acid is caused by the reduction of intracellular pH and dissipation of membrane potential (Kashket, 1987).

Octanoic acid/Caprylic acid (fatty acids) have bactericidal and anti- fungal (notably against *Candida* species) properties. Caprylic acid and its monoglyceride, monocaprylin, have shown effectiveness against major bovine mastitis pathogens *Strep. agalactiae, Strep. dysgalactiae, Strep. uberis, S. aureus, and E. coli* (Nair et al., 2005). Hexanoic acid /caproic acid, straight-chain fatty acid provides variety of flavours such as butter, milk, cream, strawberry, bread, beer and nut and is normally found in beer to contribute caprylic flavour (Clapperton, 1978).

Acetic, propionic, isobutyric, butyric, isovaleric and valeric acid have been categorized into SCFA which are mostly absorbed in the large intestine and provide energy to the host animal (Franklin et al., 2002). Butyric acid, the metabolite more abundantly found in BBAN, than other metabolites is normally produced within the intestinal lumen and exerts a wide variety of effects on intestinal function (Hamer et al., 2008). Multiple functions provided by butyric acid included well documented anti-inflammatory effects, reinforcement of colonic defence barrier by producing mucin, and antimicrobial peptides and decreased intestinal epithelial permeability by increasing the expression of tight junction proteins (Breuer et al., 1997). Anti-inflammatory activities coupled with a strengthening of mucosal barrier integrity make butyric acid of potential therapeutic value against IBD (Van Immerseel et al., 2010).

Alcohols are largest group of chemical class detected in the BBAN, followed by ester, organic acids, carbonyl compounds and miscellaneous compounds. There were more than 15 different alcohols in varying amounts detected in the BBAN. It has been reported that animals fed with metabolite combinations could yield better performance due to presence of organic acids, ethanol, bacteriocins, H_2O_2 and vitamins in the metabolite combinations (Desouky & Ibrahim, 2009; Foo et al., 2005). Ethanol was major alcohol type identified in the product. Apart from ethanol, major alcohol type included phenylethyl alcohol, phenol, isoamyl alcohol and isobutanol. Ethanol and CO_2 are the major end products of yeast fermentation and this causes inhibitory effects to many undesirable

microorganisms (Ferreira & Viljoen, 2003). Phenyl ethyl alcohol (PEA) is an aromatic alcohol with a rose-like odour mostly produced by yeasts from aromatic amino acid metabolism (Naz et al., 2013). The production of PEA has shown the ability to inhibit the growth of gram-negative bacteria, such as *Salmonella, Escherichia, Shigella, Aerobacter, Klebsiella, Pseudomonas* and *Proteus* (Lilley & Brewer, 1953). Higher alcohols (fusel alcohol) are most abundant organoleptic compounds present in beer. These higher alcohols produced in the Lager beer (Table 4.5) were also detected in BBAN including n-propanol (sweet), isobutanol (solvent), isoamyl alcohol (banana), amyl alcohol (Solvent) and 2-phenylethanol (roses).

Table 4.5 Higher alcohols present in Lager Beer Pires et al. (2014)

Compound	Threshold (mg/l)	Concentration range (mg/l)	Aroma impression
n-propanol	600	4-17	Alcohol, sweet
isobutanol	100	4-57	solvent
Isoamyl alcohol	50-65	25-123	Alcoholic, banana
Amyl alcohol	50-70	7-34	Alcoholic, solvent
2-phenylethanol	40	5-102	roses

Second largest group of chemical class identified in the probiotic formulation is ester and almost 13 different esters were detected in the product. Esters are formed in a reaction between alcohols and organic acids (Engels & Visser 1994). Therefore, with a larger number of different alcohols and organic acids in BBAN, there is considerable potential for the formation of wide range of esters. Ethyl esters are straight chain fatty acids mostly responsible for fruity aroma of fermented alcoholic beverages (Sumby et al., 2010). Amongst various ethyl ester groups, ethyl acetate (fruity, balsamic, solvent-like aroma) and ethyl butyrate (floral, fruity, strawberry, sweet) were largely found in BBAN, followed by ethyl formate, ethyl propanate and ethyl hexanoate (fruity, strawberry, green apple and anise aroma). Most of these esters are important aromatic yeast metabolites found in beer and wine including ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl acetate, isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma), phenyl ethyl acetate (roses and honey aroma), and ethyl octanoate (Pires et al., 2014; Sumby et al., 2010). Esters, are found in beer only in trace amounts and have a very low odour threshold. However, if overproduced, they can negatively affect the beer with a bitter, over fruity taste (Pires et al., 2014).Isobutyl butyrate, isoamyl butyrate and phenyl ethyl butyrate have been widely used as food grade flavouring agents .

Diacetyl (2, 3-Butanedione) is another important metabolites with characteristic buttery aroma produced by LAB detected in BBAN that have shown antimicrobial spectrum against gram-negative, gram-positive and yeast (Suskovic et al., 2010).

Dimethyl sulphide and methionol are Volatile sulphur flavour compounds (VSFC) found in BBAN in small amounts. These VSFC are commonly found in many foods and beverages as aroma compounds with low detection thresholds (Seow et al., 2010). Eugenol and (Z)-isoeugenol are type of Guaiacol which is a naturally occurring organic compound. Eugenol exhibit antibacterial, antifungal, anti-inflammatory activity, insecticidal and antioxidant properties, and are used traditionally as flavouring agent and antimicrobial material in food (Huang et al., 2002).

In addition, metabolites like (3E)-3-penten-2-one, 2, 5-dimethyl- furan (used as biofuel (Zu et al., 2014) and gamma-Amylbutyrolactone (used as cosmetic, flavour and fragrance agents-(www.thegoodscentscompany.com)) detected in the BBAN. Although these compounds are not usually found in the food products that raised safety concerns. However, the relative amounts of these metabolites found in trace amounts in the BBAN.

Although the product was not investigated for gas content, it is obvious the product contains considerable amount of CO_2 which exhibits antimicrobial activity especially, anti-fungal activity because of its ability to accumulate in the membrane lipid bilayer resulting in dysfunction in permeability (Lindgren et al., 1990). Combination of pH reduction and conversion of sugars to organic acids by LAB plus production of CO_2 and alcohol by yeast have inhibitory effect on many undesirable microorganisms in the fermented product (Ferreira & Viljoen, 2003).

Finally, metabolites such as lactic acid, ethanol, acetic acid, butyric acid, diacetyl with antimicrobial properties and various aromatic ester compounds were detected in the product. The production of organic acids and other metabolites varied with different storage days of the same product and products from different production sites. The variation in metabolite concentration amongst various storage days cannot be correlated with viable cell counts of LAB and yeast and this variation did not show clear tendency. The relative concentration we discussed above is not the true concentration, it is indicative value only. Detecting true concentration by quantification method is the next step of this study and which would validate the metabolite concentration in the BBAN.

4.5 Conclusions

Ethanol and lactic assays confirmed that the product contained considerable amount of lactic acid and ethanol in BBAN. HS-SPME/GCMS analysis was used to detect the semi-volatile and volatile

metabolites in the BBAN. Several chemical groups of volatile metabolites in varying quantities were identified and alcohols were the largest group of chemical class detected in the BBAN, followed by ester, organic acids, carbonyl compounds, sulphur compounds and miscellaneous compounds. Lactic acid, ethanol and butyric acid were primary metabolites detected in higher quantities in BBAN formulation. Lactic acid, acetic acid, ethanol, butyric acid, diacetyl were the important metabolites identified with antimicrobial properties.

Chapter 5

Quality assurance (QA) system to produce the product

5.1 Introduction

In recent years, the use of fermented liquid feed in animal nutrition has significantly been increased because of their relative advantages over other forms of delivery. The aim of reducing the use of antibiotics and the availability of liquid animal feed for cheaper prices may be attributable for increased usage (Canibe & Jensen, 2012). LAB are naturally a core part of the gut microbiota and probiotics containing LAB have shown ability to adhere to and colonise in the host gut (Spencer & Chesson, 1994) and improve animal health (Cross, 2002). Fermented liquid feed consisting of LAB have, through their beneficial effects, been shown to improve the animal health (Plumed-Ferrer & von Wright, 2009). Yeast based products have been fed to animals for more than hundred years. *S. boulardii* is an important yeast species employed in probiotics and it has been demonstrated that the probiotic contains live yeast (*S. boulardii*) preparations can stimulate enzyme activities and active against various pathogens (Czerucka & Rampal, 2002).

Molasses as animal feed has been used for many years. Since molasses contains about 50% sugars in the form of sucrose, glucose and fructose, which give readily available energy, it is directly fed to ruminants, cattle and sheep in many different ways or used in silage formation (Cleasby, 1963).

BBAN is molasses fermented animal probiotic formulation consisting of multi-species and multi-strains of LAB and yeast strains. Production of BBAN involved 3 steps fermentation and quality of each step is dependent on other steps of fermentation. This chapter explains how the BBAN satisfy the QA parameters.

5.2 QA during manufacturing processes

QA is applied to products to verify whether it meet specifications and requirements, and during manufacturing-production run by validating lot samples meet specified quality parameters. In our study we analysed more than 300 BBAN samples belonged to 25 batches from Canterbury production site. Products at many stages of production and during storage were investigated for microbiological quality testing, safety and metabolic compounds. Samples of final product from Waikato and Balclutha; Samples with extended storage and products from several days old starter culture were also analysed. Intensive sampling (monitoring every day until its expiry day) was done for first 3 batches and rest of the batches were sampled at the end of different stages and weekly during storage period.

Number of forms (BBQA01 to BBQA04) with a variety of information was prepared for data entry purposes (Appendix E1-E4). The information in the forms were useful to assess the QA parameters for the product. Generally information included sample ID, starting day of starter culture, day of starter culture transferred to molasses fermentation, mixed fermentation day, agar plate preparation day in the laboratory, pH reading, microbial counts and detection of metabolite details. In addition, information on product testing for various foodborne pathogens such as *E. coli, Staphylococcus, Coliform, Listeria and Salmonella* was included.

Sugar in stage 1, and molasses in stage 2 and 3 were used as growth media and the density of these media was maintained as 5% w/v. LAB population in stage 1 and 2 struggled to establish and it reached maximum in mixed fermentation. Cell densities (7.67 log CFU/ml) and organic acid production in stage 1 was relatively lower than in stage 2. However, due to the influence of molasses, the cell counts (9.3 log CFU/ml) and number of metabolites was found higher than stage 2. LAB population in stage 1, 2 and 3 were significantly different from each other. However, cell densities in mixed fermentation did not differ with final product at different storage days.

Yeast cell densities increased up to 10^{10} log CFU/ml at the end of stage 1 and maintained higher in the stage 2. Similar to LAB metabolites, the number and relative concentration of yeast metabolites was found to be higher in stage 2 than in stage 1. However, when the mixed fermentation started the viable counts of yeast tended to decrease. When LAB and yeast are found in a single product, growth requirement for these microbes are different and there can be negative and positive interaction between them.

The following factors observed in this study confirmed that the influence of LAB on yeast growth:

a) Ethanol production in the single culture is much more than in the co-culture.

b) The growth of yeast tended to decrease in the mixed culture relative to single culture.

Weak organic acids produced by LAB have been shown to inhibit yeast growth without interfering with development of LAB population in the liquid feed (Plumed-Ferrer & von Wright, 2011). On the other hand, there are reports of stimulating effect of LAB on yeast growth especially because of lactic acid and acetic acid production (Thomas et al., 2002). The temperature requirement for the growth of LAB and yeast are 37 ^{o}C and 25 ^{o}C respectively. However, the temperature, in the mixed culture has been set to 37 ^{o}C. The temperature rise in the mixed culture may affect the viability of the yeast cells. Moreover, LAB is mostly anaerobic or sometimes facultative in nature. Therefore during mixed fermentation and storage period product is maintained in anaerobic condition. This is unfavourable for yeast species which while not strictly aerobic, thrive more in the presence of oxygen. Therefore these factors combined together may retard yeast growth in mixed culture and final product. Obviously, this is a question of microbial trade-off. Hence, BBAN has been designed to get more benefits from LAB, Increasing conditions favourable for yeast growth would affect the LAB population in the co-culture.

Plate count method, we used for enumeration of viable counts in this study has some disadvantages like time consuming and results may be biased by poor viability or low cell densities. Currently, there are numerous modern and rapid methods including Solid-phase cytometry (SPC), polymerase chain reaction (PCR) etc. are in practice to assess microbial counts accurately. When SPC and traditional plate count were used to enumerate *S. boulardii* in several probiotic products, cell counts was significantly higher in SPC than plate count method. In addition, SPC quantified all the viable yeasts whereas plate count only enumerated viable culturalable yeast population in the products (Vanhee et al., 2010).

Study of product with extended storage and production of different lots from the same starter revealed different quality aspects. Products produced using old starter contained more alcohol based metabolites in the final product when compared with products produced using fresh starter culture whereas Product quality was higher with extended storage than the product produced using several days old starter culture.

5.3 Assessing BBAN for the QA criteria

BBAN is discussed under following quality control criteria.

5.3.1 Formulation (vehicle)

Probiotics are available in the form of powder, capsule, tablets and liquid. Viability of the probiotics in the form of powder and pellets are more affected than liquid fermented probiotics since pelleting and extrusion process require high temperature and pressure (Ananta et al., 2004; Champagne et al., 2011). Powdered and semi-solid products may heavily be subjected to processing techniques such as spray drying, freeze drying and vacuum drying. During processing, these drying processes may affect the viability of the product by applying various stresses on the product and also some dried products may contain dissolved oxygen which is toxic to probiotic bacteria (Korbekandi et al., 2011)). In recent years, the use of fermented liquid feed in animal nutrition has significantly been increased. Some of the problems associated with liquid probiotics are short term shelf-life and maintaining the storage conditions which is usually done by refrigeration. BBAN is fresh and liquid probiotics which does not need any special storage requirement and can be stored at normal environmental conditions. BBAN contains multi species and multi strain of LAB and yeast. Reduction of pH and production of lactic acid, acetic acid and ethanol prevents growth of foodborne pathogens and spoilage organisms in a fermented liquid feed (van Winsen et al., 2001). Molasses, which are rich in nutrients and cost effective energy source, have long been used in livestock feeds. Probiotics supplemented by LAB and yeast strains in molasses fermentation ensure the health benefits to the target animals.

5.3.2 Combination

Probiotics containing several different species (multi-species probiotics) or various strains of one species (multi-strains probiotics) have been demonstrated more effective than that of using mono-strain probiotics (Timmerman, 2004). Using multi-species and multi-strain probiotic products cover a broad spectrum and by their synergistic effect increases probiotic functionality and efficacy. When different probiotic formulations were tested in treating antibiotic-associated diarrhoea in children, it was found that probiotics containing lactobacilli and *bifidobacteria* (multi-species) were more effective than that of preparations with mono-strain (Timmerman, 2004). Likewise, Lema et al. (2001) demonstrated multi-species of lactobacilli showed higher rate of effectiveness in reducing *E. coli* shedding in sheep. On the other hand, studies suggested that use of multi-strain preparation effectively improved growth performance, particularly mortality in broiler chickens (Jin et al., 2000; Timmerman, 2004). Since BBAN is a multi-species and multi strains product, the functionality of this product can be more effective and consistent.

5.3.3 Viability

Viability is one of the important criterion for quality assurance of probiotic products. To maintain functionality of the probiotic product, sufficient number of viable counts should be consistently maintained throughout their shelf-life of the product. Composition of most of the commercially available probiotics do not meet a quality standard because of having inconsistent variability (Fasoli et al., 2003). As a result, there have been controversy over the functionality and efficiency of the product. Viability has been estimated by conventional plate count method in which colonies were grown on a selective growth medium. In recent years, several rapid and efficient methods are used to enumerate viable counts since conventional methods are considered time consuming and underestimation of true viable counts.

In our study we analysed 25 batches of BBAN (more than 300 samples) until its shelf-life (one month) and tested for microbiological quality and safety using conventional method. Higher number of samples and cost involved in using modern rapid methods limited us to use conventional method in our laboratory. However, results obtained from different batches and changes in the viable counts at many stages of production were consistent.

Mean viable counts of all batches analysed during different month of study has been presented in Table 5.1. The results indicated the viability of the LAB was not affected when the product was at its end of shelf-life and the average viable counts varied between 10.7 ± 0.08 and 11.3 ± 0.00. Monthly analysis of viability of the LAB were not significantly different. Therefore, the BBAN contains LAB viable counts that is more than MSL (10^6 CFU/ml) and recommended level in some developed countries (10^9 CFU/dose in Canada).

Table 5.1 Viable counts of BBAN for quality testing on monthly basis

Month of production	No. of batches	Average Lb counts (log CFU /ml)		Average yeast counts (log CFU /ml)	
		Day 7	Day 28	Day 7	Day 28
August 2013	4	9.8 ± 0.33	10.7 ± 0.08	6.4 ± 0.27	5.4 ± 0.35
September 2013	3	10.9 ± 0.05	10.8 ± 0.09	5.9 ± 0.41	5.2 ± 0.20
October 2013	3	Contamination. sampling stopped			
November 2013	6	11.4 ± 0.18	11.6 ± 0.04	6.4 ± 0.73	5.6 ± 0.37
December 2013	1	11.7 ± 0.00	11.4 ± 0.00	7.6 ± 0.00	6.0 ± 0.00
January 2014	4	11.5 ± 0.08	11.6 ± 0.14	4.7 ± 0.13	4.8 ± 0.41
February 2014	4	10.7 ± 0.80	11.4 ± 0.13	5.6 ± 0.54	5.2 ± 0.33
March 2014	1	11.3 ± 0.00	11.3 ± 0.00	5.1± 0.00	5.0 ± 0.00

Several studies revealed that the use of multi-species and multi-strain probiotic formulations have been more effective than using mono-strain (Timmerman, 2004). BBAN preparations contain different species and strains of LAB and yeast. The viable yeast population was higher at early stages of production and when the storage days increased the yeast population tended to decrease. On the whole, the viable yeast counts were just below the MSL at the end of shelf-life. Yeast population in the single culture goes up to 10^{10} CFU/ml and the population started to decrease when it is mixed with LAB culture. Both positive and negative interactions between LAB and yeast have been reported on each other. It is likely that the strong influence of LAB (especially, low pH) on the co-culture may inhibit the growth of the yeast in the BBAN. Moreover, yeast cells are inoculated at 25 ^{o}C when it is grown in the single culture. But, the temperature of mixed culture rises up to 35 ^{o}C which is not favourable for yeast growth. However, it is revealed that the health benefits from yeast culture is not necessarily limited to the viable culturable yeasts and viable non-culturable *S. boulardii* which cannot be enumerated in plate count method have also shown probiotic effects (Vanhee et al., 2010). Apart from this, end products of fermentation such as low pH from LAB fermentation and the alcohol and CO_2 produced by the yeasts in combination is responsible to inhibit many undesirable microorganisms (Ferreira & Viljoen, 2003).

BBAN production across different production sites have consistent viable counts. On average products from all manufacturing sites contained more than 10^{9} CFU/ml viable counts, which is more than MSL. Product with extended storage period did not affect the viable cell densities and therefore the product quality in terms of viability, was not affected when the product extended for 2 more weeks. BBAN is the product consisting number of favourable characteristics of individual strains that are combined in a single preparation. Therefore, BBAN as a multi-species multi-strains product, with multiple probiotic effects combined together, may act synergistically to confer benefits to the host.

5.3.4 Safety

When selecting microbial strains to be used as probiotics, strains which possess a clear history of non-pathogenicity should be taken into account. Safety evaluation of the probiotics is implemented in different countries. GRAS status regulated by Food and Drug Administration (FDA) in USA and Qualified Presumption of Safety (QPS) status implemented by European Food Safety Authority (EFSA) in Europe are important references to monitor the safety of probiotics. It is generally recommended that the probiotic strain should possess either GRAS status or QPS status. BBAN comprises 4 LAB species and 3 yeast species and all of which possess QPS status which imply that these probiotic strains are safe to use in food product. GCMS studies indicated that BBAN did not

contain any undesirable metabolites which in some instances have been reported as the end product of fermentation.

Throughout the study period product was tested for potential foodborne pathogens and test for bacterial pathogens was negative in all the occasions. High acidity of the product and metabolites produced by probiotic strains is effective and unfavourable against bacterial pathogens. However, detection of *Rhodotorula* species contamination in the product was effectively controlled during period of the study. Possible cause of the contamination source was detected and eliminated to ensure that this type of contamination would not be recurring in future.

5.3.5 Metabolites

Amongst different strategies against pathogenic and food spoilage organisms, metabolite production is regarded as an important probiotic mechanism (Gaggìa et al., 2010). Thus, probiotic product analysis for metabolic compounds have become an important in QA criteria. Furthermore, some undesirable metabolites produced as fermented end products have to be studied to makes sure product quality is maintained. Investigation of the BBAN for volatile metabolite analysis studied using SPME/GC-MS. Colorimetric enzymatic assays were used to evaluate lactic acid (non-volatile) and ethanol concentration in the BBAN samples. Lactic acid assay confirmed the major non-volatile metabolite detected in BBAN was lactic acid. Likewise, major volatile compounds detected in BBAN samples were grouped based on relative amounts, included organic acids (lactic acid, acetic acid and butyric acid), alcohol (ethanol, phenylethyl alcohol and isoamyl alcohol), esters (ethyl acetate and ethyl butyrate) and phenol. Although different stages of production having different metabolites at varying quantity, generally they have been grouped into organic acids, alcohols, esters, carbonyl compounds, sulphur compounds and miscellaneous compound.

Our results showed that important antimicrobial compounds such as lactic acid, ethanol and other alcoholic compounds, acetic acid, butyric acid, diacetyl, and etc. were identified in the BBAN. These metabolites play a key role in controlling gut pathogens and undesirable microorganisms.

5.4 Model QA system

Based on the results obtained from this study, we propose model QA system which includes testing parameters such as temperature, pH, viable counts of LAB and yeast, safety assessment and beneficial metabolites. Forms prepared for proposed QA system have been presented in Appendices E1-E4.

5.4.1 QA during Production stages

BBAN product can be monitored at the end of each stages of production. Sample have to be tested for temperature, pH and viable counts. When tested samples at many stages of production meet the requirement mentioned in Table 5.2, then it is said that product at various stages satisfy the microbiological quality parameters.

Table 5.2 QA specification for microbiological quality testing for various manufacturing stages

	Stage 1 day 1 (Sugar fermentation)	**Stage 2 day 2 (Molasses fermentation)**	**Stage 3 day 3 (Molasses fermentation)**
Sample ID			
	Yeast		
Date			
Temperature:	25 ^{0}C	25 ^{0}C	37 ^{0}C
pH :	3.3 -4.0	4.0 - 4.5	3.5 - 4.0
Viable counts:	9.0-10.0 CFU/ml	8.0-9.5 log CFU/ml	7.5 - 8.75 log CFU/ml
	LAB		
Date			
Temperature:	37 ^{0}C	37 ^{0}C	37 ^{0}C
pH :	3.8 - 4.5	4.0 - 4.5	4.0 - 4.5
Viable counts:	7.0 -7.5 log CFU/ml	8.0 - 9.3 log CFU/ml	9.5 -10.5 log CFU/ml

5.4.2 QA during storage

QA of final product is important to deliver the expected beneficial effects to the host animal. Based on our research findings we have set the benchmark microbial counts of LAB and yeast at different storage days (Table 5.3). BBAN product tested for microbiological counts meet the benchmark viable count range at different storage days are considered that they satisfy the QA criteria.

Table 5.3 QA specification for microbiological quality during storage

Sample ID:				
Date:				
Storage days	**Viable count (log CFU/ml)** **LAB**	**Yeast**	**pH**	**Temperature**
Day1	9.00 -10.69	7.00-8.02	3.5 -4.0	Room Temperature
Day7	9.00 -10.99	5.5-6.10	3.5 -4.0	
Day14	9.00 -11.14	5.00-5.82	3.5 -4.0	
Day21	9.00 -11.07	5.00-5.55	3.5 -4.0	
Day28	9.00 -11.24	5.00-5.47	3.5 -4.0	

Furthermore, form BBQA03 explain product safety testing for potential foodborne pathogens (*Staphylococcus* species, *E. coli, Coliform* and *Rhodotourula rubra*) at different manufacturing stages and storage periods (Appendix E3). Test results show negative for these foodborne pathogens would assure the quality in terms of safety of the product. Likewise, form BBQA04 illustrates testing the product for metabolites (Ethanol, lactic acid, butyric acid, acetic acid and diacetyl) in the final product. Presence of these metabolites in the final product would ensure the quality assurance for beneficial metabolites.

Chapter 6

Conclusions and recommendations

6.1 Conclusions

High quality animal feed ensures the quality of animal meat and by which it would improve consumer safety. In recent years, use of animal probiotic formulation have demonstrated prevention against zoonotic pathogens and infectious diseases by which improve the quality of animal meat. However, a high quality standard of the probiotic product should be met, in order to gain maximum health benefits from these products. BBAN is fresh animal probiotic formulation consisting of multi-species and multi-strains of LAB and yeast strains in a molasses fermented media.

In the current study we assessed the BBAN for important QA parameters including viable counts, metabolic compounds and safety assessment. In general, final product of the BBAN had more than recommended number of LAB viable counts irrespective of the storage days. However, yeast cell densities was higher in the first week of storage and started to decline towards the end of expiry date. Overall yeast counts in the product were just below the MSL.

Metabolite profiling study revealed amounts and number of metabolites detected were different in products at many stages of production. However, detection of lactic acid, ethanol, organic acids, diacetyl and CO_2 in the product suggested that the BBAN contained wider range of important antimicrobial compounds.

Product safety assessment is important to assure the quality of the product. Investigation of the product for potential foodborne pathogens indicated that the product did not have any bacterial contamination throughout the study period. However, contamination with *Rhodotorula rubra* was reported for 2 batches. Product was recalled and factors conducive for disease contamination was identified and effectively eliminated. Constant viability of the product in association with production of antimicrobial metabolites confirmed the probiotic characteristics. Furthermore, safety assessment confirmed the product is safe to use it. Based on these studies proposed QA system was developed to monitor the BBAN products manufactured in future. Proposed QA system would make sure the products produced would be consistent, high quality BBAN products.

In conclusion, BBAN satisfied the important QA parameters such as viability, metabolite compounds and safety assessment. Proposed QA system was developed to to produce consistent, high quality and safe BBAN.

6.2. Recommendations

QA parameters we investigated confirmed that the BBAN:

- had consistent viable counts throughout their shelf-life
- was safe to use
- Presence of desirable metabolites for probiotic effects.

However, these parameters assure the quality of the product before it reaches the animal gut. Investigating performance of probiotics upon administration to the farm animals is also essential for QA criteria.*in vitro* and clinical assessment can be made to study following QA parameters.

- acid and bile stability study
- Ability to adhere and colonise in the gut.
- Dose-response studies

Yeast viable counts were slightly lower than MSL in the products. Plate count, culture-dependent method used in this study was like a stepping stone in this study and rapid culture-independent methods can be used to verify the yeast counts in the product.

Contamination during production caused by *Rhodotorula rubra*. Improvement in the production site in terms of hygienic and good practices would always make sure that consistent production of the product. Furthermore, Quality of the product can be maintained by constant implementation of standard operating procedure (SOP) in the facility. Plastic containers used in production of BBAN showed more affinity with these fungal organism. Replacing plastic containers with other types (Steel) of containers may reduce the chance of being contaminated.

Unlike other probiotics, BBAN is not refrigerated but stored in room temperature. However, temperature control during production plays an important role for the microbial growth. Monthly variations in the product viable counts were observed and this may be due the effects of seasonal variations in the product. Setting up the facility in well protected and closed area may be helpful to avoid these variations.

Product quality of different lots from same starter culture look inferior to normal standard products and this can be avoided rather product expiry day can be extended up to 6 weeks.

In general, BBAN contains 4 species of LAB and 3 species of yeast. However, colony counting did not differentiate which species was dominant in the product and species level counting was not undertaken.

We analysed quantity of the metabolites in relative amount. However detecting true concentration by quantification method should be made to understand for validating the metabolite concentration in the BBAN.

Appendix A Log form of LAB and yeast population in different batches at many stages and during storage period

A.1 LAB population

	130823 L2	130829 L1	130829 L2	130906	130926 L1	130926 L2	131101	131108	131112	140107 L1	140107 L2	140123 L1	140123 L2	140123 L3	Ctrl1
stage1	8.7993	9.0607	9.0607	5.8451	8.0414	8.0414	7.9542	5.8451	6.0569	6.1903	6.1903	8.2430	8.2430	8.2430	9.2455
Stage2	8.9590	9.6990	9.6990	6.4314	6.8751	10.7324	8.1859	7.4146	9.5796	9.9445	9.9445	11.5119	11.5119	10.2253	9.1335
stage3	9.5276	9.5263	9.5465	9.7723	10.8921	10.8808	9.2148	8.1614	10.4771	11.5623	11.8808	11.4594	11.5119	11.5119	11.4216
day1	9.3636	9.6405	9.9542	9.9912	10.7160	10.8920	10.5798	8.3304	11.5132	11.7924	11.5888	11.5705	11.3838	11.4942	11.5752
day7	9.4456	9.5366	10.7634	10.9243	10.7634	10.9031	11.7076	10.6128	11.0334	11.4942	11.4314	11.7292	11.3324	11.6474	11.5798
day14	9.3766	10.7160	10.5315	10.5740	10.6758	10.8921	11.5682	11.4150	11.5051	11.7451	11.7482	11.9009	11.9325	11.1271	11.4502
day21	10.3222	10.5119	10.5366	10.7356	10.5888	10.7324	11.3424	11.5623	11.3617	11.7126	11.8987	11.7482	11.4409	10.2201	11.2923
day28	10.4914	10.6920	10.7634	10.9675	10.6684	10.8921	11.5563	11.5378	11.5051	11.9877	11.7574	11.4472	11.4048	11.1303	11.7435

A.2 Yeast population

	130823 L2	130829 L1	130829 L2	130906	130926	131101	131102	131108	131112	131121	140107 L1	140123 L1	140123 L3	Ctrl 1
stage1	9.6435	9.7076	9.7076	10.2041	10.1072	11.1072	11.0934	10.9243	9.6180	9.8573	10.8248	9.3201	9.3201	11.9877
Stage 2	9.6902	9.8129	9.8129	9.3263	9.4150	9.7441	10.0732	9.8828	9.7378	10.8156	10.4393	9.6628	9.6628	11.4579
stage3	9.4150	9.3598	8.0000	9.3909	8.9243	9.6085	9.7007	8.5378	7.6180	9.3617	8.7093	5.2900	8.3636	10.2355
day1	9.5092	9.0170	8.7993	9.0294	7.2779	9.5416	8.2923	8.1614	7.5105	8.3424	7.1303	5.3979	7.0645	7.1367
day7	6.0334	5.8751	6.7853	6.9542	4.9542	6.0414	7.2430	6.6335	7.1303	6.0492	4.9445	4.6128	6.0719	6.0682
day14	5.6128	5.8195	5.9031	7.1139	4.9243	5.4624	5.5051	7.2304	6.4914	6.3222	5.5132	4.5051	6.1271	4.9731
day21	5.3010	5.6021	6.1461	5.8513	4.8686	7.2201	5.8261	6.5798	5.6232	5.1614	4.5051	4.2553	5.7243	5.0531
day28	6.0607	4.9294	5.1072	5.4472	4.8129	6.4914	5.4393	6.5315	6.2304	4.5441	5.2095	4.8921	6.1430	4.7076

Appendix B Volatile metabolites from BBAN (Control 1) at many stages of production isolated by the SPME/GC-MS

Metabolite	CAS	KI	RT	ST7	ST0	S3D3	S2 D3 L	S2D3 Y	S1D1L	S1D1Y
Compounds	No	published	(mins)	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l
2-methyl-1-Pentene	763-29-1	-	3.54				10.5	6.1	9.4	5.8
Dimethyl sulfide	75-18-3	844	4.05	7.1	9.1	7.9	0.0	27.9	0.0	0.0
Ethyl formate	109-94-4	848	4.79	10.8	13.6	9.7	0.0	0.0	0.0	0.0
Ethyl acetate	141-78-6	886	5.74	83.8	65.9	48.0	0.0	428.5	0.0	66.0
Ethanol	64-17-5	929	6.84	1932.5	2250.3	1997.8	22.3	2696.9	0.0	1612.5
2,5-dimethyl- furan	625-86-5	943	7.15	6.2	7.7	4.8	0.0	0.0	0.0	0.0
Ethyl propanoate	105-37-3	950	7.28	0.0	0.0	0.0	0.0	11.7	0.0	0.0
Diacetyl	431-03-8	977	7.75	0.0	0.0	0.0	0.0	18.6	13.8	0.0
Ethyl butyrate	105-54-4	1028	9.50	567.4	591.5	511.1	0.0	50.3	0.0	0.0
1-propanol	71-23-8	1040	9.59	0.0	0.0	0.0	0.0	20.4	0.0	0.0
Isobutanol	78-83-1	1108	11.47	97.0	120.9	112.3	15.2	288.7	0.0	105.9
isoamyl acetate	123-92-2	1117	12.29	7.7	6.7	5.7	0.0	21.3	0.0	0.0
1-Butanol	71-36-3	1145	13.20	62.7	73.8	61.1	13.0	0.0	0.0	0.0
isoamyl alcohol	123-51-3	1205	15.44	792.5	985.2	900.7	44.5	2558.1	0.0	881.2
Ethyl hexanoate	123-66-0	1220	16.30	0.0	0.0	0.0	0.0	13.8	0.0	0.0
isoamyl butyrate	106-27-4	1267	17.45	5.9	7.5	6.6	0.0	0.0	0.0	0.0
cis-3-Hexenyl Acetate	3681-71-8	1308	19.27	-	-	-	-	-	-	-
Ethyl octanoate	106-32-1	1436	23.29	0.0	0.0	0.0	0.0	54.5	0.0	10.0
Acetic acid	64-19-7	1450	23.60	448.4	318.0	239.8	71.5	0.0	62.2	0.0
2-ethyl-1-hexanol	104-76-7	1487	25.07	0.0	0.0	0.0	0.0	6.7	0.0	0.0

unknown	-	-	26.13	0.0	0.0	0.0	0.0	0.0	11.5	0.0
2,3-Butanediol	513-85-9	1523	26.64	0.0	0.0	0.0	13.3	0.0	0.0	0.0
1-Octanol	111-87-5	1553	27.23	0.0	0.0	0.0	0.0	7.9	0.0	0.0
Butyric acid	107-92-6	1619	29.09	5401.1	6007.9	5380.4	2978.3	0.0	0.0	0.0
Menthol	1490-04-6	1626	29.81	4.5	4.7	5.7	3.3	0.0	0.0	0.0
unknown	-	-	32.68	7.0	0.0	0.0	29.5	7.3	0.0	0.0
Phenyl butyrate	4346-18-3	-	33.36	8.6	59.8	77.4	6.5	0.0	0.0	0.0
4-methylphenyl butyrate	14617-92-6	-	36.39	0.0	16.0	17.2	0.0	0.0	0.0	0.0
Phenylethyl Alcohol	60-12-8	1925	37.32	15.8	16.0	17.8	0.0	81.2	0.0	10.9
Phenyethyl butyrate	103-52-6	1930	38.76	7.6	7.5	6.3	0.0	0.0	0.0	0.0
Phenol	108-95-2	1951	39.61	36.2	36.2	38.0	29.6	20.5	0.0	0.0

Metabolites	Internal Standard	Unconfirmed ID

Appendix C Volatile metabolites from BBAN (Control 2) at many stages of production isolated by the SPME/GC-MS

Metabolite Compounds	CAS No	KI	Retention Time	St 0	S3D3	S2 D3 L	S2 D3 Y	S1 D1 L	S1 D1 Y
		published	(mins)	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l
2-methyl-1-Pentene	763-29-1	-	3.54	9.9	8.2	11.0	8.1	8.2	4.6
Dimethyl sulfide	75-18-3	844	4.05	9.6	7.4	2.7	32.4	0.0	0.0
Ethyl formate	109-94-4	848	4.79	9.1	10.6	0.0	0.0	0.0	0.0
Ethyl acetate	141-78-6	886	5.74	125.1	73.4	0.0	615.3	0.0	87.0
Ethanol	64-17-5	929	6.84	2304.5	2090.2	60.4	3364.9	0.0	1987.5
2,5-dimethyl- furan	625-86-5	943	7.15	5.1	2.3	3.3	0.0	0.0	0.0
Ethyl propanoate	105-37-3	950	7.28	0.5	0.4	0.0	20.1	0.0	0.8
Diacetyl	431-03-8	977	7.75	3.7	4.4	10.4	16.3	18.9	33.0
Ethyl butyrate	105-54-4	1028	9.50	369.4	207.9	0.0	156.1	0.0	0.0
1-propanol	71-23-8	1040	9.59	0.0	0.0	0.0	30.4	0.0	8.4
Isobutanol	78-83-1	1108	11.47	141.3	152.7	17.4	327.7	4.7	176.2
isoamyl acetate	123-92-2	1117	12.29	10.7	8.7	0.6	26.8	0.5	44.5
1-Butanol	71-36-3	1145	13.20	31.9	22.1	185.0	13.7	0.3	0.4
isoamyl alcohol	123-51-3	1205	15.44	1075.9	927.3	62.0	3121.1	1.2	1288.3
Ethyl hexanoate	123-66-0	1220	16.30	0.5	0.5	0.0	9.8	0.0	2.0
isoamyl butyrate	106-27-4	1267	17.45	5.8	4.0	0.0	8.0	0.0	0.0
cis-3-Hexenyl Acetate	3681-71-8	1308	19.27	-	-	-	-	-	-
Ethyl octanoate	106-32-1	1436	23.29	0.8	1.3	0.0	36.3	0.0	9.7

Acetic acid	64-19-7	1450	23.60	419.5	209.2	103.8	106.0	18.7	0.0
2-ethyl-1-hexanol	104-76-7	1487	25.07	5.7	6.3	5.8	6.7	3.8	5.0
unknown	-	-	26.13	2.4	1.9	1.8	1.7	2.4	1.6
2,3-Butanediol	513-85-9	1523	26.64	5.6	2.7	4.2	3.0	0.0	0.0
1-Octanol	111-87-5	1553	27.23	9.0	8.8	7.3	8.3	3.6	4.3
Butyric acid	107-92-6	1619	29.09	4626.0	3151.8	4154.6	932.5	0.0	0.0
Menthol	1490-04-6	1626	29.81	2.7	3.5	5.2	0.0	0.0	0.0
unknown	-	-	32.68	6.9	7.7	6.2	7.1	4.2	5.1
Phenyl butyrate	4346-18-3	-	33.36	15.8	15.5	12.0	0.0	0.0	0.0
4-methylphenyl butyrate	14617-92-6	-	36.39	5.9	4.8	5.4	0.0	0.0	0.0
Phenylethyl Alcohol	60-12-8	1925	37.32	18.6	15.1	1.3	93.8	0.0	26.4
Phenyethyl butyrate	103-52-6	1930	38.76	2.2	0.0	2.3	3.0	0.0	0.0
Phenol	108-95-2	1951	39.61	19.9	15.5	53.2	31.0	0.0	0.0

Metabolites	Internal Standard	Unconfirmed ID

Appendix D Total volatile compounds identified by the SPME/GC-MS in BBAN (similarity match % - Control 2 ST 28)

Metabolite Compounds	CAS No	KI Published	Retention Time (min)	Similarity match %
Dimethyl sulfide	75-18-3	844	4.04	98
Ethyl formate	109-94-4	848	4.77	99
Ethyl acetate	141-78-6	886	5.72	97
Ethanol	64-17-5	929	6.84	96
2,5-dimethyl- furan	625-86-5	943	7.13	98
Diacetyl	431-03-8	977	7.75	89
ethyl butanoate	105-54-4	1028	9.49	98
butyl acetate	123-86-4	1105	10.58	88
2-methyl- 1-propanol	78-83-1	1108	11.60	99
isoamyl acetate	123-92-2	1117	12.20	98
(3E)-3-penten-2-one	3102-33-8	1128	12.42	95
2-pentanol	6032-29-7	1130	12.46	95
1-Butanol	71-36-3	1145	13.31	99
Isobutyl butyrate	539-90-2	1152	13.49	98
isoamyl alcohol	123-51-3	1205	15.48	98
Ethyl hexanoate	123-66-0	1220	16.20	94
isoamyl butyrate	106-27-4	1267	17.37	99
cis-3-Hexenyl Acetate	3681-71-8	1308	19.26	96
1-hexanol	111-27-3	1360	20.49	96
3-octanol	589-98-0	1406	21.88	91
Ethyl octanoate	106-32-1	1436	23.24	89
Acetic acid	64-19-7	1450	23.56	98
1-Octanol	111-87-5	1553	27.32	97
Butanoic acid	107-92-6	1619	29.03	97
Menthol	1490-04-6	1626	29.77	97
meththionol	505-10-2	1738	31.93	92

Methyl salicylate	119-36-8	1786	33.65	92
Benzyl alcohol	100-51-6	1865	36.39	97
Phenylethyl Alcohol	60-12-8	1925	37.29	98
Phenol	108-95-2	1951	39.57	97
p-Ethylguaiacol	2785-89-9	2048	40.32	90
gamma-nonalactone	104-61-0	2024	40.34	95
3p-Cresol	106-44-5	2077	41.48	97
Eugenol	97-53-0	2161	43.62	93
4-ethyl-phenol	123-07-9	2195	43.72	97
(Z)-isoeugenol	5912-86-7	2298	47.68	92

Appendix E Viable counts of LAB and yeast during stages of production and storage

E.1 BBQA1 Viable counts of LAB and yeast during stages of production

	Stage 1 day 1 (Sugar fermentation)	**Stage 2 day 2 (Molasses fermentation)**	**Stage 3 day 3 (Molasses fermentation)**
Sample ID			
	Yeast		
Date			
Temperature:			
pH :			
Viable counts:			
	LAB		
Date			
Temperature:			
pH :			
Viable counts:			

E.2 BBQA2 Viable counts of LAB and yeast during storage

Sample ID:				
Date:				
Storage days	**Viable count (log CFU/ml)** **LAB**	**Yeast**	**pH**	**Temperature**
Day1				
Day7				
Day14				
Day21				
Day28				

E.3 BBQA03: Product safety testing and results

Date tested	**Production Stage**	**Sample ID**	**pH**	**T°**	**Staphylococcus species**	**E. coli**	**Coliform**	***Rhodotourula rubra***
	Stage 1 day 1							
	Stage 2 day 2							
	Stage 3 day 3							
	Storage day 7							
	Storage day 14							
	Storage day 28							

E.4 BBQA03: Product metabolites testing and results

Date tested	Production Stage	Sample ID	pH	T°	Ethanol	Lactic acid	Butyric acid	Acetic acid	Diacetyl
	Stage 3 day 3								
	Storage day 7								
	Storage day 14								
	Storage day 28								

References

Abee, T., & Wouters, J. A. (1999). "Microbial stress response in minimal processing.". *Int. J. Food Microbiol., 50*, 65–91.

Acree, T., & Arn, H. (2004). Flavornet and human odor space www.flavornet.org. pp. Database of chemicals, their odors and chromatographic kovat retention indices.

Adhikari , K., Mustapha , A., & Grun , I. U. (2003). Survival and metabolic activity of microencapsulated Bifidobacterium longum in stirred yogurt. *J. Food Sci., 68*, 275–280.

Ahn, C., Collins-Thompson, D., Duncan, C., & Stiles, M. E. (1992). "Mobilization and location of the genetic determinant of chloramphenicol resistance from Lactobacillus plantarum caTC2R." *Plasmid, 27*, 169–176.

Álvarez-Martín, P., Flórez, A. B., Hernández-Barranco, A., & Mayo, B. (2008). Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation. *Food Control, 19*(1), 62-70. doi: http://dx.doi.org/10.1016/j.foodcont.2007.02.003

Alvarez-Perez, S. A., Mateos, L., Dominguez, E., Martinez-Nevado, J. L., Blanco, & Garcia, M. E. (2010). Isolation of Rhodo-torula mucilaginosa from skin lesions in a Southern sea lion (Otaria flavescens): a case report. *Veterinarni Medicina, 55*(6), 297–301.

Ananta, E., Heinz, V., Knorr, D., Carvalho, A. S., Silva, J., Ho, P., . . . Gibbs, P. (2004). Assessment of high pressure induced damage on Lactobacillus rhamnosus GG by flow cytometry. *Food Microbiology, 21*, 567–577.

Ananta, E., & Knorr, D. (2004). "Evidence on the role of protein biosynthesis in the induction of heat tolerance of Lactobacillus rhamnosus GG by pressure pre-treatment.". *Int. J. Food Microbiol. , 96*, 307–313.

Arthur, C., Salminen, O. S., & Isolauri, E. (2002). "Probiotics: an overview of beneficial effects.". *Antonie van Leeuwenhoek, 82*, 279–289.

Arturo, A., Maria, R. M., Maria, A. M., & (2006). "Probiotics for animal nutrition in the European Union.Regulation and safety assessment.". *Regul. Toxic. Pharm., 45*, 91-95.

Aruo, S. K. (1980). Necrotizing cutaneous rhodotorulosis in chickens in Uganda. *AvianDiseases, 24*(4), 1038–1043.

Babu, V., Mital, B. K., & Garg, S. K. (1992). "Effect of tomato juice addition on the growth and activity of Lactobacillus acidophilus.". *Int. J. Food Microbiol. , 17*, 67–70.

Banzatto, D., de Freita, L. A., & Mutton, M. J. R. (2013). Carotenoid production by Rhodotorula rubra cultivated in sugarcane juice, molasses, and syrup. *Food Science and Technology, 33*, 14-18.

Baum, B., Liebler-Tenorio, E. M., Enss, M. L., Pohlenz, J. F., & Breves, G. (2002). "Saccharomyces boulardii and bacillus cereus var. Toyoi influence the morphology and the mucins of the intestine of pigs.". *Z Gastroenterol, 40*, 277-284.

Bennett, G., Rajan, R., Bunt, C. R., & Hussain, M. A. (2013). Microbiological assessment of four probiotic feed supplements used by the dairy industry in New Zealand. *New Zealand veterinary journal, 61*(2), 119-120. doi: 10.1080/00480169.2012.716359

Bontempo, V., Di Giancamillo, A., Savoini, G., Dell'Orto, V., & Domeneghini, C. (2006). Live yeast dietary supplementation acts upon intestinal morpho-functional aspects and growth in weanling piglets. *Animal Feed Science and Technology, 129*(3), 224-236. doi: 10.1016/j.anifeedsci.2005.12.015

Boza, Y., Barbi, D., & Scamparini, A. R. P. (2004). "Survival of Beijerinckia sp. microencapsulated in carbohydrates by spray-drying.". *J. Microencapsul., 21*, 15–24.

Brashears, M. M., Galyean, M. L., Loneragan, G. H., Mann, J. E., & Killinger-Mann, K. (2003). " Prevalence of Escherichia coli O157:H7 and performance by beef feedlot cattle given Lactobacillus direct-fed microbials.". *Journal of Food Protection, 66*, 748–754.

Breuer, R. I., Soergel, K. H., Lashner, B. A., Christ, M. L., Hanauer, S. B., Vanagunas, A., ... (1997). Short-chain fatty acid rectal irrigation for left-sided ulcerative colitis: a randomized, placebo-controlled tria. *Gut, 40*, 485–491.

Brki, B., Sukovic, J., Matosic, S., & Gjurasic, K. (1995). Ability of chosen lactic acid bacteria to produce antibacterial substances. *Prehrambeno-tehnol. biotehnol. rev., 33* 145–150.

Broome, M. C., Thomas, M. P., Hillier, A. J., Horwood, J. F., Jago, G. R., & . (1979). The effect of linoleic acid on the growth and metabolisms of Streptococcus lactis. *Australian Journal of Dairy Technology, 34*, 163–168.

Buts, J. P., & de Keyser, N. (2006). Effects of Saccharomyces boulardii on intestinal mucosa. *Dig Dis Sci, 51*, 1485–1492.

Callaway, T. R., Edrington, T. S., Anderson, R. C., Harvey, R. B., Genovese, K. J., Kennedy, C. N., . . . Nisbet, D. J. (2008). "Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease.". *Anim Health Res Rev, 9*(2), 217-225.

Canani, B. R., Cucchiara, S., Cuomo, R., Pace, F., & Papale, F. (2011). Saccharomyces boulardii: A summary of the evidence for gastroenterology clinical practice in adults and children. *European Review for Medical and Pharmacological Sciences, 15*, 809–822.

Canibe, N., & Jensen, B. B. (2012). Fermented liquid feed—Microbial and nutritional aspects and impact on enteric diseases in pigs. *Animal Feed Science and Technology, 173*(1–2), 17-40. doi: http://dx.doi.org/10.1016/j.anifeedsci.2011.12.021

Cannon, J. P., Lee, T. A., Bolanos, J. T., & Danziger, L. H. (2005). Pathogenic relevance of Lactobacillus: a retrospective review of over 200 cases. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 24*(1), 31-40. doi: 10.1007/s10096-004-1253-y

Castanon, J. I. R. (2007). "History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds". *Poultry Science, 861*, 2466–2247.

CFIA. (2009). (Canadian Food Inspection Agency)Probiotic claims.

Champagne, C. P., Ross, R. P., Saarela, M., Hansen, K. F., & Charalampopoulos, D. (2011). Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. *International Journal of Food Microbiology, 149*(3), 185-193. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2011.07.005

Chen, T., Wu, Q., Li, S., Xiong, S., Jiang, S., Tan, Q., . . . Wei, H. (2014). Microbiological quality and characteristics of probiotic products in China. *Journal of the Science of Food and Agriculture, 94*(1), 131-138. doi: 10.1002/jsfa.6221

Choct, M. (2009). " Managing gut health through nutrition.". *British Poultry Science, 50*, 9–15.

Clapperton, J. F. (1978). Fatty acids contributing to caprylic flavour in beer.The use of profile and thershold data in flavour research. *Journal of the Institute of Brewing, 84*(2), 107-112. doi: 10.1002/j.2050-0416.1978.tb03849.x

Cleasby, T. G. (1963). The feeding value of molasses *Proceedings of The South African Sugar Technologists' Association-April 1963*, 113-117.

Coeuret, V., Gueguen, M., & Vernoux, J. P. (2004). Numbers and strains of lactobacilli in some probiotic products. *International Journal of Food Microbiology, 97*(2), 147-156. doi: 10.1016/j.ijfoodmicro.2004.04.015

Cogliani, C., Goossens, H., & Greko, C. (2011). Restricting antimicrobial use in food animals: A lesson from Europe. *Microbe, 6*, 274-279.

Collado, M. C., Meriluoto, J., & Salminen, S. (2007). Measurement of aggregation properties between probiotics and pathogens: In vitro evaluation of different methods. *J.Microbiol. Methods, 71*, 71-74.

Corcionivoschi, N., Drinceanu, D., Pop, I. M., Stack, D., Ştef, L., Julean, C., & Bourke, B. (2010). The Effect of Probiotics on Animal Health:REVIEW. *Scientific Papers: Animal Science and Biotechnologies,, 43*(1).

Corcoran, B. M., Ross, R. R., Fitzgerald, G. F., Dockery, P., & Stanton, C. (2006). "Enhanced survival of GroESL-overproducing Lactobacillus paracasei NFBC 338 under stressful conditions induced by drying.". *Appl Environ Microbiol, 72*, 5104-5107.

Crittenden, R., Laitilia, A., Forssell, P., Matto, J., Saarela, M., Mattila-Sandholm, T., & Myllarinen, P. (2001). "Adhesion of bifidobacteria to granular starch and implications in probiotic technologies.". *Appl. Environ. Microbiol., 67*, 3469–3475.

Cross, M. L. (2002). Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunol. Med. Microbiol., 34*, 245–253.

Cutting, S. M. (2011). Bacillus probiotics. *Food Microbiology, 28*, 214–220.

Czerucka, D., Nano, J. L., Bernasconi, P., & Rampal, P. (1989). Effect of Saccharomyces boulardii on cholera toxin-induced cAMP levels in rat epithelial intestinal cell lines. *Gastroenterol Clin Biol, 3*, 383–387.

Czerucka, D., & Rampal, P. (2002). Experimental effects of Saccharomyces boulardii on diarrheal pathogens. *Microbes Infect., 4*, 733–739.

Czinn, S. J., Blanchard, S. S., & (2009). " Probiotics in foods and supplements. In: Michail, S., Sherman, P.M. (Eds.), Nutrition and Health: Probiotics in Pediatric Medicine.". *Humana Press, Totowa, NJ:*, 299–306.

Dave, R. I., & Shah, N. P. (1997). Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter culture. *Int. Dairy J., 7*, 31–41.

De Jong, C., & Badings, H. T. (1990). Determination of free fatty acids in milk and cheese procedures for extraction, clean up, and capillary gas chromatographic analysis. *Journal of High Resolution Chromatography, 13*(2), 94-98. doi: 10.1002/jhrc.1240130204

De Man, J. C., Rogosa, M., & Sharpes, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*, 130-135.

Desouky, S. G., & Ibrahim, S. M. (2009). Effect of antimicrobial metabolites produced by lactic acid bacteria (Lab) on quality aspects of frozen tilapia (Oreochromis niloticus) fillets. *World Journal of Fish and Marine Sciences, 1*(1), 40-45.

Dessart, S., & Steenson, L. R. (1991). " High frequency intergeneric and intrageneric transfer conjugal transfer of drug resistance plasmids in Leuconostoc mesenteroides ssp. cremoris.". *J Dairy Sci, 74*, 2912–2919.

Dibner, J. J., & Richards, J. D. (2005). "Antibiotic growth promoters in agriculture: history and mode of action.". *Poultry Science, 84*, 634–643.

Doores, S. (1993). Organic acids. In P. M. Davidson and A. L. Branen (ed.), Antimicrobials in foods. *Marcel Dekker, Inc., New York, N.Y.*, 95–136.

EFSA. (2012). "Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update).". *EFSA Journal, 10*(12), 3020.

El-Sayed, A. (2014). The Pherobase: Database of Pheromones and Semiochemicals. *www.pherobase.com*.

Elder, R. O., Keen, J. E., Siragusa, G. R., Barkocy-Gallagher, G. A., Koohmaraie, M., & Lagreid, W. W. (2000). Correlation of enterohemorrhagic Escherichia coli O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. USA, 97*, 2999–3003.

Engels , W. J. M., & Visser , S. (1994). Isolation and comparative chracterization of components that contribute to the flavour different types of cheese. *NETHERLANDS MILK AND DAIRY JOURNAL, 48*(3), 127-140.

FAO/WHO. (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. . *Report of a Joint FAO/WHO Expert Consultation. Food and agriculture organization of the United Nations and World Health Organization. Córdoba, Argentina*.

Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., & Torriani, S. (2003). Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int. J. Food Microbiol., 82*, 59– 70.

Ferreira, A. D., & Viljoen, B. C. (2003). Yeasts as adjunct starters in matured Cheddar cheese. *International Journal of Food Microbiology, 86*(1-2), 131-140. doi: 10.1016/S0168-1605(03)00252-6

Filho-Lima, J. V., Vieira, E. C., & Nicoli, J. R. (2000). "Saccharomyces boulardii and Escherichia coli combinations against experimental infections with Shigella flexneri and Salmonella enteritidis subsp. Typhimurium." *J Applied Microbiol., 88*, 365-370.

Foo, H. L., Lim, Y. S., Loh, T. C., Saleh, N. M., Raha, A. R., & Rusul, G. (2005). Characterization of bacteriocin produced by Lactobacillus plantarum I-UL4 isolated from Malaysian fermented tapioca, Tapai Ubi. *Proceeding of 4th NIZO Dairy Conference,Papendal, Netherland*, 33.

Franklin, M. A., Mathew, A. G., Vickers, J. R., & Clift, R. A. (2002). Characterization of microbial populations and volatile fatty acid concentrations in the jejunum, ileum, and cecum of pigs weaned at 17 vs 24 days of age. *Journal of Animal Science, 80*, 2904-2910.

Fujiwara, Y., Masutani, C., Hanaoka, F., & Iwai, S. (1997). " Detection, purification and characterization of a protein that binds the (6-4) photoproduct-containing DNA in HeLa cells.". *Nucleic Acids Symp Ser*, 277-278.

Fuller. (1989). Probiotics in man and animals *Journal of Applied Bacteriology, 66*, 365–378.

Fuller. (1999). "Probiotics for farm animals :Probiotics: A Critical Rev*., 15*(8), 15-22.

Gaggìa, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology, 141 Suppl 1*, S15-S28. doi: 10.1016/j.ijfoodmicro.2010.02.031

Gardiner, G. E., O'Sullivan, E., Kelly, J., Auty, M. A., Fitzgerald, G. F., Collins, J. K., . . . Stanton, C. (2000). "Comparative survival of human-derived probiotic Lactobacillus paracasei and L. salivarius strains during heat treatment and spray drying." *Appl. Environ. Microbiol., 66*, 2605–2612.

Garg, D. P. M. a. D. N. (1980). Ovine pulmonary infection
caused by Rhodotorula rubra. *Mykosen, 23*(4), 208–211.

Gevers, D., Danielsen, M., Huys, G., & Swings, J. (2003). Molecular characterization of tet(M) genes in Lactobacillus isolates from different types of fermented dry sausage. *Applied and Environmental Microbiology, 69*(2), 1270-1275. doi: 10.1128/AEM.69.2.1270-1275.2003

Giang, H. H., Viet, T. Q., Ogle, B., & Lindberg, J. E. (2010). Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. *Livestock Science, 129*(1), 95-103. doi: 10.1016/j.livsci.2010.01.010

Gueimonde, M., Garrigues, C., van Sinderen, D., de los Reyes-Gavilán, C. G., & Margolles, A. (2009). Bile-inducible efflux transporter from Bifidobacterium longum NCC2705,conferring bile resistance. *Applied and Environmental Microbiology, 75*, 3153–3160.

Hamer, H. M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F. J., & Brummer, R. J. (2008). the role of butyrate on colonic function. *Aliment Pharmacol Ther 27*, 104-119.

Harish, K. I., & Varghese, T. V. (2006). Probiotics in humans evidence based review. *Calicut Med., J4(4): e3 openmed.nic.in*, 1856.

Hatoum, Rima, Labrie, Steve, Fliss, & Ismail. (2012). Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Frontiers in microbiology, 3*, 421. doi: 10.3389/fmicb.2012.00421

Heyman, M. (2000). Effect of lactic acid bacteria on diarrhoeal diseases. *Journal of the American College of Nutrition, 19*(2), 137-146.

Higgins, S. E., Higgins, J. P., Wolfenden, A. D., Henderson, S. N., Torres-Rodriguez, A., Tellez, G., & Hargis, B. (2008). "Evaluation of a Lactobacillus-based probiotic culture for the reduction of Salmonella enteritidis in neonatal broiler chicks. *Poult Sci*(87), 27-31.

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., & Huis int' Veld, J. H. J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology, 41*(2), 85-101.

Huang, Y., Ho, S.-H., Lee, H.-C., & Yap, Y.-L. (2002). Insecticidal properties of eugenol, isoeugenol and methyleugenol and their effects on nutrition of Sitophilus zeamais Motsch. (Coleoptera: Curculionidae) and Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae). *Journal of Stored Products Research, 38*(5), 403-412. doi: 10.1016/S0022-474X(01)00042-X

Huff, B. A. (2004). "Probiotics" might not be what they seem " *Canadian Family Physician, 50*, 583–587.

Hugas, M., Tsigarida, E., Robinson, T., & Calistri, P. (2009). The EFSA Scientific Panel on Biological Hazards first mandate: May 2003–May 2006. Insight into foodborne zoonoses. . *Trends in Food Science and Technology, 20*, 188–193.

Hussain, M. A., & Dawson, C. O. (2013). Economic Impact of Food Safety Outbreaks on Food Businesses. *Foods, 2*(4), 585-589. doi: 10.3390/foods2040585

Huys, G., Vancanneyt, M., D'Haene, K., Vankerckhoven, V., Goossens, H., & Swings, J. (2006). Accuracy of species identity of commercial bacterial cultures intended for probiotic or nutritional use. *Research in Microbiology, 157*(9), 803-810. doi: 10.1016/j.resmic.2006.06.006

Ibrahim, S. A., & Carr, J. P. (2006). Viability of bifidobacteria in commercial yogurt products in North Carolina during refrigerated storage. *International Journal of Dairy Technology, 59*(4), 272-277. doi: 10.1111/j.1471-0307.2006.00282.x

Ifeanyi, V. (2013). Assessment of Microbiological Quality of Yogurt Sold By Street Vendors in Onitsha Metropolis, Anambra State, Nigeria. *British Microbiology Research Journal, 3*(2), 198-205. doi: 10.9734/BMRJ/2013/1801

Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H., & Salminen, S. (2001). Probiotics: Effects on immunity. *Am. J. Clin. Nutr., 73*, 444-450.

Jin, L. Z., Ho, Y. W., Abdullah, N., & Jalaludin, S. (2000). Digestive and bacterial enzyme activities in broilers fed diets supplemented with Lactobacillus cultures. *Poult. Sci., 79*, 886– 891.

Kashket, E. R. (1987). Bioenergetics of lactic acid bacteria: Cytoplasmic pH and osmotolerance,. *FEMS Microbiol. Rev., 46*, 233–244.

Kopsahelis, N., Nisiotou, A., Kourkoutas, Y., Panas, P., Nychas, G. J. E., & Kanellaki, M. (2009). Molecular characterization and molasses fermentation performance of a wild yeast strain operating in an extremely wide temperature range. *Bioresour Technol, 100*(20), 4854-4862. doi: 10.1016/j.biortech.2009.05.011

Korbekandi, H., Mortazavian, A. M., & Iravani, S. (2011). Technology and stability of probiotic in fermented milks. In Probiotic and Prebiotic Foods: Technology, Stability and Benefits to the human health, Shah, N., A.G. Cruz and J.A.F Faria (Eds.) Nova Science Publishers, New York. 131-169.

Kosin, B., & Rakshit, S. K. (2006). Criteria for Production of Probiotics. *Food Technol. Biotechnol., 44*(3), 371–379

Lavermicocca, P. (2006). Highlights on new food research. *Digestive and Liver Disease, 38*(Suppl. 2), S295–S299.

Lee , K. G., & Shibamoto, T. (2000). Antioxidant Properties of Aroma Compounds Isolated from Soybeans and Mung Beans. *Journal of Agricultural and Food Chemistry 48*, 4290-4293. doi: 10.1021/jf000442u.

Lefteris, M., Vagelis, T., Domitille, F., Tom, A., Georgia, Z., & Effie, T. (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards Salmonella enterica serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Research in Microbiology, 157*(3), 241–247.

Lema, M., Williams, L., & Rao, D. R. (2001). Reduction of fecal shedding of enterohemorrhagic Escherichia coli O157:H7 in lambs by feeding microbial feed supplement. *Small Rumin. Res., 39*, 31– 39.

Leroy, F., & Pidoux, M. (1993). Detection of interactions between yeasts and lactic acid bacteria isolated from sugary kefir grains. *The Journal of Applied Bacteriology, 74*, 48–53.

Leroy, F., Pidoux , M., & (1993). Detection of interactions between yeasts and lactic acid bacteria isolated from sugary kefir grains. *The Journal of Applied Bacteriology, 74*, 48–53.

Licciardi, P. V., Wong, S.-S., Tang, M. L., & Karagiannis, T. C. (2010). Epigenome targeting by probiotic metabolites. *Gut pathogens, 2*(1), 24-24. doi: 10.1186/1757-4749-2-24

Lievin, V., Peiffer, I., Hudault, S., Rochat, F., Brassart, D., Neeser, J. R., & Servin, A. L. (2000). " Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity.". *47, Gut :* , 646-652.

Lilley, B. D., & Brewer, J. H. (1953). The selective antibacterial action of phenylethyl alcohol. *J Am Pharm Assoc Am Pharm Assoc (Baltim)*(42), 6–8.

Lin, W.-H., Hwang, C.-F., Chen, L.-W., & Tsen, H.-Y. (2006). Viable counts, characteristic evaluation for commercial lactic acid bacteria products. *Food Microbiology, 23*(1), 74-81. doi: 10.1016/j.fm.2005.01.013

Lindgren, S. E., & Dobrogosz, W. J. (1990). Antagonistic activites of lactic acid bacteria in food and feed fermentations *FFEMS Microbiology Letters, 87*(1-2), 149-163.

Lindgren , S. E., Dobrogosz , W. J., & (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiol. Rev., 87*, 149-164.

Liong, M. T. (2007). "Probiotics: A critical review of their potential role as antihypertensives, immune modulators, hypocholesterolemics and perimenopausal treatments. *Nut. Rev, 65*, 316-328.

Ljungh, A., & Wadström, T. (2006). Lactic acid bacteria as probiotics. *Current Issues in Intestinal Microbiology, 7*(2), 73–89.

Marshall, V. M. E., & Tamine, A. Y. (1997). Physiology and biochemistry of fermented milks. In B. A. Law (Ed.). *Microbiology and chemistry of cheese and fermented milk, London: Blackie Academic and Professional*, 153–192.

Marteau, P., & Boutron-Ruault, M. C. (2002). "Nutritional advantages of probiotics and prebiotics". *Br. J. Nutr., 87*, 153-157.

Mathur, S., & Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria—a review. *International Journal of Food Microbiology, 105*(3), 281-295. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2005.03.008

Nair, M. K. M., Joy, J., Vasudevan, P., Hinckley, L., Hoagland, T. A., & Venkitanarayanan, K. S. (2005). Antibacterial effect of caprylic acid and monocaprylin on major bacterial mastitis pathogens. *Journal of Dairy Science, 88*(10), 3488-3495. doi: 10.3168/jds.S0022-0302(05)73033-2

Naz, S., Cretenet, M., & Vernoux, J. P. (2013). Current knowledge on antimicrobial metabolites produced from aromatic amino acid metabolism in fermented products. *Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.) FORMATEX*, 337-346.

Neal-McKinney, J. M., Lu, X., Duong, T., Larson, C. L., Call, D. R., Shah, D. H., & Konkel, M. E. (2012). Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. *PloS one, 7*(9), e43928. doi: 10.1371/journal.pone.0043928

Neish. (2004). " Bacterial inhibition of eukaryotic pro-inflammatory pathways." *Immunol Res :, 29*, 175-186.

Niamsup, P., Sujaya, I. N., Tanaka, M., Sone, T., Hanada, S., Kamagata, Y., . . . Yokota, A. (2003). "Lactobacillus thermotolerans sp. nov., a novel thermotolerant species isolated from chicken faeces." *Int. J. Syst. Evolut. Microbiol. , 53*, 263–268.

Nielsen, M. S., Frisvad, J. C., & Nielsen, P. V. (1998). Protection by fungal starters against growth and secondary metabolite production of fungal spoilers of cheese. *International Journal of Food Microbiology, 42*, 91–99.

Nurmi, E., & Rantala, M. (1973). " New aspects of Salmonella infection in broiler production." *Nature, 241*, 210–211.

Olbrich, H. (2006). THE MOLASSES. *Biotechnologie-Kempe GmbH*.

Olivier, T., Bunt, C., & Hardy, G. (2008). The quality of probiotics in New Zealand, Asia Pacific Critical Care 2008 Congress, Sydney, Australia.

Pascual, M., Hugas, M., Badiola, J. I., Monfort, J. M., & Garriga, M. (1999). "Lactobacillus salivarius CTC2197 prevents Salmonella enteritidis colonization in chickens." *Appl. Environ. Microbiol., 65*, 4981–4986.

Pires, E. J., Teixeira, J. A., Brányik, T., & Vicente, A. A. (2014). Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology, 98*(5), 1937-1949. doi: 10.1007/s00253-013-5470-0

Plumed-Ferrer, C., & von Wright, A. (2009). Fermented pig liquid feed: nutritional, safety and regulatory aspects. *Journal of Applied Microbiology, 106*(2), 351-368. doi: 10.1111/j.1365-2672.2008.03938.x

Plumed-Ferrer, C., & von Wright, A. (2011). Antimicrobial activity of weak acids in liquid feed fermentations, and its effects on yeasts and lactic acid bacteria. *Journal of the Science of Food and Agriculture, 91*(6), 1032-1040. doi: 10.1002/jsfa.4278

Purohit, S., Ruchika, S., Jodhawat, N., & Kaur, S. (2012). "Evaluation of probiotic potential of Lactobacillus fermentum strain. International Journal of Pharmaceutical Sciences rev and Research." *14*(2), 142-144.

Ray, B., & Sandine, W. E. (1992). Acetic, propionic, and lactic acids of starter culture bacteria as biopreservatives. *In B. Ray and M. Daeschel (ed.), Food preservatives of microbial origin. CRC Press, Boca Raton, Fla*, 103–136.

Ray, B., Sandine , W. E., & Daeschel , M. (2000). Acetic, propionic, and lactic acids of starter Culture bacteria as biopreservatives *Food bio preservatives of biological origin. CRC press, Inc. Boca Raton, Florida 103-136*.

Reid, G. (2008). Probiotics and prebiotics – progress and challenges. *International Dairy Journal of Agricultural and Food Chemistry, 18*, 969–975.

Research and Markets. (2013). Probiotics Market By Products, Applications & Ingredients - Global Trends and Forecasts to 2017. Retrieved 02 August 2013, 2013

Roostita, R., & Fleet, G. H. (1996). The occurrence and growth of yeast in Camembert and blue-veined cheese. *International Journal of Food Microbiology, 28*, 293–404.

Ross Crittenden, R. W., Sanguansri, L., & Augustin, M. A. (2006). "Synbiotic Microcapsules That Enhance Microbial Viability during Nonrefrigerated Storage and Gastrointestinal Transit.". *Applied and Environmental Microbiology*, 2280–2282.

Rycroft, C. E., Jones, M. R., Gibson, G. R., & Rastall, R. A. (2001). "A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides.". *J. Appl. Microbiol., 91*, 878–887.

Sanders, M., Gibson, G. H., Gill, & Guarner, F. (2007). Probiotics: Their potential to impact human health. *Council for Agricultural Science and Technology (CAST) Issue Paper, 36*(CAST,Ames, IA).

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., & Biavati, B. (2010). Characterization of probiotic strains: an application as feed additives in

poultry against Campylobacter jejuni. *International Journal of Food Microbiology, 141 Suppl 1*, S98-S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Sazawal, S., Hiremath, G., Dhingra, U., Malik, P., Deb, S., & Black, R. E. (2006). "Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials." *Lancet Infectious Diseases, 6*, 374–382.

Schierack, P., Wieler, L. H., Taras, D., Herwig, V., Tachu, B., Hlinak, A., . . . Scharek, L. (2007). "Bacillus cereus var. toyoi enhanced systemic immune response in piglets.". *Vet Immunol Immunopathol, 118*, 111-114.

Seow, Y. X., C.O., P. K., & Liu, S. Q. (2010). Production of flavour-active methionol from methionine metabolism by yeasts in coconut cream. *International Journal of Food Microbiology, 143*(3), 235-240. doi: 10.1016/j.ijfoodmicro.2010.08.003

Shah, N. P., Lankaputhra, W. E. V., Britz, M., & Kyle, W. S. A. (1995). "Survival of L. acidophilus and Bifidobacterium bifidum in commercial yoghurt during refrigerated storage." *Int. Dairy J, 5* 515–521.

Shimamura. (1982). "Milk products containing bifidobacteria.". *Jap. J. Dairy Food Sci., 31*, A245–253.

Siggers, R. H., Thymann, T., Siggers, J. L., Schmidt, M., Hansen, A. K., & Sangilda, P. T. (2007). "Bacterial colonization affects early organ and gastrointestinal growth in the neonate.". *Livestock Science 109*, 14–18.

Snydman, D. R. (2008). The safety of probiotics. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 46 Suppl 2*(S2), S104-S111. doi: 10.1086/523331

Sow, N. M., Robin, D. D., Guiro, A. T., & Thonart, P. (2005). "Polyphasic identification of a new thermotolerant species of lactic acid bacteria isolated from chicken faeces.". *Afric. J. Biotechnol., 4*, 409–421.

Spencer, R. J., & Chesson, A. (1994). The effect of Lactobacillus spp. on the attachment of enterotoxigenic Escherichia coli to isolated porcine enterocytes. *J. Appl. Microbiol., 77*(2), 215–220.

Sullivan, A., & Nord, C. E. (2006). "Probiotic lactobacilli and bacteraemia in Stockholm.". *Scand J Infect Dis, 38*, 327–331.

Sumby, K. M., Grbin, P. R., & Jiranek, V. (2010). Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chemistry, 121*(1), 1-16. doi: 10.1016/j.foodchem.2009.12.004

Suskovic, J., Kos, B., Beganovic, J., Pavunc, A. L., Habjanic, K., & Matoic, S. (2010). Antimicrobial Activity - The Most Important Property of Probiotic and Starter Lactic Acid Bacteria. *FOOD TECHNOLOGY AND BIOTECHNOLOGY, 48*(3), 296-307.

Svensson, U. (1999). "Industrial perspectives. Probiotics: A Critical Review.". *15*(8), 57-64.

Tharmaraj , N., & Shah , N. P. (2009). "Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips." *International Food Research Journal, 16*, 261-276.

Tharmaraj, N., & Shah, N. P. (2009). "Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips.". *International Food Research Journal, 16*, 261-276.

Thomas, K. C., Hynes, S. H., & Ingledew, W. M. (2002). Influence of Medium Buffering Capacity on Inhibition of Saccharomyces cerevisiae Growth by Acetic and Lactic Acids. *Applied and Environmental Microbiology, 68*(4), 1616-1623. doi: 10.1128/AEM.68.4.1616-1623.2002

Thu , T. V., Loh, T. C., Foo, H. L., Yaakub, H., & Bejo, M. H. (2011). Effects of liquid metabolite combinations produced by Lactobacillus plantarum on growth performance, faeces characteristics, intestinal morphology and diarrhoea incidence in postweaning piglets

Trop Anim Health Prod, 43, 69–75. doi: DOI 10.1007/s11250-010-9655-6

Timmerman, H. (2004). Monostrain, multistrain and multispecies probiotics?A comparison of functionality and efficacy1. *International Journal of Food Microbiology, 96*(3), 219-233. doi: 10.1016/j.ijfoodmicro.2004.05.012

Torres-Rodriguez, A., Donoghue, A. M., Donoghue, D. J., Barton, J. T., Tellez, G., & Hargis, B. M. (2007). " Performance and condemnation rate analysis of commercial turkey flocks treated with a Lactobacillus sp.-based probiotic.". *Poult. Sci., 86*, 444-446.

Tuomola , E. R. C., Playne , M., Isolauri , E., & Salminen , S. (2001). "Quality assurance criteria for probiotic bacteria." *Am J Clin Nutr 73:*, 393S–398S.

Umano, K., Nakahara, K., Shoji, A., & Shibamoto, T. (1999). Aroma Chemicals Isolated and Identified from Leaves of Aloe arborescens Mill. Var. natalensis Berger. . *Journal of Agricultural and Food Chemistry, 47*, 3702-3705. doi: 10.1021/jf990116i.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S. D., & Maguin, E. (2002). Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek, 82*, 187–216.

Van Immerseel, F., Flint, H. J., Ducatelle, R., De Vos, M., Boon, N., Van De Wiele, T., . . . Louis, P. (2010). Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. *Journal of medical microbiology, 59*(Pt 2), 141-143. doi: 10.1099/jmm.0.017541-0

van Winsen, R. L., Lipman, L. J. A., Biesterveld, S., Urlings, B. A. P., Snijders, J. M. A., & van Knapen, F. (2001). Mechanism of Salmonella reduction in fermented pig feed. *J. Sci. Food Agric., 81*, 342–346.

Vandenbergh, P. A. (1993). Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiology Reviews, 12*(1–3), 221-237. doi: http://dx.doi.org/10.1016/0168-6445(93)90065-H

Vanhee, L. M. E., Goemé, F., Nelis, H. J., & Coenye, T. (2010). Quality control of fifteen probiotic products containing Saccharomyces boulardii. *Journal of Applied Microbiology, 109*(5), 1745-1752. doi: 10.1111/j.1365-2672.2010.04805.x

Vanhee , L. M. E., Goemé , F. H. J., Coenye , T., & Nelis. (2010). Quality control of fifteen probiotic products containing Saccharomyces boulardii. *Journal of Applied Microbiology, 109*(5), 1745-1752.

Verstegen, M. W. A., & Williams, B. A. (2002). " Alternatives to the use of antibiotics as growth promoters for monogastric animals." *Animal Biotechnology, 13*, 113–127.

Vinderola, C. G., Bailo, N., & Reinheimer, J. A. (2000). Survival of probiotic microflora in Argentinian yogurths during refrigerated storage. *Food Res. Int., 33*, 97–102.

Voetsch, A. C., Van Gilder, T. J., Angulo, F. J., Farley, M. M., Shallow, S., Marcus, R., . . . Tauxe, R. V. (2004). FoodNet estimate of the burden of illness caused by nontyphoidal Salmonella infections in the United States. *Clin Infect Dis, 38*(suppl 3), 127-134.

Wannaprasat, W., Koowatananukul, C., Ekkapobyotin, C., & Chuanchuen, R. (2009). "Quality analysis of commercial probiotic products for food animals.". *Southeast Asian Journal of Tropical Medicine and Public Health, 40*, 1103–1112.

Wassenaar, T. M., & Klein, G. (2008). " Safety aspects and implications of regulation of probiotic bacteria in food and food supplements.". *Journal of Food Protection, 71*, 1734–1741.

Weese, J. S., & Martin, H. (2011). Assessment of commercial probiotic bacterial contents and label accuracy. *The Canadian veterinary journal. La revue vétérinaire canadienne, 52*(1), 43-46.

Wirth, F., & Goldani, L. Z. (2012). Epidemiology of Rhodotorula: an emerging pathogen. *Interdisciplinary perspectives on infectious diseases, 2012*, 465717. doi: 10.1155/2012/465717

www.chemspider.com.

www.flavornet.org.

www.pherobase.com.
www.thegoodscentscompany.com. from http://www.thegoodscentscompany.com/data/rw1006752.html
Younts-Dahl, S. M., Galyean, M. L., Loneragan, G. H., Elam, N. A., & Brashears, M. M. (2004). " Dietary supplementation with lactobacillus- and propionibacterium-based direct-fed microbials and prevalence of Escherichia coli O157 in beef feedlot cattle and on hides at harvest." *Journal of Food Protection, 67*, 889–893.
Yu, P., Huber, J. T., Theurer, C. B., Chen, K. H., Nussio, L. G., & Wu, Z. (1997). "Effect of steam-flaked or steam commercial rolled corn with or without Aspergillus oryzae in the diet on performance of dairy cows fed during hot weather.". *J. Dairy Sci., 80*, 3293-3297.
Zu, Y. H., Yang, P. P., Wang, J. J., Liu, X. H., Ren, J. W., Lu, G. Z., & Wang, Y. Q. (2014). Efficient production of the liquid fuel 2,5-dimethylfuran from 5-hydroxymethylfurfural over Ru/Co3O4 catalyst. *APPLIED CATALYSIS B-ENVIRONMENTAL, 146*, 244-248. doi: 10.1016/j.apcatb.2013.04.026