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Technological benefits and potential of incorporation of probiotic bacteria and inulin in soft cheese

By

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Technological benefits and potential of incorporation of probiotic bacteria and inulin in soft cheese

Abstract

There is an increasing consumer demand for dairy products which are safe and free from additives. Microbial starter strains, in combination with other factors, were studied for their contribution to the control of unwanted microbes, and maintaining the quality of soft cheese. The technological and functional characteristics of the starter culture strains *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris,* and probiotic bacterial strains *Bifidobacterium animalis* subsp*. lactis* BB12, *Lactobacillus acidophilus* LA-5 and *Lactobacillus casei* Shirota were investigated. The tests included the milk fermentation, resistance to salt and heat, bile and acid resistance, and growth at a range of temperatures. The probiotic strains differed in their resistance to salt, bile salts and acid. Inhibitory interactions between probiotic bacterial strains with each other and with starter culture strains were not detected. The probiotic bacteria and starter culture strains used have an ability to grow together on homofermentative and heterofermentative differential agar and fermentation of fructose in different levels. Non-starter cheese (NSC), cheese with starter strains (SCS), and cheese with starter and probiotic strains (PSC) were manufactured. The levels of mesophilic aerobic and lactic acid bacteria, moulds and yeasts, and *Enterobacteriaceae* were evaluated in all cheeses. Their contents of fat, total solids, salt and pH value were tested during 21 days of storage at 2-5°C. Starter culture strains contributed to maintaining the quality of all cheeses, through decreasing the viable count of some undesirable microbes. Cheeses differed in the intensity of the crumbliness attribute, and in preference and intensity of colour attribute. The colour of starter soft cheese, which was tested using a colorimeter, was closer to the colour of probiotic soft cheese than those cheeses which were manufactured without starter culture. The microbial status, storage conditions,

rancidity, and the sensory characteristics of unripened soft cheese, which was manufactured with starter culture strains only, were determined during the storage for 50 days at 2-5°C, as well as during their shelf life for the product. Modified Atmosphere Packaging (MAP) contributed to slowing the growth of unwanted microbes, and decreased the values of TBA, TVB-N and TMA in soft cheese. Consequently, delaying the undesirable changes and maintaining the quality of the product and extending its shelf life, when compared with vacuum, brine, and air packaging methods, under the same storage conditions. Potential effects of inulin on the cheese quality and sensory characteristics of probiotic soft cheese were investigated. The cheeses differed in their loads of lactic acid bacteria, in addition to the total solids and water activity. The levels of probiotic bacterial strains were higher in probiotic soft cheese that manufactured with inulin than in cheese without inulin, with a potential in the formation synbiotic between the probiotic strains LA-5 and BB12 and inulin. Both cheeses were recorded to have high acceptance in the cheese attributes, in terms of appearance, aroma, colour texture and the overall acceptance. The presence of inulin increased the hardness of cheese under vacuum packaging, after storage for 14 days at 2-5°C.

List of Contents

List of tables

List of figures

List of abbreviations

Abbreviations	Terms used
APC	Aerobic Plate Count
AP	Air Packaging
ANOVA	Analysis of Variance
BB12	*Bifidobacterium animalis* subsp. *lactis* BB12®
BA	Biogenic Amines
BSA	Bovine Serum Albumen
BP	Brine Packaging
CFS	Cell-Free Supernatants
°C	Centigrade
cm	Centimetre
cm^2	Centimetre Squared
COPS	Cholesterol Oxidation Products
CIP	Clean-in-Place
CFU	Colony Forming Unit
CIE	Commission International d'Eclairage
cm^3	Cubic Centimeter
MRS	De man, Rogosa and Sharpe
ΔEab*	Difference in Colour
DNS	3, 5-dinitrosalicylic acid
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FFA	Free Fatty Acids
GIT	Gastrointestinal Tract
GRAS	Generally Recognized As Safe
GMP	Good Manufacturing Practices
g	Gram
GLMA	Growth in Liquid Media Assay
HP	High-Performance
HPLC	High-Performance Liquid Chromatography
HHD	Homofermentative and Heterofermentative
IFST	Institute of Food Science and Technology
IDF	International Dairy Federation
Kg	Kilogram
LAB	Lactic Acid Bacteria

LA-5	*Lactobacillus acidophilus* ®LA-5
LcS	*Lactobacillus casei* Shirota
L.S.D	Least Significant Difference
L*	Lightness
L	Litre
Log	Logarithm
LX	Luminous Flux
MAD	Malondialdehyde
MRD	Maximum Recovery Diluent
MAB	Mesophilic Aerobic Bacteria
MABC	Mesophilic Aerobic Bacteria Count
µl	Microliter
µm	Micrometre
mg	Milligram
ml	Millilitre
mm	Millimetre
mmol	Millimole
min	Minute
M17	M17 Media
MAP	Modified Atmosphere Packaging
M	Molarity
Mol	Mole
M&Y	Moulds and Yeasts
nm	Nanometre
−Ve	Negative
NSLAB	Non-Starter Lactic Acid Bacteria
N	Normality
OD	Optical Density
%	Percentage
ΔpH	pH Difference
PBS	Phosphate Buffer Saline
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
+Ve	Positive
PDA	Potato Dextrose Agar
PSC	Probiotic Soft Cheese

PBC	Psychrotrophic Bacteria Count
RSM	Reconstituted Skimmed Milk
a*	Redness
RH	Relative Humidity
SD	Standard Division
SE	Standard Error
SPA	Standard Plate Agar
SPC	Standard Plate Count
SC	Starter Culture
SCS	Starter Soft Cheese
TEP	1,1,3,3-Tetraethoxypropane
TPA	Texture Profile Analysis
TPC	Total Plate Count
TS	Total Solids
TVB-N	Total Volatile Base Nitrogen
TBA	Thiobarbitutric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic Acid
TMA	Trimethylamine
TMNO	Trimethylamine-Noxide
UHT	Ultra High Temperature
VP	Vacuum Packaging
VRBGA	Violet Red Bile Glucose Agar
V/V	Volume / Volume
a_w	Water Activity
W/V	Weight/Volume
WHO	World Health Organization
b*	Yellowness

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- Jawad E, Beal, J. Kuri V. (2012). Effect of inulin on the viability and activity of starter cultures and probiotic bacterial strains (Poster). Young Researchers Food Sector Event, Biosciences Knowledge Transfer Network, KTN, 2012, Edinburgh, Scotland, 14 November.
- Jawad E, Beal, J. Kuri V. (2012). Microbial quality and sensory properties evaluation of Middle Eastern style soft cheese produced without and with starter culture and probiotic bacteria (Poster). The Postgraduates and Early Career Scientists' (PECS) Research Conference, 'Molecular and Microbiological Techniques', 25 October, London, UK.
- Jawad E, Beal, J. Kuri V. (2012). Effect of prebiotic ingredient (inulin) on the sensory properties of a probiotic soft cheese (Poster). Centre of Agricultural Researches Symposium (CARS), University of Plymouth, 10 December 2012.
- Jawed E, Beal, J. Kuri V. (2012). Microbiological quality and sensory Evaluation of Middle Eastern style soft cheese produced without and with starter culture and probiotic bacteria (Oral Presentation). Centre for Agriculture and Rural Sustainability Symposium, Duchy College, Plymouth, 5th July 2012.
- Jawad E, Beal, J. Kuri V. (2012). Application of probiotic bacteria to Middle Eastern style soft cheese (Poster). Society for General Microbiology, Autumn Conference 2012, University of Warwick, 3-5 September 2012.
- Jawad E, Beal, J. Kuri V. (2011). Effect of salt (NaCl) on the viability and activity of cheese starter culture (Poster). Society of Chemical Industry, Bio Resources Young Researchers, University of reading, July 2011.

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Chapter 1

General introduction, aims and objectives, main hypotheses and research questions

1.1 Introduction

Soft cheese is one of the most popular dairy products in the Middle Eastern countries. Soft white cheese is commonly produced from pasteurized or raw milk, using rennet. These products have different pH values, moisture content (>50%), and has a considerable variety in its content of protein, fat, and added salt. It is manufactured originally from cow's milk, buffalo's milk, sheep's milk and goat's milk by adding rennet. Extending the shelf-life and conserving the nutritional components of milk are the primary objectives for cheese manufacturing. It has an excellent nutritional value, and therefore, constitutes a suitable environment for the growth of many pathogens, and it has frequently been associated with several food-borne diseases, particularly in Iraq, where the product is still manufactured without starter culture. In this sense, the physiochemical and microbiological characteristics of soft cheese increase the potential risk that is caused by pathogens (Beresford *et al.*, 2001). The quality and yield of soft cheese depend on the quality of milk used.

Cheese is consumed by many people, and the nutritional value of cheese, in addition to a variety of cheese types all over the world has contributed to market growth for probiotic cheeses (Karimi *et al.*, 2012). Consequently, it is important to improve the functionality, safety, and the quality of soft cheese, and protect the consumer's health, through the incorporation of specific strains of selected probiotics, which have been defined by FAO/WHO (2002) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". The incorporation of probiotic bacteria in cheese contributes to potential improvement of the health status

of the products. For this reason, the range of probiotic products has widely increased the response to the market requirements (Ong *et al.*, 2006). Cheese has a number of advantages over other fermented dairy products, such as yoghurt and fermented milk, as a carrier for delivering live probiotic bacteria to the GIT. It has a high pH value, buffering capacity, and a more solid consistency. Therefore, it offers excellent conditions for the survival and growth of probiotics during storage, and during its passage through the GIT. Cheese manufacturing steps, the storage at low temperatures, and the short ripening period makes soft cheese a good carrier for the probiotics, and deliver them to the consumers (Heller *et al.*, 2003). Development of probiotic cheese require selected strains of probiotics, the ability to maintain their viability in the cheese during the steps of manufacturing, and in the storage period until consumption (Phillips *et al.*, 2006). Shah (2007), stated that the probiotics should be present in a dairy food to a minimum level of 10^6 cfu g^{-1}, or the daily intake should be about 10^8 cfu g^{-1}. The aim is to compensate for the possible drop in the number of the probiotic microorganisms during their passage through the upper gut. The quality parameters, texture, and the sensory attributes of soft cheese, which are considered to be most important for the consumers, may be changed during the application of the probiotics strain to cheese (Buriti *et al.*, 2005a). Due to the short shelf life of soft cheese, it is essential to extend it, through the use of appropriate packaging system, such as modified atmosphere packaging, which demonstrates a good performance with some types of cheese. MAP system could play an important role in preventing or decreasing the growth of unwanted microorganisms, which contribute to the occurrence of undesirable changes in the physio-chemical properties of cheese. These changes could negatively affect the consumer's acceptability of the product (Khoshgozaran *et al.*, 2012). The recent trend in food technology is to use 1-combination of probiotic bacterial strains and prebiotic

ingredients, such as inulin, lactulose, and oligosaccharides “Synbiotic”, so that they work together to stimulate 2-the growth and/or activity of one or a limited number of bacteria. In addition, prebiotics may be more effective when used in appropriate combinations, and consequently, improving the gut health of the consumers (Boylston *et al.*, 2004; Wang, 2009). The increased interest in the incorporation of probiotics and prebiotic ingredients in foods, especially in the dairy products and the new trend focus on cheese, increasingly makes this a very interesting topic to work with. Although there are many studies related to the incorporation of probiotic bacterial strains as an adjunct culture to the starter culture during the cheese manufacture of some Middle Eastern cheeses. There is a comparatively limited number relating to unripened soft cheese, such as Iraqi unripened soft cheese, is considered to be a suitable medium for the growth, carrying, and the delivery of probiotic bacteria to the consumers, and consequently, the development of probiotic soft cheese. It has suitable components for the growth and survival of these microorganisms. In this study, therefore, the research was focused for dissolving the problems of the unripened soft cheese, which is manufactured currently in Iraqi dairy plants without any starter culture, and contribute to maintain the quality, safety, sensory and functional characteristics for the product, through the incorporation the starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. Lactis* subsp. *cremoris*. In addition, probiotic bacterial strains *B. animalis* subsp. *lactis* BB12, *Lb. acidophilus* LA-5 and *Lb. casei* Shirota as adjunct cultures, as well as the prebiotics ingredient (inulin).

1.2 Aims and objectives

The aim of this study is to study some factors that contribute to improve the quality, safety, and functionality of “Middle Eastern“ style soft unripened cheese, which is manufactured without any starter culture. Not only that, probiotic bacteria levels are important, but also the growth of other microbial groups, including the spontaneous

and starter lactic acid bacteria, and pathogen and spoilage indicators, will be of interest. The effect of starter culture strains, the type of milk, and possibly the novel fibers or prebiotic ingredient will be studied, in relation to the sensory acceptability, acidity development, safety, and shelf life. Also, investigating the effect of the manufacturing stages of cheese, such as heat treatment, storage temperature and packaging methods, including vacuum, modified atmosphere, and brine packaging, with regards to maintaining the quality of product and extending the shelf life, is required.

1.3 The main hypotheses

1- Application of a combination of probiotics bacteria, starter culture strains, and prebiotic ingredient in unripened soft cheese, may contribute in the control of the risk of pathogens, the extension of the shelf-life, and maintaining the sensory properties of the product.

2- The manufacture of unripened soft cheese with starter culture strains, and storage the product under the controlled storage conditions with suitable packaging method, would be effective in extending the shelf life and maintaining the sensory quality of the product.

1.4 Research questions

The research questions are:-

1- Are probiotic bacterial strains, when used as adjunct cultures with starter culture strains in the manufacture of soft cheese under the study, able to maintain a level required after the end of storage period and at time of consumption of the product in order to confer health benefits to consumers?

2- Could the manufacture of non-starter cheese, with the starter culture and probiotic bacterial strains under study, contribute to ensuring its positive effect on the consumer's health?

3- Which combination of cultures can affect the quality, safety and the acceptance of non-starter soft cheese? And which ones do not? In order to see what is essential to obtain the desired results, what measures can be taken?

4- Could the incorporation of a combination of starter culture and probiotic bacterial strains in the unripened soft cheese with and without inulin affect the microbiological, physicochemical, sensory characteristics of the product, and the consumer's acceptance?

5- Do salt additions during the manufacture of probiotic soft cheese at a level up to 3.0%, affect the performance of the starter culture and probiotic bacterial strains, and maintain the cheese quality and consumer acceptability?

6- Which is an appropriate packaging method, amongst the methods used for maintaining and /or protecting the quality, safety, sensory attributes, and for extension of the shelf life of the product, in order to selection that method for achieving a target?

1.5 Outline of the thesis

An introduction to how this study will be carried out is given in the following points:

1- Before their application to cheese, evaluation of the technological characteristics of the starter culture and probiotic bacterial strains used, *in vitro*. This includes the acidification activity, their ability to ferment of milk, salt resistance, growth at a range of temperatures, and their ability to grow on different media, in the presence of inulin and lactose together and separately. In addition to evaluating the functional characteristics of probiotic bacterial strains which are used in this study, several factors are to be considered, including their survival during exposure to the

conditions that could be encountered in the GIT. In addition, investigating the possible competitive inhibition between individual probiotic bacterial strains, and with starter culture strains, needs implementation.

2- Manufacture of non-starter soft cheese, soft cheese with starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, with starter culture and probiotic bacterial strains *Lb. acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB12 and *Lactobacillus casei* Shirota, and evaluation of the performance of starter culture and probiotic bacterial strains used in relation to their effect on the microbiological, as well as the physicochemical and sensory characteristics of unripened soft cheese under cold storage.

3- Assess the role of packaging methods, including VP, MAP and BP, in extending the shelf life of unripened soft cheese, which is manufactured with the starter cultures. Through measuring the levels of thiobarbituric acid reactive substances (TBARS), trimethylamine (TMA) and total volatile base nitrogen TVB-N. Also, evaluate the sensory attributes, including the odour and colour of the product. In addition, evaluate the effectiveness of packaging methods on the physicochemical properties of the product, during the storage shelf life.

4- Evaluate the ability of probiotic strains *Lb. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB12 and *Lb. casei* Shirota (LcS), to survive after their incorporation as an adjunct in soft cheese, which is manufactured with and without inulin, and the storage under cold conditions. In addition, their performance, with regards to the consumer acceptability of a product, needs to be noted. Furthermore, examine the microbiological, physicochemical characteristics, the sensory properties and the texture profile analysis (TPA) of probiotic soft cheese, with and without inulin.

Chapter 2

Literature review: the technological benefits and potential of the incorporation of probiotic bacteria and inulin in soft cheese

2.1 Introduction

Cheese manufacture remained an art rather than a science until relatively recently. With the development of knowledge on chemistry and microbiology of milk and cheese, it became possible to direct the changes involved in cheese manufacture in a more controlled way. Although few new varieties have involved as a results of this knowledge, the existing varieties have become distinct and their quality more consistent. Its production involves a complex interaction between milk, rennet, and bacteria (Fox and McSweeney, 2004a). This primarily consists of milk fat and coagulated proteins, and controlling the pH and water activity plays an important role in the preservation of the product. Cheeses differ widely in their compositions, which have a major effect on the microbial quality of the final product (Beresford and Williams, 2004). The microbiology of 'cheese' is, therefore, differ, and its study naturally requires a familiarity, not only of the composition of the product, but also the conditions of manufacture and the storage of the product. Consequently, different types of cheeses convey different levels of microbiological risk. The survival or growth of pathogens are greater in soft cheeses than in the harder varieties, because the environment of soft cheeses are more suitable for the growth of these microorganisms than harder cheeses, which have low water activity in comparison to the soft cheeses, but there is significant correlation between the milk production and the cheese manufacturing in relation to the quality of the final product (Neaves and Williams, 1999; Farkye, 2004). Technologically, the positive influences of probiotics on the quality and sensory characteristics of probiotic products are one of the essential requirements for their application into foods, which depend on their

viability and activity during the manufacture stages and storage. Consequently, conferring their beneficial effects to the consumers (Saarela *et al.*, 2000). Keeping probiotic bacterial strains in a survivable state during the manufacture of functional foods is a major challenge (Desmond *et al.*, 2002). In the development of the probiotic cheese, irrespective of viability of probiotics until the time of consumption and within the GIT, other quality factors of cheese, including, sensory acceptance, chemical stability, and microbial quality throughout the shelf life of this product, technological respects in industrial production, in addition, its price to the consumer have substantial importance (Karimi *et al.*, 2012). Fermented foods, such as cheese, help promote the positive health image of probiotics, since the health image of dairy products in general is positive, and consumers are familiar with the fact that fermented foods contain live microbes (bacteria). In addition, the use of probiotic bacteria as starter microorganism combines the positive images of the fermentation process and probiotic cultures (Heller, 2001). Factors that affects the stability of probiotic bacterial strains in cheese, including, the physiologic state of strain (whether during the logarithmic or stationary phase of growth), the types of inoculation, inhibition activities of strains, the pH and titratable acidity of cheese. In addition, food additives, salt, the temperature of incubation and storage, heat treatment, and the packaging system used (Karimi *et al.*, 2011) affect the stability. The application of probiotic bacteria into milk-based food systems, including cheese, creates challenges, in relation to maintaining the viability and probiotic functionality during manufacture and the shelf-life of the product (Ross *et al.*, 2002a). During the manufacture of fresh foods, such as soft cheese, the use of contaminated raw materials, insufficient heat treatment, and uncontrolled natural fermentation significantly affects the quality of the final product. In addition, use of ineffective starter and unsuitable storage conditions contribute to the reduction of the safety of

fermented foods. Furthermore, ensuring good manufacturing conditions, safe starters which have the ability to inhibit pathogenic microorganisms are more important question (Nout, 1994; Shalaby, 1996; Johnson, 2001; Adams and Mitchell, 2002; Irkin, 2010). The use of different methodologies in cheese manufacturing, such as direct acidification with lactic acid and by ultrafiltration causes differences in physicochemical and microbiological qualities of the final products, may contribute to make the environmental conditions favourable for developing spoilage and pathogenic organisms (Carvalho *et al.*, 2007; Planzer Jr *et al.*, 2009). It is difficult to manufacture cheese in sterilized conditions and keep the quality of cheese intact for a long period. With regards to unwanted microbial growth, the ability of a cheese maintain good quality for long time depends on the degree of contamination, method of manufacturing, cheese composition, and the capacity of contaminants to grow in the product (Johnson, 2001).

2.2 Characteristics of probiotic bacterial strains

The functionality and safety of probiotics depend upon the strains used. There has to be a focus, therefore, on the species and on the level at which they are used, in order to control the quality of the probiotic products for human consumption, and to protect consumers (Coeuret *et al.*, 2004b). Some strains of probiotic bacteria, such as *Lb. casei* Shirota and *Lb. fermentum* have several functions, including antioxidative properties, which are important for the prevention of lipid peroxidation, and for the secretion of hydrogen peroxide. Their antimicrobial activity combined with antioxidative properties may serve as a protective system in the intestinal microbial ecosystem and overcome exogenous and endogenous oxidative stress. Furthermore, the production of organic acids and carbon dioxide (CO_2) is evident (Yuki *et al.*, 1999; Kullisaar *et al.*, 2002; Meurman, 2005). The differences in the characteristics of probiotic bacterial strains can be significant, even when the same strains from

different sources are used (Grzeskowiak *et al.*, 2011). The technological and functional characteristics of the same probiotic strains can vary during their incorporation with different food ingredients (Silveira Rodríguez *et al.*, 2003).

Technologically, probiotic bacterial strains should be capable of growing in milk-based media and survive during the manufacturing processes and the shelf-life period without negatively affecting the quality and sensory properties of the final product (Stanton *et al.*, 2003). The beneficial characteristics of probiotic bacteria are their ability to survive in acidic conditions, and their resistance to heat, oxygen, and digestive stress, in addition to metabolize prebiotics (Charteris *et al.*, 1998b; Shah *et al.*, 2000b; Desmond *et al.*, 2001; Gibson *et al.*, 2004; Homayouni *et al.*, 2008). The actual safety criteria of successful probiotics have been reviewed in several studies (Donohue and Salminen, 1996; Collins *et al.*, 1998; Salminen *et al.*, 1998; Zhou *et al.*, 2000; Song *et al.*, 2012). The ability of bifidobacteria to survive in desirable numbers after their application to foods and during the period of shelf life without adding to production costs represents an important innovation in the food industries (Doleyres and Lacroix, 2005).

Because of the use of heat in the manufacturing and storage of cheese, detailed knowledge of the technical properties of these strains, such as their ability to grow at a range of temperatures, is useful (Christiansen *et al.*, 2006).

2.3 Selection of the appropriate probiotic strains in the production of probiotic cheese

LAB strains have a long history of safety, and most of these strains are commensal microbes with no pathogenic potential (Soomro *et al.*, 2002). A commensal bacterium evolves with their hosts, but, under certain environmental conditions, they are contribute outdo defensive the responses of the host and conferring pathological influences (Tlaskalova- Hogenova *et al*., 2004). Recently, the application of probiotic bacteria to foods has aimed to improve their functional and health properties.

Charteris *et al.* (1998b), reported that the selection criteria for probiotics strains, in addition to technological characteristics, includes medico-scientific properties. Initial tests, for example, on the acid and bile tolerance of probiotic strains before their application to the food product provide useful information for predicting their capacity to transit through the GIT. The tolerance of the strains to the acidic environment could also be a useful predictor for the selection of probiotic strains for application to fermented foods, with regards to their performance in these products. The main apprehensions over the addition of probiotics to foods include what type or form of ingredient/probiotic should be selected, how much must be added to have a beneficial effect, and whether supplementation contributes to changes sensory properties. Table 2.1 shows the issues that should be considered when applied probiotics into food.

Table 2.1. Factors that should be considered when applying probiotics into food

The species or the formula of probiotics selected for the application.
The essential levels of probiotics needed in order to have beneficial effects.
Classification of toxicity of probiotics.
Stability and ability of probiotics facing each stage of manufacture.
The proportion of probiotics in relation to the totality of cells before and after manufacture.
The stability of probiotics during transportation and storage.
The level of their beneficial effects on the sensory properties of the product.
The relationship between probiotics and other fermenting microorganisms, as there may be synergistic or antagonistic effects between them.

Sources: (Pouwels and Leer, 1993; Holzapfel *et al.*, 2001; Holzapfel and Schillinger, 2002b; Champagne *et al.*, 2005; Champagne, 2009).

A number of studies have shown that the ability of probiotic bacterial strains to survive in acidic conditions varies between species and strains (Ross *et al.*, 2005). Selection of new probiotic bacterial strain requires not only a focus on safety, but also the risk to the benefits ratio should be evaluated (Salminen *et al.*, 1998). The growth properties and the requirements for the application of bifidobacteria strains to dairy foods, such as cheese, need to be studied and understood in relation to their survival

and activity during the manufacture and storage, and with regards to the possible negative effects on the quality and sensory properties of the final product (Boylston *et al.*, 2004).

2.4 Fermented dairy probiotic and the functional foods

The fermentation process play an important role in the extension of the shelf life and an increase in the microbiological safety of foods, and it may also contribute to making some foods more digestible (Caplice and Fitzgerald, 1999; Soomro *et al.*, 2002; Heller *et al.*, 2003). Stanton *et al.* (2005) stated that the functional foods are "foods that through specific beneficial physiological action contribute to the health of the consumer". They provide health benefits, through the creation of equilibrium of microbiota in the GIT, as well as energy and nutrients (Shah, 2004; Granato *et al.*, 2010a). The activity of probiotic bacterial strains depends on special characteristics of a single strain (Reuter et al., 2002). The good flavour and texture of probiotic foods contribute to promoting success their applications into foods (Champagne *et al.*, 2005). A study by Fritzen-Freire *et al.* (2010), stated that the incorporation of the probiotic culture *Bifidobacterium* BB-12 to Minas frescal cheese directly after acidification using lactic acid, contributed to the production of cheese with great potential as a functional food after the storage for 28 days at 5 ± 1°C.

The physicochemical properties and ingredients of carrier foods affect the growth and survival of probiotics during gastric transit, and also bile and acid tolerance and adherence to the gastrointestinal epithelium (Silveira Rodríguez *et al.*, 2003). In most of the cheeses that were tested, the viability of probiotic bacterial strains was maintained at the end of storage periods >10^6 cfu g^{-1}, such as *Lb. acidophilus* LA-5 in Menas fresh cheese stored at 4-5°C for 14 days, which was generally recommended as the minimum therapeutic level for probiotics (De Souza *et al.*, 2008). In other research, the final viable counts of probiotic bacteria were >10^7 cfu g^{-1}, such as *Lb.*

casei in Fresco cheese stored 10 days (Fernandez *et al*. 2005) and in many >10^8 cfu g^{-1}, such as *B*. *lactis* BB12 in Cheddar cheese stored at 8 °C for 6 months (Mc Brearty *et al*. 2001). In order to offer probiotic health benefits, probiotics must remain viable in food products over than 10^6 cfu g^{-1} at the time of consumption, without negative changes in the sensory attributes (Karimi *et al.,* 2011). The manufacture of probiotic cheese requires essential probiotic bacterial strains that survive adequately in relatively lengthy cheese production and storage (Karimi *et al.*, 2012). Probiotic bacterial strains *Bifidobacterium* spp., *Lb. acidophilus*, and *Lb. casei*, are extensively applied to dairy foods (Shah, 2007). Controlling the storage conditions of probiotic dairy products is essential for maintaining high survival of probiotics and increase the product stability (Shah *et al.*, 1995; Roy *et al.*, 1997). According to Gomes *et al.* (2009), the manufacture of a probiotic cheese should have minimum variations in comparison to traditional products. In addition, the physicochemical parameters that affect the quality of these products must be determined, aiming at process optimization. The development of new functional food products is very complicated, with regards to the consumer's preferences for the product (Shah, 2007; Granato *et al.*, 2010a). Sensory evaluations of such food products must be conducted throughout the whole process in order to avoid and prevent problems affecting the quality of the final product, and during its marketing (Granato *et al.*, 2010a).

2.5 Probiotic bacteria, prebiotics and synbiotic and the development of dairy products

Gibson *et al.* (2010) defined a prebiotic as ''a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health". Inulin and oligofructose are functional food ingredients that offer the best combination of nutritional properties and important technological benefits. Industrially, they can be obtained from chicory

roots. In food preparations, they play an important role in the stimulation of the growth of beneficial microbes in the intestine, and improve the organoleptic characteristics. Inulin is a heterogeneous blend of fructose polymers stored in plants as a carbohydrate (Niness, 1999; Franck, 2002a). It has been applied to various types of foods, like confectionery, fruit preparations, milk desserts, yogurt and fresh cheese, as well as into "functional foods" e.g. probiotic cheese (Roberfroid, 1997; Roberfroid and Delzenne, 1998; Van Loo *et al.*, 1999; Kaur and Gupta, 2002). Inulin is a non-digestible oligosaccharide, while, nutritionally, is classified as a dietary fibre (Gibson *et al.*, 2010). Rastall and Maitin (2002) stated that the new researches in regards to prebiotic ingredients and synbiotic combinations with probiotics plays important roles in increasing functional food ingredients. Consumption of inulin and fructo-oligosaccharide products, can be have significant health benefits, particularly in regards to their supposed anti-cancer properties, the effect on mineral absorption, lipid metabolism, and anti-inflammatory and other immune effects such as atopic disease. In many cases, prebiotics seem to be more effective when used as part of a synbiotic (Macfarlane *et al.*, 2008).

Selection of suitable prebiotic ingredients for their application to probiotic foods must be careful and should be metabolized by probiotics, in addition, they affects the food acceptability, through the effect on the sensory characteristics. They also affect the survivability of probiotics in the food product (Jonson *et al.*, 2004; Vorobjeva *et al.*, 2008; Wang, 2009). In a study by Modzelewska-Kapitula (2007), it was shown that the use of a high performance inulin (HP) at level 2.5g $100g^{-1}$ cheese, in the production of probiotic soft cheese, affected the numbers of potential probiotic bacteria with desirable properties after storage for 45 days at 6°C. Prebiotic ingredients are important for maintaining mucosal growth and mucosal functions, keeping the balance of water and electrolytes, providing energy and nutrients for the

host, and conferring the body's resistance against invasion of pathogens (Bengmark, 2003). The survival of probiotic bacteria passing through the GIT depends on their resistance to acid and bile, and the existence of prebiotics may afford them greater performance/protection during cultivation, processing, and ingestion (Charalampopoulos *et al.*, 2003; Corcoran *et al.*, 2007; Ooi and Liong, 2010). Wang (2009), reported that the criteria for the classification of food ingredients as prebiotics are 1-their resistance to the upper gut tract, 2-fermentation by intestinal microbiota, 3-having health benefits to the host, and 4-selective stimulation of probiotic microorganisms.

2.6 Factors affecting the soft cheese safety and quality during manufacture and storage

Milk is a complex fluid consisting of water, lactose, fat, protein, organic acids and minerals. It is an important food commodity, often used in the manufacture of a variety of different dairy products. It has a high nutritive value, not only for infants of mammal and for human consumption, but also the microorganisms (Wouters *et al.*, 2002; Walstra *et al.*, 2010). Cheese is a name that refers to a group of food products based on the fermentation of milk, produced in several flavours and forms in a wide range around the world. The objective of cheese manufacturing is to keep the essential components of milk (Fox and McSweeney, 2004a). Cheese manufacturing includes the formation of curd by the action of chymosin on casein micelles, followed by acidification, salting, packaging and cooling (Everett and Auty, 2008). The quality of the milk used affect the quality of the cheese produced (Green and Manning, 1982; Guinee *et al.*, 2000). Caseins in cheese are important sources for peptides, which have biological significance, through their effect on human health and the manufacture of novel functional food ingredients (Silva and Malcata, 2005). The levels of calcium used in the cheese manufacturing affect the coagulation of milk by the addition of rennet, and the

subsequent stages of the curd. The critical factor in cheese-making is the pH value, which is adjusted inadvertently by the addition of starter culture at level 1.5 – 2.0 % of cheese milk, which contributes to the reduction of the pH value of milk results in a decrease in the negative charge of the micelles due to titration of negative charges with H^+ ion. Acidification process occur with a number of physicochemical changes, including, stimulating hydration/dispersion or dehydration/accumulation effects on the casein micelle, with the ratio of these influences changing as the pH declines from the milk fermentation (Fox, 2000a; Guinee and O'Brien, 2010). During soft cheese manufacture, the composition of the milk, including protein, calcium content and the pH value, affect the rennet coagulation, gel firmness and the syneresis (Fox *et al.*, 2004b). The main premise of the dairy industry is dairy products quality depend on the raw materials which they are made (Clark *et al*., 2009). Undesirable changes which occur in the food during storage may result from physical or chemical changes in food, or from microbial activity in or on the product (Wilbey, 1997). According to Fox *et al.* (1990) and Johnson and Law (1999), the milk fermentation process by the metabolism of starter culture strains, is an essential primary reaction during the manufacture of all types of cheese. Dropping the pH value of curd, which depend on the cheese type, affects at least the following properties of curd and cheese, such as syneresis and later cheese composition, retention of calcium which affects cheese texture, retention and activity of coagulant which affect the amount and type of proteolysis during ripening process and the growth of contaminating bacteria. Furthermore, it causes the dissolution of colloidal calcium phosphate from the casein and slows the growth of contaminating bacteria, including some pathogens. The milk salts, Ca and PO_4 and their balance in milk are essential during cheese-making. The Ca ingredient reduces the rennet-coagulation time of the milk that is due to the neutralization of negatively charged residues on casein, through increasing the

aggregation of micelles (Fox and McSweeney, 2004a). The use of low levels of Ca also increases gel firmness and the degree of acid production and the pH value of the drained whey are the critical factors that regulate the mineral content of cheese. In addition, both the pH value and the amount of undissolved milk salts significantly affect the cheese texture (Lucy and Fox, 1993). Natural rennet is a complex enzyme-containing ingredient which is added to milk. It contributes to the curd formation and to the consequent separation of liquid as whey. The levels of rennet used in cheeses are variable. Some cheeses need a more stable curd than others, and some of the others need a longer time period for milk coagulation (Fox and Mc Sweeney, 2004a).

In addition to chymosin, rennet could contain other enzymes, such as pepsin and lipase. The proteolytic processes in cheeses manufactured with rennet are commonly dependent on the activity of two main proteases, which are chymosin and plasmin. Chymosin is active at low pH while plasmin is active at higher pH value reach around 7.5. Therefore, chymosin activity during ripening is highest in mature cheeses, such as Cheddar cheese. Plasmin has the opposite activity profile; it is mainly responsible for the hydrolysis of ß-casein which is weakly hydrolysed by chymosin (Lawrence *et al*., 1984; Fox and McSweeney, 1997; Mullan, 2005).

The high temperature, short time pasteurisation is insufficient to inactivate milk enzymes completely, as some of the latter are heat-resistant and active at refrigeration temperatures (Di Cagno *et al.*, 2006). Because of the low cost and varied functions of salt, it is widely used in the preservation of foods, including cheese. Its functions in cheese are as a preservative, a contributor to the cheese flavour, and a source of dietary sodium. Salt contributes to cheese preservation through inhibiting the growth of pathogens and overcoming spoilage in the presence of desired pH, water activity and the redox potential. Generally, salt concentrations in cheeses ranges between ~0.7% (w/w) in Swiss-type and ~6% (w/w) in Domiati cheese

(Guinee, 2004; Albarracín *et al.*, 2011). Numerous public health agencies worldwide are interested in the reduction of the salt content of foods in order to reduce the population's blood pressure values (Durack *et al.*, 2008). Any reduction in the salt content of processed foods, however, must not affect the quality, safety, and sensory properties of the product. In the dairy industry, the detection and control of psychrotrophic bacteria and their heat-resistant enzymes include lipases and proteases are considered a major concern of the quality control work and ongoing research (Sørhaug and Stepaniak, 1997; Breseford, 2007). Cheese makes small contribution to dietary sodium intake, except in the case of the consumption of some types of cheeses, such as Feta and Domiati cheese (Freitas and Malcata, 1996; Guinee, 2004). The deterioration of soft or unripened cheeses is rapid, due to their higher pH and moisture content and lower salt- to- moisture ratios. The maximum shelf life is 10-12 days, compared to ripened cheese, which has lower water activity, pH value, and redox potential and long shelf life, which reaches 3-6 months in some types of cheeses under refrigerated conditions, such as Swiss cheese. It has been stated that the variations in the milk composition used for cheese manufacturing cause differences in the sensory properties of the cheese. This is because of the wide range of practices involved in animal feeding, milk production and cheese manufacturing (Cornu *et al.*, 2009).

Salaün *et al.* (2005), stated that the buffering capacity of milk products is a main physicochemical property. This is linked to the colloidal Ca phosphate in the product. In study by Gomes *et al.* (2009) identified significant problems, which may occur during probiotic cheese-making, includes the following: (a) the negative effect of the interaction between starter cultures and probiotic strains, (b) some viable probiotics may be lost during the draining of whey, (c) the sensitivity of probiotic bacteria to the level of salt used, (d) the presence of oxygen during the packaging of cheese, (e) the

survival of probiotic bacteria during the ripening period, and (f) the effect of storage conditions on the survival of probiotic bacterial strains used.

2.7 Cheese starter cultures

Dairy starter cultures are defined as species of lactic acid bacteria that are deliberately added to milk, where their main functions are acid production, through the fermentation of carbohydrates into lactic acid for cheese manufacturing, in addition to the production of ATP from lactose by the enzymes of starter cultures. The byproduct of ATP production is a lactic acid. The selection of starter culture strains for application to cheese is based on tradition, the flavour required, the rate and degree of acid development during cheese-making and in the final product. The starter culture strains differ in their resistance to salt, temperature and pH value, which are all considered important factors during the cheese manufacturing procedure (Caplice and Fitzgerald, 1999; Johnson and Law, 1999; Broome and Weimer, 2007). In fermented dairy products, the starter culture strains have an extremely positive effect on the quality of the product. Dairy manufacturers are eager to explore new prospects for promoting a large variety of their products (Wouters *et al.*, 2002). The safety of cheese depends not only on pasteurization and heat treatment of cheese milk, but also on the management of the quality of milk and starter cultures, the control of pH, the addition of salt, and the curdling conditions. Furthermore, antibacterial materials and natural inhibitory substances are present in milk, e.g. nisin and lysozyme (Johnson *et al.*, 1990). In the dairy industry, diversity in the characteristics of the starter cultures used has diminished due to a concern with the uniformity of cheese quality. The industry has also had to respond to the consumers' demand for presenting tasty, high quality products, which requires a greater interest in the strains with novel properties (Picon *et al.*, 2010). Cheese contains a complex group of microbes that change with time. These groups initially

contain SLAB, and during ripening, the numbers of the NSLAB increase (Ross *et al.*, 2002a). Factors affect the determination of cells metabolic responses, during the fermentation process, includes temperature, oxygen and yield, in addition to the pH value, and the quantity and composition of the starter cultures used (Serrazanetti *et al.*, 2009).

2.8 Vacuum packaging (VP) modified atmosphere packaging (MAP) and brine packaging (BP) enhance the cheese quality and safety

Physical, chemical, biochemical and microbiological processes can all affect the quality of food. The unfavourable characteristics caused by microorganisms include numerous types of spoilage, which significantly affect food quality, and make the food commercially undesirable (Lee and Kang, 2004). VP is defined as an uncomplicated method of packaging that removes air from the package prior to sealing. This method involves placing food in the selected package, removing air from inside, and sealing the package. The purpose of VP is usually to remove oxygen from the inflexible container to extend the shelf life of food products, and with flexible package forms, to reduce the volume of the contents and package (Yam, 2010). MAP is a process by which the shelf life of the product is improved significantly, by placing it in an atmosphere which contributes to the delay of the destructive processes which result in microbial growth and, in addition, it enhances beneficial actions; for example, the retention of desirable properties, such as white colour in cheese and red colour in meat (Inns, 1995; Marsh and Bugusu, 2007; Galić *et al.*, 2011). It is defined as a system in which air is replaced, either totally or partially, with other gases, which are used for the packaging of perishable food products (Lampila, 1991; Robertson, 2012). To obtain the maximum positive effect and minimum negative effect on packaged foods under MAP, the optimum level of each gas for each food product should be

determined (Floros and Matsos, 2005). The potential advantages and disadvantages of using MAP as reported by Farber (1991) and Parry (1993) are listed in Table 2.2.

Table 2.2. Advantages and disadvantage of the application of MAP into food packaging

Advantages	Disadvantages
The possibility of increasing the shelf-life is between 50% and 400%	Opening or leaking in pack causes a loss of the benefits
Provides high quality	Control of temperature is essential for maintaining quality of product
Longer shelf-life and less spoilage decreases costs	Each food type needs a different formulation of gases to be used in the process
Sliced products can be separated easily	Process need special equipment and training
Improving the presentation of food through a clearer view of the product	Additional cost for use visible is needed
Chemical preservatives are used in tiny quantities or not used at all.	Stabilization of product safety necessary during process

Sources: (Farber, 1991; Parry, 1993)

Champagne *et al.* (1994), mentioned that the defects in milk and its products are caused by the action of heat resistant enzymes in milk, the growth of heat resistant psychrotrophic bacteria, and contamination after pasteurization. Use the combined preservative factors for food preservation, such as pH and salt will have greater effectiveness at inactivating microorganisms than the use of single factor, such as pH solely. Thus, will contribute to increasing the microbial safety for food (Lee and Kang, 2004). The three main gases used in MAP are oxygen (O_2), Nitrogen (N_2) and carbon dioxide (CO_2). The role and activity of each gas in MAP are related to its properties. Nitrogen is used primarily to displace oxygen and prevent packages collapsing. The main factor which contributes to limit the shelf life of cheese is mould growth, which could control by reducing or excluding the O_2 from the headspace of package (Parry, 1993; Subramanian, 1993). The application of MAP has increased the range of food products which are packaged, such as cheese (Jayas and Jeyamkondan, 2002). The recommended MAP conditions for Soft cheese are 20-40% CO_2, 0% O_2 and at 1-4°C. The use of VP in packaging of some cheeses has specific disadvantages For example.

the package cannot be opened easily and VP for soft cheeses or cheeses that have a crumble texture, may damage the product. The application of MAP contributed to overcome these problems by using pack could be opened easily and extending the shelf life. The development of easy-open and resalable packs was an important development. The range of cheeses packaged under MAP also expanded, bringing to the market many traditional and difficult-to-package soft cheeses with extended shelf life (Subramanian, 1998). Taniwaki *et al*. (2001) studied the growth of fungi and mycotoxin production, on commercial sliced Cheddar cheese packaged under MAP. The authors reported that the reduction in the growth of fungi was between 20 and 80% depending on species, and formation of aflatoxins B1 and B2 were greatly decreased when level of O_2 decreased from 5% to < 0.5% and increasing the level of CO_2 from 20% to 40%. Because of the large number of cheese types, which are different in their composition, consequently they differ in their shelf-life. Therefore, the packaging of each type of cheese should be considered separately and a mixture of 20-40% CO_2 and 40-80% N_2 is suitable for soft cheeses under MAP, to prevent package breakdown (Floros and Matsos, 2005). The effect of CO_2 on foods packaged under MAP is influenced by several factors, such as the pH value of food, the gas/product ratio, the initial percentage of CO_2 in the gas-phase, the fat content and storage temperature, in addition to the age, the initial viable count of bacterial groups, and the type of food which is to be packaged (Farber, 1991; Devlieghere *et al.*, 1998). Factors influences the extension of the shelf life of the product during packaging are (1) the ratio of gases used, (2) the types of packaging material, (3) the bacterial species, (4) the composition of the food, (5) the storage temperature and (6) the size of packaging for each product. The various types of cheese require different types of packaging methods. Some factors that affect the composition of the package atmosphere, includes permeability to O_2 and CO_2, and the water vapour transmission

rates of the packaging films, which can play an important role in the rate of damage (Farber *et al.*, 2003). The packaging materials must be chosen carefully, in order to maintain the quality and safety of the product during its shelf life (Hotchkiss, 1997). Castle (2007), stated that the temperatures and storage period are important, as the high storage temperatures accelerates the transfer, whilst the storage for long duration, contributes to raising the quantity of unwanted flavours which are transferred. Therefore, the control of the storage temperatures under MAP is considered most important, with regards to the effectiveness of this method, since the activity of CO_2 is greatly enhanced by low temperatures. The inhibitory activity of CO_2 depends on the initial viable count of microbial groups in cheese (Moir *et al.*, 1993). Garabal *et al.* (2010), where it is stated that the best sensory attributes for San Simón da Costa cow's milk cheeses packaged using MAP, were obtained by using 100% N_2 or N_2:CO_2 (50:50). Gammariello *et al.* (2009b) stated that the application of MAP into packaging of Stracciatella cheese, and stored at 8°C for 8 days using gases at levels CO_2:N_2. 50:50 and 95:5, contributed to delay the growth of spoilage microorganisms. Several factors influence the food quality during packaging, including, the food manufacturing method, the period and temperatures of storage, which cause an effect on the attributes of the flavour and colour, losing the vitamins and the microbial activity (Hotchkiss, 1997). Oxidative reactions contribute to the changes in the qualities of odours and producing 'off' flavours', as well as causing unwanted changes in colour and a decline of nutritional quality (Kerry *et al.*, 2006). In packaged foods, the chemical metabolites in foods, the volatile package components, the microbial metabolites, the respiration of the product, or the off flavours in raw foods affect the flavour and odour attributes of the product (Rooney, 2005).

The shelf-life of foods is defined by the Institute of Food Science and Technology (IFST) in the United Kingdom as, "the period of time during which the food

product will remain safe; be certain to retain desired sensory, chemical, physical, microbiological and functional characteristics; and comply with any label declaration of nutritional data when stored under the recommended conditions" (Robertson, 2009). It is also defined as the length of time for maintaining the quality of the product under storage (Wilbey, 1997). With regards to the packaging of cheese, the use of the MAP method may not provide sufficient control, and also the shelf life of the product may be inconsistent under high storage temperature (15°C) in comparison to 5°C. Inhibition by CO_2 was greater at 5°C than at 15°C and greater at the surface than deeper in the cheese (Moir *et al.*, 1993).

During the manufacture and storage of food, such as cheese, some changes occur in the internal components and external environmental factors, and these may cause a deterioration of the product and a decrease in its shelf life. MAP is capable of extending the shelf life of food, and creating a delay or prevention of occurrence of undesirable changes during the storage and marketing of food products (Church, 1994). In a study by Gonzalez-Fandos *et al.* (2000), it has been shown that the shelf life of Cameros cheese can be extended by the application of MAP using CO_2:N_2 50:50 and 40:60 at 3-4°C. Temiz *et al.* (2009), stated that the storage of "Lor" cheese under MAP at 4°C, contributed to the extension of the shelf life of cheese for 45 days, through delaying the microbial growth while retaining good odour and taste attributes, comparison with VP and air packaging (control), where the samples were unacceptable after 10 days of storage. In a study by Kristensen *et al.* (2000) on sliced Havarti cheese stored using MAP at 5°C for up to 21 days, with and without exposure to light, the results showed that exposing the product to light contributed to an increase of redness without significant effect on lightness, and a trend of decrease in yellowness measured

using colour parameter a*, L* and b*, respectively.Trobetas *et al.* (2008) studied the quality of Graviera cheese stored under MAP, and stated that the light, gas mixture, and the storage period significantly affected the taste and odour attributes, and the best sensory characteristics of cheese samples were observed by using gas mixtures of 100% N_2 or 50:50 N_2:CO_2, and storage of the product in the dark place.

2.8.1 Biogenic amines (BA) and the quality and safety of dairy products

Biogenic amines are organic and basic nitrogenous compounds with biological activity, which are mainly formed by the decarboxylation of amino acids. BA are present in a wide range of foods, including dairy product (Linares *et al.*, 2011). Trimethylamine (TMA) is an aliphatic amine, which is a volatile tertiary, resulting from consumption of foods containing TMA, directly or through the intake of food containing some precursor of TMA, such as trimethylamine-Noxide (TMNO), choline and L-carnitine. Following oral absorption in humans, TMA undergoes effective N-oxidation to TMNO, a reaction catalysed by the flavin-containing monooxygenase Generally TMA and TMNO are considered non-toxic materials, and clinically important, because of their potential to form the carcinogen N-nitrosodimethylamine (Bain *et al.*, 2005).

Biologic amines are nitrogen compounds of biological significance in vegetables, microbial and animal cells. The amount of these compounds can be estimated in both raw and processed foods. With regards to food microbiology, biogenic amines are sometimes associated with spoilage and fermentation processes. The control of factors in their formation is essential to the improvement of the quality and safety of food (Santos, 1996). High concentrations of histamine in foods and beverages are a source of a microbial contamination and is considered a challenge

for the food industry to produce foods with the lowest possible levels of histamine (Bodmer *et al.*, 1999). Biogenic amines are important because of their contribution to the causes of a number of food poisoning incidents. In the dairy industry, the organism *Lb. buchneri* is important because of its involvement in cheese-related outbreaks of histamine poisoning. The presence of other biogenic amines in foods contribute to enhancing the inhibition of histamine-metabolizing enzymes in the small intestine, and the occurrence of histamine toxicity (Stratton *et al.*, 1991).

2.8.2 Oxidative rancidity (lipid oxidation of milk and milk products)

Lipid oxidation commonly occurs at different levels in several food products. Although these levels are generally low, the problem of lipid oxidation strongly affects the quality and shelf life of some foods. The changes in foods caused by lipid oxidation include the development of an off flavour and unwanted changes in colour and nutritive value, in addition to the possible production of toxic compounds. Increasing the levels of these compounds in foods may cause an effect in the health of the consumers, such as coronary heart disease (Jadhav *et al.*, 1995). Foods that contain lipids be more predisposed to occurrence oxidation, which is considered one of the primary processes contributing to the deterioration of food quality (Kofakowska, 2010). A study by Decker and Faraji (1990) shown that the chemical spoilage in foods mainly occurs through lipid oxidation (peroxidation). Milk and dairy products containing lipid are prone to oxidative damage. Light sources may contribute to the enhancement of certain chemical reactions, which cause the development of an off-flavour and the breakdown of pigments and vitamins in the products (Sattar *et al.*, 1975). The concentration of cholesterol oxidation products (COPS) significantly correlate with thiobarbituric acid reactive materials (Angulo *et al.*, 1997). Free radicals

oxidative play an important role in the deterioration of foods and remain a great challenge in the preservation of food quality. Understanding mechanisms of free radical oxidation and their control through critical chemical interactions contributes to providing a solution to the problem (Vercellotti *et al.*, 1992). Both autoxidation and thermal oxidation of unsaturated fatty acids occurs via a free radical chain reaction that proceeds through three steps of initiation, propagation, and termination. A simplified scheme explaining the mechanism of autoxidation is given below:

1-Initiation

RH + (reactive oxygen species) ·OH -->R· + H_2O

2-Propagation

R· + O_2 ------> ROO·, ROO· + RH ------> R· + ROOH, ROOH -------> RO· + HO

3-Termination

R· + R· ------> RR, R· + ROO· ------> ROOR, ROO· + ROO· ------> ROOR + O_2

It was stated by Johnson and Law (1999) that cheese made from the milk of animals infected with mastitis are susceptible to developing rancidity, even when the milk is pasteurized by 72°C for 15sec, which is commonly used in cheese manufacturing. The release of fatty acids, glycerol and mono or diacylglycerides by enzymatic hydrolysis of triacylglycerides contributes to the development of flavour in some varieties of cheese. Lipolysis results directly in the formation of flavour compounds by liberation of free fatty acids (FFA), which may also be metabolized to an alkane-2-ones and fatty acid lactones. Proteolysis of the caseins to a small and intermediate-sized peptides and free amino acids (FAA), may contribute to formation of flavour ingredients in most types of cheeses. But FAA are important precursors for numerous catabolic reactions, which contribute to the production of fundamental volatile compounds for cheese flavour

(McSweeney and Sousa, 2000). A major product produced from the degradation of lipid hydroperoxides in foods, is malondialdehyde, which is often used as an indicator for detecting the level of lipid peroxidation in food (Raharjo and Sofos, 1993).

2.9 Cheese as a probiotic food carrier for probiotic bacterial strains

Dairy products play an important role in the delivering of probiotic bacteria to the human body, in order to confer their beneficial effects, because these products offer a suitable environment for probiotic bacteria, which support their growth and viability (Gardiner *et al.*, 1999; Phillips *et al.*, 2006). Selection of the suitable food system to deliver probiotic bacteria is a vital factor. Fresh cheese is considered typical for preserving the probiotic bacteria as a carrier, because it is unripened with limited shelf-life, with a resistance to cold temperature during storage and a high recommended daily intake. Therefore, it is a suitable food with a high potential for usage as a carrier for probiotic microorganisms (Heller *et al.*, 2003). Grattepanche *et al.* (2008), stated that although cheese has specific characteristics, such as high buffer capacity, closed matrix, high cell viability compared to other fermented dairy products, such as fermented milk and yoghurt, it is underused as a carrier for the delivery of probiotic bacteria, and for conferring health benefits on the host. Kılıç *et al.* (2009), stated that the combination of *Lb. fermentum* and two strains of *Lb. plantarum* could be used in the production of Turkish beyaz cheese, without any negative effect on the quality and sensory properties during storage for 120 days at 4°C.

Numerous studies have demonstrated desirable results regarding the application of probiotic bacteria in several types of cheese, as illustrated in Table 2.3. Many probiotic bacterial strains have been successfully applied in different types of cheese, such as *Lb. acidophilus*, *Lb. plantarum*, *Lb. casei*, *Lb. paracasei*, *Lb.*

rhamnosus and *Lb. gasseri* and *Bifidobacterium* spp., such as *B. animalis* subsp. *lactis, B. longum*, *B. bifidum* and *B. infantis*. Argentinian Fresco cheese is considered to be a good carrier for the probiotic bacteria *B. lactis*, *Lb. acidophilus* and *Lb. casei,* when applied as an adjunct culture in cheese manufacturing, with good survival rate during refrigerated storage for 60 days (Vinderola *et al.*, 2000b). Four strains of probiotic bacteria, including, *Lb. acidophilus*, *Lb. paracasei*, *B. animalis*, and *Lb. brevis*, were incorporated in the whey cheese matrix. The results indicated that the cheese matrix nature positively affect the viability of probiotic strains during storage of 28 days at 7°C (Madureira *et al.*, 2005).

2.10 Effect of probiotic strains on the sensory properties of cheese

The required minimum levels of probiotic microorganisms in food products at the time of consumption, without adversely affecting the sensory properties of the product, such as flavour, texture and appearance, may vary. So, for example, the Canadian Food Inspection Agency (2009), requires that a serving of stated size of a product, should contain a minimum level 10^9 cfu ml^{-1} or g^{-1} of one or more of the suitable microorganism(s). On the other hand, the recommendation of European Food Safety Authority (2010) stated that the level 10^8 cfu/serving was to be provided for the alleviation of lactose intolerance. The influence of probiotics on the flavour of probiotic cheese is essentially dependent on the species and strains used and the metabolic activities during cheese manufacturing and storage. LAB play an important role in the development of the flavour and texture attributes of cheese, through the enzymatic potential of the strains used in cheese manufacturing (Karimi *et al.*, 2012). Texture and colour attributes are used in the evaluation of cheese quality, and they are often a primary consideration for the consumers, when taking the decision for purchasing the product (El-Nimr *et al.*, 2010). Escobar *et al.* (2012), stated that the inclusion of probiotic strains *Lactobacillus* and *Bidobacterium* in fresh-style Panela

cheese did not affect the taste and appearance of cheese during its storage for 4 weeks at 4°C. The product could be suitable for the incorporation of probiotic bacterial strains used. Souza and Saad (2009), found that the incorporation of *Lb. acidophilus* LA-5 solely, and in combination with *S. thermophilus* positively, affected the quality of Minas fresh cheese. The use of a traditional yoghurt culture strains, with probiotic strain *Lb. acidophilus* LA-5, could also be useful in improving the quality of the product. Ong *et al.* (2007) stated that the probiotic strains *Lb. acidophilus* 4962, *Lb. casei* 279, *B. longum* 1941, *Lb. acidophilus* LAFTI, *Lb. paracasei* LAFTI and *B. lactis* LAFTI were applied successfully in Cheddar cheese. After 6-month ripening period at 4°C, the viable count of strains in the product was in high levels with higher concentrations of a free amino acid (FAA) and acetic acid. In a previous study by Buriti *et al.* (2005a), it was observed that the incorporation of *Lb. acidophilus* into Minas fresh cheese, conferred better sensory attributes, which indicates that this cheese is a suitable food system for the delivery of this probiotic strain to consumers.

Not only is the preservation of the viability of probiotic bacteria during their incorporation into cheese is an important matter, but also, maintaining expected sensory characteristics, such as, the flavour, texture and appearance of the traditional cheeses, is deemed significant. However, the application of probiotics can contribute to maintaining the desired flavour and texture properties (Urala and Lähteenmäki, 2004). In this respect, and according to Ryhänen *et al.* (2001), *Lb. acidophilus* and bifidobacteria with cheese starter cultures were applied to ripened low-fat cheese and the product was then stored for four months. The product was tasty, with strong flavours, and it was found that the product, after ripening period, contains bioactive peptides, which have potential influences on human health, such as antihypertensive.

Table 2.3. Selected publications on the application of probiotic bacteria in cheese

Type of cheeses	Probiotic bacterial groups	References
Gouda	*Bifidobacterium* spp. Bo and *Lb. acidophilus* Ki	(Gomes *et al.,* 1995)
Creamed cottage	*B. infantis*	(Blanchette *et al*., 1996)
Iranian white-brined	*B. bifidum; S. thermophilus* and *Lb. delbrueckii or Lc. lactis*	(Ghoddusi and Robinson, 1996)
Fresh cheese	Bifidobaceria	(Roy *et al.*, 1997)
Crescenza	*B. bifidum; B. infantis* and *B. longum*	(Gobbetti *et al*., 1998)
Cheddar	*B. infantis*	(Daigle *et al*., 1999)
Argentinean fresco	*B bifidum, B. longum*, *Lb. acidophilus* and *Lb. casei*	(Vinderola *et al*., 2000b)
Canestrato pugliese	*B. bifidum* Bb02 and *B.longum* Bb46	(Corbo *et al*., 2001)
Soft cheese	*Lb. plantarum* UCMA 3037	(Coeuret *et al*., 2004a)
Turkish white	*Lb. acidophilus*	(Kasımoğlu *et al*., 2004)
White-brined	*Lb. acidophilus* LA-5 and *B. bifidum* BB - 02	(Yilmaztekin *et al*., 2004)
Probiotic goat	*Lb. delbrueckii* subsp. *lactis*	(Fernández *et al*., 2005)
Cheddar	*Lb. acidophilus*, *Bifidobacterium* spp., *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*	(Phillips *et al*., 2006)
Petit suisse	*Lb. acidophilus*	(Cardarelli *et al*., 2008)
Menas fresh	*Lb. acidophilus*	(De Souza *et al*., 2008)
Cheddar	*Lb. casei 334e*	(Sharp *et al*., 2008)
Pategrás	*Lb. acidophilus, Lb. paracasei* and *B. lactis*	(Bergamini *et al*., 2009)
Crescenza	*Lb. paracasei* A13 and *Lb. acidophilus* H5	(Burns *et al*., 2009)
Turkish beyaz	*Lb. fermentum* and *Lb. plantarum*	(Kılıç *et al.*, 2009)
White-brined	*Lb. acidophilus* LA-5 and *B. bifidum* BB12	(Özer *et al*., 2009a)
Fresh-style panela	*B. breve* and *Lb. rhamnosus*	(Escobar *et al*., 2012)

A number of studies have shown that the changes in the textural properties of some cheeses were positive when compared to the control, like, for example, the application of *Lb. casei* subsp. *rhamnosus* with starter cultures strains *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* into low-fat Kefalograviera-type cheese by Katsiari *et al.* (2002b). The low-fat cheeses made with the adjunct cultures received significantly higher scores for flavour intensity and body and texture than the control low-fat cheese after 90 and 180 days of ripening. As well, the application of *B. lactis* BB12 and *B. longum* BB536 into Cheddar cheese as starter adjuncts by Mc Brearty *et al.* (2001), after six months of ripening, improved flavour were observed in the *B. lactis* BB-12 cheese when compared with the control cheese. Gobbetti *et al.* (1998), stated that the sensory evaluation of the cheeses with incorporated probiotic

bacterial strains *B. bifidum*, *B. infantis*, and *B. longum* was very similar to that of Crescenza cheese produced by the conventional method. Dinakar and Mistry (1994), stated that although the viable count of probiotic strain *B. bifidum,* which was applied into Cheddar cheese, increased during storage period of 24 weeks at 4°C. But, it did not affects cheese attributes of flavour, texture or appearance, also the flavour intensity, in comparison with those observed in control cheese.

2.11 Probiotic bacteria and human health

The importance of beneficial bacteria on human health was first hypothesised by the bacteriologist and Nobel Prize laureate *Elie Metchnikoff* in the turn of 20th century. He concluded after studying the longevity and good health of Bulgarian farmers who basically feed on dairy products, that the presence of LAB in yogurt may prevent harmful influences of pathogens in GIT thus extending their life span. The scientific name of the species of LAB in the traditional diary product consumed is *Lb. bulgaricus*, which is considered one of the two main yoghurt starter cultures. This is considered the basic step for beginning the modern dairy industry.

With regards to the definition of term probiotics, Lilley and Stillwell (1965) described it as “a substances secreted by one microorganism which stimulated another microorganism”. Whilst a definition of Sperti (1971) was as “tissue extracts which stimulated microorganisms”. In 1974, the description of Parker was as “organisms and substances which contribute to intestinal microbial balance”. After that, it was defined by Fuller (1989) as “a live microbial food supplement which beneficially affects the host animal by improving its microbial balance”. In this regards, numerous definitions probiotics were suggested by several researchers. The definition drafted by the experts of FAO/WHO, (2001) was as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". The human GIT in is a micro ecosystem containing a diverse microbiota of up to 1000 various

species. The balance amongst these groups is necessary for carrying out vital host functions. The manipulation of the GIT microbiota through consumption of cheese containing a probiotic strains is an engaging way for maintaining and renovate human health (de Almada *et al*., 2015b).

Numerous mechanisms of action of probiotic strains in the human body have been proposed, including, production of inhibitory materials (organic acids, H_2O_2, bacteriocins), competition for nutrients and/or promotion of an immune response (Stanton *et al*., 2005; Jungersen *et al*., 2014). The influences of probiotic are strain specific, thus, the beneficial effects conferred to the host by specific strain is not necessarily the same as that of other strains, even if they are from the same species (Senok *et al*., 2005). In regards to the effect of probiotic cheese on the human health, Ibrahim *et al*., (2010) said "The intake of probiotic bacteria has been reported to enhance the immune response through other products and now we have discovered that cheese can be a carrier of the same bacteria". The team believes that the daily intake of probiotic cheese can fight the age-related decline of the immune system known as immunosenescence which refer to age-related changes in the immune system. The application of probiotic strains into cheese not only for maintaining and / or improvement its quality and sensory properties of the product, but also in their contribution to prevention and treating numerous health situations and diseases in the human body, such as gastrointestinal infections, inflammatory bowel disease, lactose intolerance, and also in oral health such as prevention of dental caries, periodontal diseases (Singh et al., 2013). A probiotic bacterial strain contributes to enhance the intestinal microbiota, through replenishing damaged bacteria and preventing the growth of numerous pathogenic flora (Salminen and Deighton, 1992). In this regards, a great number of probiotic strains *B. animalis* subsp. *lactis* and *B. lactis* inhabit the intestines and the colon. Their jobs are break down body waste and

the absorption of different vitamins and minerals. Volatile fatty acids which produced by the indigenous microbiota which main contents are LAB responsible for controlling the colonization of gut by Enteropathogenic *E. coli*. Functions of *Bifidobacterium* in human body, besides fighting the pathogenic organisms, include the production of glutamine, which is considered an important amino acid for the intestinal epithelium and maintaining mucosal safety, in addition to glutamic acid. Butyrate is also essential for intestinal epithelial cells as a supplement to promote production of normal epithelium and plays important role in maintaining the stability of mucosal barrier (Anuradha and Rajeshwar, 2005; Tappenden *et al*., 2007).

The purpose of the consumption of probiotic soft cheese involves providing enough essential nutrients for completing the metabolic requirements in the body, in addition to confer numerous body modifying functions. On the other hand, probiotic cheese that containing prebiotic ingredients, such as inulin in combination with probiotic bacteria (synbiotic) contributes to support the viability of these microorganisms. The greatest level of bacteria in the human body is present in the colon, where reach 10^{12} cfu g^{-1} GIT contents. Some factors that affect the colonization of friendly bacteria in the colon are the change of food tradition and lifestyle which encouraged on consumption the processed and sterile foods, in addition to use some antibacterial ingredients, such as vinegar and antibiotics (Anuradha and Rajeshwar, 2005). The effect of intestinal microorganisms on the human health is positively, negatively or neutrally. The beneficial strains of probiotic bacteria are strains *Bifidobacterium* and *Lactobacillus*, and are considered the main beneficial microorganisms and contribute to digestion, immune promotion and fighting the pathogens in the human body (Stanton *et al*., 2003).

Probiotic bacterial strains *Lb. acidophilus*, *Bifidobacterium* spp., and *Lb. casei*, have been demonstrated numerous health benefits for human body. Some these benefits,

are anticarcinogenic characteristics, improvement in lactose metabolism, reduction in serum cholesterol, and immune system promotion (Shah, 2007). In this respect, Maragkoudakis et al., (2006) found that the probiotic bacterial strain *Lb. casei* Shirota has the capacity to inhibit the adhesion of *E. coli* and *S. typhimurium* to Caco-2 cells, and stimulated the human circumferential blood mononuclear cell secretion of pro- and anti-inflammatory cytokines. With regards to contribution of cheese that containing probiotic bacterial strains and prebiotic ingredients into maintaining the consumer's health, according to Reid (2004), the regular consumption of cheese that contains both lactic acid probiotic strains, with and without inulin, contributes to reduction the risk of *Campylobacter enteritis*. Incorporation of probiotic strain *Lb. casei* Shirota in soft cheese, may confer health benefits to consumers due to its beneficial effects, through the prevention and treatment of certain GI infections, including, infantile viral diarrhoea and antibiotic associated diarrhea. In addition, its application orally, contributed to reduce the superficial bladder carcinoma in humans (Aso and Akazan, 1992; Aso *et al*., 1995; Lee and Salminen, 1995; Salminen and Wright, 2004). With regards to the health benefits of a probiotic strain *Lb. acidophilus* LA-5, which is one of strains that were applied in soft cheese in this study, Medellin-Pena and Griffiths (2009) found that this organism is a gut-colonizing bacteria and established that becomes an important part of the microbiota of GIT. It has inhibitory activity against enterohemorrhagic *E. coli* O157:H7 infection. On the other hand, there is relationship between nutrition and dental health, cheese contains compounds play important role in reduction the risk of dental caries. These compounds are calcium and phosphorus which plays important role in stimulating the cariostatic mechanism of cheese. Furthermore, both of casein and whey protein in cheeses contribute to reduce coating demineralization. Moreover, probiotic strains are considered one of the new means which are applied into their therapeutics function

(Herod, 1991; Moynihan *et al*., 1999; Çaglar *et al*., 2005; Bhushan and Chachra, 2010). In this regards, Ahola *et al*., (2002), found that the short term consumption of cheese containing probiotic bacterial strain *Lb. GG* and *Lb. rhamnosus* LC 705 might reduce the risk of *S. mutants* and salivary yeasts. In order to promote the beneficial microbes in our colon, the prebiotic ingredients selected for applications into probiotic soft cheeses need to have three major characteristics, including, being able to resist the high acidity in gut, the bacteria in our colon needed to be able to ferment it and it needs to benefit selective bacteria which are beneficial for human health (Roberfroid, 2007; Slavin, 2013). Both prebiotic ingredients and probiotic bacterial strains in cheese are working in harmony with one another and the cheese that contain adequate levels of either them beneficially affect the health and immune function of consumers.

2.12 Conclusions

The quality of soft cheese is affected by two factors: intrinsic factors, which include the quality of raw materials, composition, formulation, pH, viscosity, water activity and redox potential, and extrinsic factors, such as hygiene, particularly the downstream of heat treatment, packaging systems, storage conditions, and especially temperature. Probiotic bacteria have beneficial health effects through their application to food, food ingredients and as supplementations. Their effectiveness depends on the strains used and the conditions of application, for example, manufacturing processes, their interaction with other microorganisms when used as adjunct starter, and the presence of inhibitors in cheese milk e.g. nisin and lysozyme. Selection of an appropriate combination of starter cultures and probiotic bacterial strains is important and merits careful study, through using appropriate assays for the detection of their interactions if they are present together. The incorporation of probiotic bacteria has been successful in many types of cheeses when compared to

other dairy products, such as fermented milk, yoghurt, and improve the properties of many varieties of cheese.

Cheese is a good carrier for delivering probiotic bacteria to the consumers due to its composition, which is regarded as a suitable environment for the growth of probiotic bacterial strains. Through its stable pH and high buffer capacity, this helps probiotic bacteria during their transit through the GIT, and consequently improves their viability and survival in the final product. Use of starter culture strains in cheese manufacturing plays an important role in the fermentation of milk, which is considered a crucial step, through its contribution to reducing the contamination in pathogens, and thus, aiding in the manufacture of cheese with high quality and safety. The use of probiotic bacterial strains with prebiotic ingredient, such as inulin, may contribute to increase their effectiveness, viability, and survival in cheese, and improve the functionality and sensory characteristics of the product. In this regard, the application of prebiotic ingredients, such as inulin, to cheese, is essential. It is important to maintain the quality of cheese during storage by using suitable packaging method, such as MAP. This method was successfully applied to many types of cheeses, and it could delay or prevent the occurrence of unwanted changes which can affect the quality of the product, such as the formation of biogenic amines, which are responsible for numerous food poisoning incidents, and occurrence of oxidative rancidity, and consequently extend the shelf life of the product, particularly unripened soft cheese, which have a short shelf life.

Chapter 3

Technological and functional characteristics of probiotic bacterial strains *Bifidobacterium animalis* subsp. *lactis* BB12, *Lactobacillus casei* Shirota (LcS) and *Lactobacillus acidophilus* LA-5

3.1 Introduction

Lactic acid bacteria (LAB) are one the most important groups of microorganisms usually associated with the human gastrointestinal tract. The microbes are Gram-positive, non-spore forming, catalase-negative, lacking of cytochromes, aerotolerant, have complex nutritional requirements, are acid-tolerant, and are able to fermentation of various carbohydrates. Lactic acid is the final major product of sugar fermentation. The most typical active constituents of probiotic products are bifidobacteria, lactobacilli and enterococci, and have the ability to grow at a pH in range 4.5 – 8.5 (Holzapfel *et al.*, 2001; Holzapfel and Schillinger, 2002b; Axelsson, 2009). Bifidobacteria ferment carbohydrates actively, and the main products are acetic acid and lactic acid in a molar ratio of 3:2 (v/v), without carbon dioxide, butyric acid, or propionic acid. In food applications, probiotic bacterial cultures are mostly supplied in frozen or dried form, as either freeze-dried or spray-dried powders (Ross *et al.*, 2005). Lactobacilli are considered the most important LAB among bacteria used in food production and are now increasingly applied in the area of probiotics (Tannock, 2004).

Traditionally, in fermented dairy products, probiotic cultures were used in combinations of different bacterial strains belonging to the genera *Lactobacillus, Streptococcus* and *Bifidobacterium* (Prasad *et al.*, 1998; Dunne *et al.*, 1999). The microorganisms were selected and incorporated on the basis of medical, scientific and technological parameters (Collins *et al.*, 1998). The viability of probiotics in

fermented dairy products are influenced by several factors, including titratable acidity, pH, hydrogen peroxide, dissolved oxygen content, storage temperature, species and strains of microorganisms associated in fermented dairy products, concentration of lactic and acetic acids, and the whey protein concentration (Lankaputhra *et al.*, 1996; Dave and Shah, 1997). *Lb. acidophilus, Bifidobacterium* spp. and *Lb. casei* are increasingly incorporated into dairy foods, and their viability in foods is important, in order to provide health benefits (Shah, 2000a). Maintaining the survival of probiotic bacterial strains in the final product during their specified shelf-life is vital, in order to provide health benefits, and consequently, it contributes to the development of these products. The profiles of metabolic products during fermentation were very different, and they depend on the control of the temperature and time of fermentation (Østlie *et al.*, 2005).

The classification and identification of a probiotic strain could be the best indication of its typical environment and origin. The species, or even genus name, would also contribute to indicate the safety of the strains and their abilities to the application into probiotic products (Holzapfel *et al.*, 2001). Because of their ability to ferment foods and their promotion of health properties, LAB are one of the most main groups of bacteria that are applied in the food industry. Identification of the LAB species correctly is considered to be a very important matter from the ecological, technological, and safety point of view. Stability of probiotic bacteria in harsh conditions, such as acid, bile salts, and enzymes during their passage through the GIT, represents a great challenge for the survival of probiotic bacteria, which should reach the intestine in a viable form. So, maintaining the viability of probiotic strains in the product is an important key for probiotic viability during gastric exposure.

Criteria for selection of probiotic bacteria *in vitro* may reflect specific impacts *in vivo* such as modification of the GIT microbiota (Dunne *et al.*, 2001), in addition to other

aspects, including safety, functional and technological characteristics, which should also be considered during the selection of probiotic microorganisms. Safety aspects include specifications such as origin, non-pathogenicity and antibiotic resistance, while functional aspects include their viability and persistence in the GIT. With regards to technological function characteristics, they must be capable to be manufactured under industrial conditions, able to survive and maintain their functionality during storage, and in the target foods without undesirable changes in the product (Saarela *et al.*, 2000). Moreover, a strain could be chosen for a product, to be applied to promote a specific process, such as acid production. The final food product must be acceptable in as shelf-life and sensory properties (colour, flavour, taste and texture), and performance on level and activity of the probiotic strain over and beyond a best before date (Holzapfel *et al.*, 1998). The use of different combinations of starter lactic and probiotic cultures permit the production of fermented dairy products, with purposed technological characteristics and potential nutritional and health benefits (Vinderola *et al.*, 2002b). Therefore, an assessment of the technological characteristics of probiotic bacteria, such as salt tolerance, growth at a range of temperatures and milk fermentation, in addition to their functional characteristics, such as bile and acid tolerance before their application as adjunct cultures with starter culture during soft cheese manufacture, are the basic requirements which probiotic strains must fulfil. Moreover, the suitability of the starter culture and probiotic bacteria strains must be assessed before their incorporation together into cheese.

The objective of this work are to investigate the effect of some factors which accompany mostly probiotic bacterial strains, when used as adjunct cultures with starter culture in the manufacture of soft cheese, such as salt concentration, temperature in relation to their viability and activity, like their ability to ferment milk,

and on functional characteristics after consumption of cheese, such as the resistance to the acids and bile salts. This work aims to evaluate the ability of probiotic bacterial strains *B. animalis* subsp. *lactis* BB12, *Lb. acidophilus* LA-5, and *Lb. casei* Shirota to survive and ferment milk when exposed to salt, temperature, bile salts, and acid, before their application to unripened soft cheese.

3.2 Materials and methods

3.2.1 Isolation, biochemical test and maintenance of *Lb. casei* Shirota (LcS)

The probiotic bacterial strain *Lactobacillus casei* Shirota was isolated from fermented a commercial product (Yakult, UK). One ml of the product was suspended, serial dilutions in sterilize PBS, to obtain 10^{-1} -10^{-7} dilutions. One ml of diluents was poured on MRS agar and incubated at 37°C under 5% CO_2 for 72 hours. After incubation, a loop-full of microbial culture was picked from different colonies and transferred to MRS broth (CM0359A, Oxoid Ltd., Basingstoke, Hampshire, UK), and then purified by streaking twice on MRS agar-gelled sterilized media, after which, the plates were incubated at 37°C for 48 - 72 hours (Patil *et al.*, 2010). The colony characteristics, such as colour, appearance, and the shape of the isolate, were recorded. Further, morphological and biochemical tests were carried out in the laboratory.

For purification of culture, a loop-full of microbial culture was picked up from different colonies, and then inoculated into 20 ml of autoclaved MRS broth (CM0359A, Oxoid Ltd., Basingstoke, Hampshire, UK). MRS broth with strain was incubated overnight under 5% CO_2 at 37°C, and after incubation, the cells were harvested by centrifugation (5000 x g, 10 min) at 4°C using centrifuge (Harrier 18/80, MSC) and washed three times in sterilized PBS. The bacterial strain was maintained as a frozen culture in MRS broth, supplemented with 30% glycerol at - 20°C for further use (Mathara *et al.*, 2004).

3.2.1.1 Characterizations of *Lb. casei* (Shirota) strain

Overnight culture of isolated cells was tested using Gram staining technique and examined microscopically, morphology, and phenotype. The following tests were carried out on strain:-

3.2.1.1.1 Indole test

The test was carried out using the methods of the Health Protection Agency (2010a) by Placing several spots of indole reagent (Sigma Aldrich, UK) on a piece of filter paper. A portion of overnight culture of the isolated colony of *Lb. casei* Shirota was selected from the surface of MRS agar and smeared onto reagent saturated area of the filter paper by using a sterile inoculating loop or wooden applicator sticks. The result of test recorded immediately. Change of colour to blue or blue green with 10 sec is positive, negative reactions remain colourless or light pink.

3.2.1.1.2 Catalase test

The test was carried out by applying the methods of Health Protection Agency (2010b) by placing 4 to 5 drops of 3-6 % hydrogen peroxide in a test tube. After that, overnight culture of isolated strain of *Lb. casei* Shirota was picked from the surface of MRS agar using a wire/loop or disposable alternative. The colony on the inside wall of the bottle, just above the surface of the hydrogen peroxide solution, was scrubbed. Then, the tube slope was stoppered in order to allow the hydrogen peroxide solution to cover the colony. Vigorous bubbling would indicate the presence of catalase (positive). No bubbling would indicate the absence of catalase (negative).

3.2.1.1.3 Oxidase test

The test was achieved on an isolated strain, by applying the method of Health Protection Agency (2004c), which was instructed to soak a piece of filter paper in a Petri dish with 2 – 3 drops of oxidase reagent 1% tetra-methyl–p-phenylenediamine

dihydrochloride. Using glass rod, colony of isolated strain was picked from the surface of MRS agar and rubbed carefully on moistened area on filter paper. A positive reaction is indicated by the appearance of a dark purple colour within 10 seconds.

3.2.1.2 Biochemical characterizations of probiotic strain *Lb. casei* Shirota

The biochemical characterizations of the strain were assessed as follows:

3.2.1.2.1 Growth of isolated strain at a range of temperatures

Growth of isolated strain at a range of temperatures was carried out through grown of isolated strain at 20, 25, 30, 37 and 42°C by inoculation of strain on MRS broth, which was adjusted at pH values 4.5 and 6.5, using NaOH and HCl. The tubes were incubated at 5% CO_2 .The growth of strain was clear at A^{600nm} through the changes in turbidity of MRS broth after 24 hours of incubation using spectrophotometer (Unicom Hexois, spectrophotometer, UK) (Azadnia and Khan Nazer, 2009).

3.2.1.2.2 Salt tolerance of strain

Salt tolerance was assessed using method of Vinderola *et al.* (2002a), by the incorporation of overnight culture of isolated strain into MRS broth containing 5% (w/v) sodium chloride and incubation at 5% CO_2 and 37°C. After 24 hours, the growth density of the strain at A^{600nm} was determined as optical density using spectrophotometer (Unicom Hexois, spectrophotometer, UK).

3.2.1.2.3 Ability of isolated strain to ferment milk

The test was assessed by inoculation of overnight culture of strain at level 2% (v/v) onto pasteurized skimmed milk 9.5% (w/v). Milk was incubated at 37°C under 5% CO_2. The difference in the pH value of milk before and after the incubation of 24 hours was determined and expressed as pH (Ayad *et al.*, 2006).

3.2.1.2.4 Preparation of MRS broth without citrate for testing ability of isolated strains on gas production

MRS broth without citrate, was prepared by dissolving the following ingredients in 1.0 litre distilled water.(1)Peptone 10g, (2)`Lab-Lemco' powder 8.0g, (3)Yeast extract 4.0g, (4) Glucose 20g, (5) Sorbitan mono-oleate 1 ml, (6) Dipotassium hydrogen phosphate 2.0g, (7) Magnesium sulphate $7H_2O$ 0.2g, (8) Manganese sulphate $4H_2O$ 0.05g and (9) Sodium acetate $3H_2O$ 5.0g. The mixture was heated to dissolve the ingredients completely, after that, media was dispensed into test tubes, with inverted Durham test tubes and sterilized by autoclaving at 121°C for 15 minutes, pH = 6.2 ± 0.2 at 25°C. Overnight culture of isolated *Lb. casei* Shirota was inoculated in MRS broth with inverted Durham test tubes. Tubes were incubated at 37° C at 5% CO_2. After 24 hours of incubation, the test tubes were tested for the gas production (Reddy *et al.*, 2007). All experiments were carried out in triplicate.

3.2.2 Salt tolerance test of probiotic bacterial strains BB12, LA-5 and LcS

3.2.2.1 Growth-in-liquid-medium assay (GLMA)

The sensitivity of probiotic bacterial strains to salt was determined using the method of Vinderola *et al.* (2002a). Five tubes for each strain, four tubes contain 3 ml of MRS broth (CM0359A, Oxoid Ltd., Basingstoke, Hampshire, UK), plus salt at levels 3, 5, 8 and 10% (w/v), and the 5^{th} tube was as a control (MRS broth without salt). Overnight cultures of probiotic bacterial strains *Lb. acidophilus* LA-5 and *Lb. casei* Shirota were inoculated in tubes at levels 2% (v/v) separately. For probiotic strain *B. animalis* subsp. *lactis* BB12, 0.05 % L-cysteine was added to MRS broth. Samples were incubated at 5% CO_2 at 37°C for strains LA-5 and LcS and anaerobically for strain BB12. After 24 hours, the growth of strains was monitored. The relative growth of strains was expressed as the percentage of optical density at A^{600nm} of the culture plus

MRS broth without salt, with respect to a control culture using a spectrophotometer (Unicom Hexois, spectrophotometer, UK). The value of a relative growth, lower than 30% or higher than 70%, were considered negative or positive results, respectively. The value of a relative growth, ranging within these limits, was regarded as weak. The relative growth was calculated as follows: % Relative growth = OD (A^{600nm}) of sample/OD (A^{600nm}) of control X 100. The experiment was carried out twice in triplicate.

3.2.2.2 Reconstituted skim milk assay (RSM)

Milk samples prepared from reconstituted skimmed milk (RSM) (9.5% w/v) were pasteurized at 100°C for 30 min and cooled to 30°C (Chr.Hansen, 2001). Sterilized salt was added to the tubes containing pasteurized skimmed milk, at levels 0, 3, 5, 8 and 10 (w/v), under aseptic conditions. Milk samples were inoculated in 1% (v/v) of overnight cultures separately. Samples were incubated at 37°C under 5% CO_2 for probiotic strains LA-5 and LcS, and anaerobically for probiotic strain BB12 for 24 hours. After 24 hours, the pH value of milk samples with salt were measured and compared with the pH of control (RSM 9.5% (w/v) without salt)(Chr.Hansen, 2001). The experiment was carried out twice in triplicate.

3.2.3 Test the ability of probiotic strains on the milk fermentation

Fifty ml of 10% (w/v) RSM was pasteurized at 100°C for 30 minutes and cooled to 37°C, and then inoculated with 2% (v/v) overnight culture of probiotic bacteria BB12, LA-5 and LcS, separately. Milk samples were incubated at level 5% CO_2 for LA-5 and LcS and anaerobically for strain BB12 at 37°C. The pH value was measured after 0, 2, 4, 6, and 24 hours, using a pH meter. Fermentation rate was calculated as ΔpH(ΔpH= pH zero time - pH at time)(Ayad *et al.*, 2006).

3.2.4 Ability of probiotic bacterial strains to growth at a range of temperatures

3.2.4.1 Growth-in-liquid media method

Overnight cultures of probiotic bacterial strains were inoculated separately at 1% (v/v) in MRS broth (CM0359A Oxoid Ltd., Basingstoke, Hampshire, UK) for strains LcS and LA-5 and in MRS broth with 0.05% L-cysteine for strains BB12 and incubated at 20, 25, 30, 37, and 42°C, at 5% CO_2, for strains LA-5 and LcS, and anaerobically for BB12 for 24 hours. Growth of strains was followed by measuring the OD at A^{600nm} using a spectrophotometer (Unicom Hexois, spectrophotometer, UK) (Ayad *et al.*, 2002). The experiment was carried out twice in triplicate.

3.2.5 Bile salt tolerance test of probiotic bacterial strains

The ability of probiotic bacterial strains to grow in the presence of bile salt was determined by the method of Vinderola and Reinheimer (2003). Overnight cultures of each strain was inoculated 2% (v/v) into MRS broth (CM0359A Oxoid Ltd., Basingstoke, Hampshire, UK), containing 0.3, 0.5 and 1% (w/v) of bile salts (LP0056, Oxoid Ltd., Basingstoke, Hampshire, UK), separately. Samples were incubated under 5% CO_2 at 37°C for 24 hours, for strains LA-5 and LcS and strain BB12 was incubated anaerobically. After 24 hours, the growth of strains were monitored at A^{600nm} using a spectrophotometer (Unicom Hexois, spectrophotometer, UK). The results were expressed as the percentage of growth in the presence of bile salts, compared to the control (MRS broth without bile salts). After 72 hours of incubation, the viable count of cultures (log cfu ml^{-1}), were enumerated on MRS agar for strains LA-5 and LcS and on MRS agar with 0.05% L-cysteine for strain BB12. Samples were incubated at 37°C at 5% CO_2 for strains LA-5 and LcS, and anaerobically for BB12.

3.2.6 Testing the tolerance of probiotic bacterial strains to stimulated gastric juice

To determine the transit tolerance through simulated gastric juice, the method of Vinderola and Reinheimer (2003) was used. Simulated gastric juice was a solution of pepsin (Sigma Aldrich Ltd., UK) 0.3% (w/v) and NaCl 0.5% (w/v), adjusted to pH2 and pH3 using HCl and a pH meter. Overnight cultures (40 ml) were centrifuged under cold condition (6000 X g, 20 min, 5°C), washed triplicate in 80 mM K_2HPO4 (29608, BDH, UK) (pH 6.5), and suspended in 3 ml of the same buffer. One ml of washed cells is harvested and suspended by centrifugation under cold conditions (12,000 X g, 5 min) at 5°C using a centrifuge (Harrier 18/80, MSC), and resuspended in 10ml of gastric solution pH2 and pH3. Total viable count (log cfu ml^{-1}), were carried out before and after incubation period of 3 hours at 37°C on MRS agar at 5% CO_2 for strains LA-5 and LcS, and on MRS agar with 0.05% L-cysteine and incubated anaerobically for strain BB12. The results were expressed as the difference in colony counts.

3.2.7 Testing the acid tolerance of probiotic bacterial strains

Overnight cultures were inoculated separately (1%v/v) in MRS broth (CM0359A Oxoid Ltd., Basingstoke, Hampshire, UK), previously adjusted to pH values 2.0 using HCl and a pH meter (Hanna, 2011 Portugal) and 6.2 using 1N NaOH or HCl. The cultures were incubated at 5% CO_2 at 37°C for strains LA-5 and LcS, and anaerobically for strain BB12. A viable count of probiotic bacterial strains at pH2 and pH6.2 was enumerated before the incubation, and after 3 hours at 37°C (Vinderola and Reinheimer, 2003). MRS Broth adjusted to pH 6.2 was used as a control. The experiment was conducted in triplicate.

3.2.8 Detection of the interactions between probiotic strains BB12, LA-5 and LcS with each other, and with starter culture strains

3.2.8.1 Preparation of cell-free supernatants

Cell-free supernatants were prepared by employing the method of Vinderola *et al.* (2002b). Probiotic bacterial strains were grown in both MRS broth and 10% RSM (Merck, Darmstadt, Germany). Starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* were grown in both M17 broth and 10% (w/v) RSM and incubated aerobically for 24 hours at 30°C. Probiotic strains LA-5 and LcS incubated under 5% CO_2 and strain BB12 incubated anaerobically using the gas pack system for 24 hours at 37°C. Cell-free supernatants (CFS) were obtained by centrifugation (3300 x g, 20 min, 5°C) of overnight cultures and sterilization by filtration through a 0.45-µm sterilized filter (Sigma-Aldrich, Ltd, UK). Cell-free supernatants were kept frozen (−40°C).

3.2.8.2 Well-diffusion agar assay

The interactions between probiotic bacterial strains together, and with starter culture strains, were investigated using the method of Vinderola *et al.* (2002b). Twenty millilitres of autoclaved M17 agar (CM0361 Oxoid Ltd., Basingstoke, Hampshire, UK) were melted and tempered at 42-45°C, and carefully mixed with 200µl of each overnight culture of starter culture and probiotic bacterial strains poured into petri dishes separately and the mixture was then allowed to solidify. Under aseptic conditions, holes of 10 mm in diameter were made in the agar layer for each strain, using sterilized stainless steel cylinder. 180µl of the cell-free supernatant of starter culture (SC) and probiotic strains were placed into holes in agar layer for each strain. Probiotic strains BB12 and LA-5 and starter culture were placed into holes in agar layer for LcS, probiotic strains LA-5, LcS and starter culture strains were placed into holes in agar layer for strain BB12. Probiotic strains LcS, BB12 and starter

culture strains were placed into holes in agar layer for strain LA-5. Probiotic strains LA-5, BB12 and LcS were placed into holes in agar layer for starter culture. The plates were incubated using gas pack, with CO_2 and H_2 atmosphere which used in incubation aerobic and anaerobic microorganisms together (10ml distilled water was added to the pack before inserting it into jar according to the instruction of manufacturer) (AG0025 Oxoid Ltd., Basingstoke, Hampshire, UK) at 37°C. After 24 hours of the incubation, the presence or absence of inhibition zone around the holes was observed. There are three behaviours with this methodology and as follows: - complete (a clear absence of growth of the test strain around the hole), weak inhibitions (the presence of a partial inhibition halo around the hole), and absence of interaction. Also, the experiment was applied with MRS agar only with probiotic bacterial strains BB12, LA-5 and LcS.

3.2.8.3 T-cross inhibition method

For testing the possible interactions between strains before application into cheese manufacturing, overnight cultures for all strains, were prepared using M17 broth for monitoring their ability to grow in M17 agar which will applying in the test. The method of Velho-Pereira and Kamat (2011), with minor modification, was applied. Picking colonies of overnight starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, the strains were grown on M17 agar, and the overnight cultures of probiotic bacterial strains *Lb. casei* Shirota (LcS) and *Lb. acidophilus* LA-5, the strains grown on MRS agar, and of strain *B. animalis* subsp. *lactis* B12, strains was grown on MRS agar with 0.05% L- cysteine. Four streaks were made down the four edges of the plate containing M17 agar, one streak of each strain was made by transferring a loop-full of overnight culture of each strain, and each strain streak was to be a cross with the streaks of other three strains. The cultured plates were incubated at 37°C using gas pack with CO_2, H_2 atmosphere (AG0025 Oxoid Ltd.,

Basingstoke, Hampshire, UK). After 24 hours of the incubation, if there is interaction between strains, it will be clear in the place of cross between strains. Presence of inhibition zone in cross place shows that the result is positive, and absence of an inhibition zone indicate an absence the interaction between strains and the result is negative.

3.2.9 Growth and enumeration of starter culture and probiotic bacterial strains LA-5, BB12 and LcS on homofermentative and heterofermentative differential broth and agar (HHD)

3.2.9.1 Preparation of HHD broth and agar media

Homofermentative and heterofermentative differential broth and agar media were prepared by applying the protocol of Camaschella *et al.* (1998), by dissolving 2.5g fructose (F2543, Sigma Aldrich Ltd, UK), 2.5g KH_2PO4 (29608, BDH, UK), 10g trypticase peptone(25039, Fluka, Switzerland), 1.5g phytone peptone (LP0042T, Oxoid Ltd., Basingstoke, Hampshire, UK), 3.0 g casamino acids (BPE, Fisher chemical, UK) and 1 g yeast extract (LP0021A Oxoid Ltd., Basingstoke, Hampshire, UK) in 500 ml of distilled water. After heating tween 80 (R 21276, Oxoid Ltd., Basingstoke, Hampshire, UK) using a water bath, 1.0 ml was added to the solution. Twenty ml of bromocresol green (Sigma Aldrich Ltd., UK) (Stock solution prepared by dissolving 100mg of bromocresol green (Sigma-Aldrich, UK) in 30ml of 0.01N NaOH) was added as indicator and complete the preparation of the HHD broth. For preparation of HHD agar media, agar No.1 (LP0011, Oxoid Ltd., Basingstoke, Hampshire, UK), was added at level 2% (w/v), and the completion of the volume of solution was made with distilled water to 1liter, using a magnetic stirrer. The pH value of media was adjusted to 7.0 ± 0.02, then autoclaved at 121°C for 15 min.

3.2.9.2 Growth of starter culture and probiotic bacterial strains BB12, LA-5 and LcS separately and together in HHD broth and enumeration them separately on HHD and references agar

Before inoculation of starter culture and probiotic bacterial strains on HHD agar, overnight cultures of strains were inoculated in HHD broth separately and incubated aerobically for starter strains and anaerobically for strain BB12, and at 5% CO_2 for strains LA-5 and LcS at 37°C. A combination of starter culture (SC) and probiotic strains BB12, LA-5 and LcS was inoculated in HHD broth and tubes were incubated at 37°C using gas pack with CO_2 and N_2 atmosphere. After 24 hours, the ability of the strains on the fermentation of fructose in HHD broth and change the colour of broth from blue to yellow or yellowish-green was monitored. Overnight cultures of starter culture and probiotic bacterial strains were grown separately on HHD agar and reference agar. Probiotic bacterial strains LA-5 and LcS were grown on MRS agar as a reference agar (Vinderola and Reinheimer, 1999). Probiotic strain BB12 was grown on MRS agar with 0.05% L-cysteine (Gobbetti *et al.*, 1998). Plates were incubated at 5% CO_2 at 37°C for strains LA-5 and LcS, and anaerobically for strain BB12. After 48–72 hours, the viable count of the strains was counted. Starter culture strains were grown on M17 agar. Plates were incubated aerobically at 30°C, and the viable count of strains was recorded after 48 hours of incubation. Colony morphology was tested on HHD and references agar. After that, images of strains individually and together, were seen clearly on HHD agar, also, individual images were seen on references media(MRS and M17 agar), which were taken using a digital camera of Spectro-photo3 microscope.

3.2.9.3 Growth of starter culture and probiotic strains BB12, LA-5 and LcS together on HHD agar

A combination of overnight cultures of starter culture and probiotic bacterial strains were inoculated at level (1%) in 40 ml reconstituted skimmed milk (LP0031, Oxoid Ltd., Basingstoke, Hampshire, UK) 9.5% (w/v) plus 1% (w/v) yeast extract and 0.5% (w/v) glucose, which was previously autoclaved at 121°C for 15 min. Samples were incubated overnight at 37°C, using gas pack with CO_2 and H_2 atmosphere. A decimal dilutions of cultured skimmed milk were made using maximum recovery diluent (MRD), 100µl of appropriate dilution were poured on the HHD agar and incubated at 37°C, using a gas pack with CO_2 and H_2 atmosphere (Oxoid Ltd., UK). After 72 hours of incubation, the morphology of colonies for starter strains and probiotic bacterial strains BB12, LA-5 and LcS were identified on HHD agar (Camaschella *et al.*, 1998). Then, images of bacterial strains individually and together on HHD agar were taken, using a digital camera of Spectro- photo 3 microscope.

3.3 Statistical analysis

Unless stated otherwise, all the numerical data were analysed using MINITAB version 16 (Minitab Ltd, Coventry, UK). One-way analysis of variance (ANOVA) and balanced ANOVA were used. (T test) was used for basic statistical to compare different treatment groups, followed by comparison test Least significant difference (LSD). Data are shown as means as (± SE), and ($P < 0.05$), which are considered significant**.**

3.4 Results and discussion

3.4.1 Characterizations of probiotic bacterial strain *Lb. casei* Shirota

LAB are a heterogeneous family of microbes that can ferment a variety of nutrients. They are the most important probiotic microorganisms, typically associated with the human GIT (Holzapfel *et al.*, 2001; Poolman, 2002). Traditionally, LAB has been classified on the basis of phenotypic properties, e.g. morphology, mode of glucose

fermentation, growth at different temperatures, lactic acid formation, and fermentation of various carbohydrates. As shown in Table 3.1, the characterizations of probiotic strain *Lb. casei* Shirota, isolated from Yakult products (UK), which is considered the main product, contained this probiotic bacterial strain.

Table 3.1 Morphological and biochemical properties of probiotic strain *Lb .casei* Shirota (LcS) isolated from Yakult products (UK)

Tests	Characteristics	Response
Morphological	Gram staining	+
	Motility	–
	Cell Shape	Rod shape
	Colony Morphology	Circular
	Colour	White
	Cell arrangement and cell size	Thin, single, pair or chains and1.0-1.5mm diameter
Biochemical and Carbohydrates Utilization	Catalase	–
	Indole	–
	Oxidase	–
	Growth at salt	
	5%	Weak
	Growth at temperatures (° C)	
	20	+
	37	+
	42	+
	Gas production of glucose	–
	Fermentation of milk	+
	Carbohydrate Utilization	
	Lactose	+
	Glucose	+
	Growth at different pH values	
	4.5	+
	6.5	+

+= Positive, –=Negative.

The negative reaction results of biochemical tests, including indole, catalase and oxidase test, were observed on the strain in microbiological laboratories.

The morphological properties of isolated strain, such as Gram stain, motility, shape and colour of strain and growth at different pH values. In addition, the arrangement of growth and size of cells were noted. With regards to the parameters used in isolating and study the strains of LAB. These parameters were applied in isolation and in the study of probiotic bacteria strain *Lb. casei* Shirota, as well, in maintaining the strain for the further use. Similar procedure have been applied by Jordan and Cogan (1993), Reddy *et al.* (2007), Azadnia and Khan Nazer (2009) and Patil *et al.*

(2010) in regards to isolate and identification of LAB strains depending on their morphological, biochemical, physiological characteristics and the fermentation of carbohydrates. On the other hand, Maragkoudakis *et al*. (2006) found that the probiotic strain *Lb. casei* Shirota showed desirable probiotic properties *in vitro*. This should be considered as a positive trait for probiotic bacteria which is used in manufacturing of soft cheese. However, the isolated strain needs to be further investigated using *in vivo* experiments to establish their potential health benefits.

3.4.2 Salt tolerance of probiotic bacterial strains BB12, LA-5 and LcS

3.4.2.1 Growth-in-liquid-medium assay (GLM)

The effects of salt levels on each strain of the probiotics tested is shown in Table 3.2, the high effect of salt on the growth of strains was at levels above 3%. Growth density of strain *Lb. acidophilus* LA-5 strain in the presence of salt at levels 3, 5, 8, and 10% (w/v), were 0.67 ± 0.035, 0.26 ± 0.029, 0.11 ± 0.012 and 0.07 ± 0.017 respectively, in comparison to its growth in control media (MRS without salt) where the growth density was 0.84 ± 0.064. The same result with other strains *B. animalis* subsp. *lactis* BB12 and *Lb. casei* Shirota LcS was also observed, when the growth density of these strains were 0.61 ± 0.052 and 0.04 ± 0.020, at salt level 3% and 10% respectively for strain BB12, and 0.23 ± 0.098 and 0.01 ± 0.00, respectively for strain LcS.

The sensitivity of probiotic bacteria to salt is considered one of the most important aspects for the application of probiotic bacterial strains in fermented foods, with regards to their effect on the product, particularly on the sensory properties (Saarela *et al.*, 2000).

Table 3.2. Growth density of probiotic strains on MRS broth in the presence of salt after 24 hours of anaerobic incubation for strain BB12 and at 5% CO_2 for strains LA-5 and LcS at 37°C (mean ± se) (n=3)

Probiotic bacterial strains	Salt (%)	OD (A^{600nm})^
Lb. acidophilus **LA-5**	0.0	0.84 ± 0.064[a]
	3.0	0.67 ± 0.035[b]
	5.0	0.26 ± 0.029[c]
	8.0	0.11 ± 0.012[d]
	10.0	0.07 ± 0.017[d]
B. animalis **subsp*. lactis* BB12**	0.0	0.81 ± 0.052[a]
	3.0	0.61 ± 0.052[b]
	5.0	0.23 ± 0.035[c]
	8.0	0.07 ± 0.010[d]
	10.0	0.04 ± 0.020[d]
Lb. casei **Shirota (LcS)**	0.0	0.78 ± 0.064[a]
	3.0	0.33 ± 0.098[b]
	5.0	0.16 ± 0.050[c]
	8.0	0.02 ± 0.010[d]
	10.0	0.01 ± 0.000[d]

Mean values of OD in the same column for each strain bearing the different superscripts differ significantly ($P < 0.05$). ^ Mean of three determinations. Control = MRS broth without salt.

The sensitivity of probiotic bacterial strains to salt, was assessed through detection the growth density of strains, which reflects level of live strains after exposure to concentrations of salt commonly used during the cheese manufacture. The presence of salt in high levels (between 5% and 10%) contributes to the decrease in live microorganisms, and increasing the damaged microorganisms, through the effect on the cell membrane, which consequently affect the metabolism of the strain. Thus, dropping the growth density of microbial strains which results from the growth of live microbes in the media used.

Probiotic strain LcS was highly sensitive to salt levels used in the test, by comparison to probiotic strains LA-5 and BB12. In this regards, Uraz *et al.* (2001) observed that the salt at levels between 4.5 and 5.5% significantly affect the growth of *Lb. casei,* isolated from milk and Centeno *et al.* (1996a), found that the high levels of salt affect the growth and activity of *Lb. casei* and *Lb. acidophilus* strains, isolated from Arzu´a cows' milk cheese. The probiotic bacterial strain BB12 displays high tolerance to salt amongst the tested strains.

According to the criteria of Vinderola *et al.* (2002a), the relative growth of probiotic bacterial strains was positive under 3% salt, weak at 5%, and negative at 8% and 10%. Similar findings have been mentioned by Jordan and Cogan (1993) and Vinderola *et al.* (2002a), when found that the best growth of these strains was at salt level up to 3%, and in line with a previous study by Gobbetti *et al.* (1998), when stated that the application of probiotic bacterial strains to cheese in presence of high levels of salt endanger the survival and activity of these strains. In this regards, a study of Gandhi and Shah (2015) showed that the activity of esterase in the probiotic strains *Lb. acidophilus* and *Lb. casei* were significantly reduced at NaCl concentrations higher than 3.5%. On the other hand, El-Dieb *et al*., (2009) found that the salt at 9% significantly affect the viability of probiotic bacterial strains *Lb. acidophilus* LA-5 and *Lb. casei*-01. The viable count of both strains was < 5 log cfu ml^{-1}, in comparison to the count at 0% salt, which was 8.3 and 8.4 log cfu ml^{-1}, respectively. Similar findings have been stated by Ayyash *et al*., (2013) who found that 10% salt strongly affected the inhibitory and proteolytic activities of probiotic strains *Lb. acidophilus* and *Lb. casei* after incubated aerobically at 37°C for 22 hours.

3.4.2.2 Reconstituted skim milk assay (RSM)

The ability of probiotic bacterial strains on the fermentation of lactose in milk is an important technological characteristic (Zárate and Chaia, 2012). Detection of the effect of salt on the probiotic strains using RSM, which is considered close to the environment of cheese milk, reflects the performance of probiotic strains in cheese milk, in regards to the fermentation of lactose in the presence of salt during cheese manufacturing. Data in Table 3.3 shows that the pH values of fermented milk containing levels of salt between 3% and 10% after incubation 24 hours at 37°C at 5% CO_2 for strains LA-5 and LcS and anaerobically for strain BB12 were higher than the pH values of control samples (RSM without salt). The pH values ranged between 4.10

± 0.012 for control samples and 6.13 ± 0.006 for samples with 10% salt. There were Significant differences (P <0.05) between the pH values for control samples and the samples with all salt levels used for each strain. Salt, is one of the natural food preservers used globally (Cai *et al.*, 1997). It is one of the ingredients of cheese, hence, studying the effect of salt on the activity of probiotic bacterial strains used is an essential aspect, with regards to the fermentation of milk, which is considered to be an important step during cheese manufacturing (Guinee, 2004). But, using salt in high levels, however, has negative effects, not only on the growth and activity of the Gram negative bacteria, but also on the beneficial Gram positive bacteria, such as the probiotic bacterial strains used. In this regards, the effectiveness of the high concentration of salt at 10% on the activity of probiotic bacteria at 37°C was very clear, with significant effect on the pH value of milk used in the test, in comparison to the control (RSM without salt). On the other hand, the pH value of milk with 3% salt were 5.98 ± 0.017, 5.80 ± 0.012 and 6.02 ± 0.017 for probiotic strains LA5, BB12 and LcS, respectively. No significant differences between the strains, with regards to salt resistance, were found (P> 0.05).

Table 3.3. Milk fermentation by probiotic strains in the presence of salt after incubation 24 hours of anaerobic incubation for strain BB12 and at 5% CO_2 for strains LA-5 and LcS at 37°C using RSM (mean ± se) (n=3)

Probiotic bacterial strains	**Salt (%)**	**pH values^**
***Lb. acidophilus* LA-5**	0.0	4.10 ± 0.012[b]
	3.0	5.98 ± 0.017[a]
	5.0	6.07 ± 0.006[a]
	8.0	6.11 ± 0.017[a]
	10.0	6.12 ± 0.012[a]
***B. animalis* subsp. *lactis* BB12**	0.0	5.43 ± 0.006[c]
	3.0	5.80 ± 0.012[b]
	5.0	6.05 ± 0.006[a]
	8.0	6.06 ± 0.017[a]
	10.0	6.08± 0.012 [a]
***Lb. casei* Shirota (LcS)**	0.0	4.30 ± 0.012 [b]
	3.0	6.02 ± 0.017 [a]
	5.0	6.03 ± 0.023[a]
	8.0	6.10 ± 0.012 [a]
	10.0	6.13 ± 0.006 [a]

Mean of the pH values in the same column for each strain bearing the different superscripts differ significantly (P <0.05). ^Mean of the pH value of RSM of three determinations with and without salt (Control 0.0% salt) after incubation 24 hours.

The high levels of salt affect activity of the bacterial enzymes which are responsible for the fermentation of lactose to acid, consequently decreasing the pH value of milk. Lactose in milk hydrolyses to glucose and galactose, by enzymes of probiotic strains. The galactose is converted to glucose by the 'Leloir pathway', and together with glucose, is fermented by glycolysis. Presence of high levels of salt affects the activity of these enzymes and the fermentation process becomes more complex (Gandhi, 2006). Similar results have been reported in the previous studies by Lanyi (1974), Wood (1999) and Denich *et al.* (2003), which stated that the high levels of salt was harmful for microbes, including the microorganisms that are considered safe for particular applications. However, it is not the microorganisms that are considered GRAS, but rather their conventional use in the food industry through their effect on the bacterial cell membrane, and on its components, which cause inhibition of the microbe. During cheese manufacturing, use of salt at high level over than 4%, could affect not only on the starter culture and probiotic bacterial strains, but also the lactose utilization, and consequently, on the production of acid, which is considered an important factor that affects cheese manufacturing. The suitable levels of salt in cheese for this target are up to 3%, to ensure the activity of strains during the manufacture of cheese. With regards to cheese, Gomes *et al.* (1998b) and Gomes and Malcata (1998c), found that goat cheese manufacturing could be optimized by using salt at level 3.5%(w/w), and the survival of *B. lactis* and *Lb. acidophilus* in a semi-hard Gouda cheese was desirable at levels 2-4% (w/w).

Using hazardous levels of salt affects the viability of the strains in the product. As well, Antonio *et al*., (2012), found that the salt at a level of 6.5%, significantly affected the viability of probiotic strain *B. animalis* subsp. *lactis.* Too, in this respect, Turner and Thomas (1980) found that the lactose consumption and lactic acid production by the starter culture, during the manufacture of Cheddar cheese, were affected at salt

levels between 4 and 6%. Furthermore, the starter cultures were more active in lactic acid production in the product, after one day of cheese manufacturing, by using low level of salt.

3.4.3 Growth of probiotic strains at a range of temperatures

With regards to comparison between the growth density for each probiotic bacterial strain at the temperatures used. The growth of probiotic bacterial strains was at temperatures ranging between 20°C and 42°C. Probiotic strains grew at all temperatures tested, but the growth levels of each strain differ according to the temperature, and between the strains.

Table 3.4. Growth density of probiotic strains at 20, 25, 30, 37 and 42°C at ^{A600nm}, after 24 hours of anaerobic incubation for strain BB12 and at 5% CO_2 for strains LA-5 and LcS (mean ± se) (n=3)

Probiotic bacterial strains	Temperature (°C)	Growth density (A^{600nm})^
Lb. acidophilus **LA-5**	20	0.44 ± 0.028^{c}
	25	0.77 ± 0.023^{b}
	30	0.87 ± 0.034^{a}
	37	0.89 ± 0.023^{a}
	42	0.57 ± 0.046^{c}
B. animalis **subsp.** ***lactis*** **BB12**	20	0.35 ± 0.023^{c}
	25	0.78 ± 0.017^{b}
	30	0.88 ± 0.023^{a}
	37	0.92 ± 0.028^{a}
	42	0.75 ± 0.034^{b}
Lb. casei **Shirota (LcS)**	20	0.51 ± 0.023^{b}
	25	0.80 ± 0.023^{a}
	30	0.86 ± 0.017^{a}
	37	0.90 ± 0.028^{a}
	42	0.60 ± 0.017^{b}

Mean values of growth density for each strain in the same column bearing the different superscripts differ significantly ($P < 0.05$).
^ Mean of three determinations.

Growth of BB12 was highest at 42°C, growth density was at (A^{600nm}) 0.75 ± 0.034 than LA5 0.57 ± 0.017, and LcS 0.60 ± 0.017, where the optimum temperature of growth for all strains was at 37°C, the growth density was 0.92 ± 0.028, 0.89 ± 0.023, and 0.90 ± 0.028 for BB12, LA5 and LcS respectively (Table 3.4).

The ability of probiotic bacteria to grow at a range of temperatures is considered one of the important parameters for the selection of an appropriate strains for industrial

application (Sanders, 2000). In this respect, the results of probiotic bacterial strains, which were tested, have the ability to grow at a range of temperatures used, but their abilities are different with regards to their growth on temperatures.

The higher growth and density correlate positively with the optimum temperature for microbial growth. In this sense, the degree of growth for all strains was at minimum levels under incubation at 20°C, where the means of optical density were 0.35 ± 0.023, 0.51 ± 0.023 and 0.44 ± 0.028 for BB12, LcS and LA-5, respectively (Table 3.4). On the other hand, the typical temperatures range between 25 and 42°C, and the optimum temperature for their growth was 37°C, which is the temperature mostly used for the activation of these strains in the dairy industry.

Similar results have been obtained by Gomes *et al.* (1995) and Axelsson (2009), with regards to the application of probiotic strains to dairy products and the factors which affect their survivability in the products. Therefore, the incubation of strains at 37 °C is mostly used during subculture of strains under different levels of oxygen. On the other hand, Antonio *et al*., (2012) found that the probiotic strain *B. animalis* subsp. *lactis* showed good growth at 25˚C and 30˚C.

3.4.4 Bile salt tolerance of probiotic bacterial strains

One of the important functional characteristics of probiotic bacterial strains is their ability to survive the bile salts in the intestine (Shah *et al.*, 2000b; Khalil *et al.*, 2007). Resistance to bile salts is considered an important functional characteristic for selecting probiotic strains (Boke *et al.*, 2010).

Data in Table 3.5 shows that the viable count of strains decrease with the increase of bile salt level in MRS broth, after incubation for 24 hours at 37°C, where the viable count of strain BB12 decreased from 9.89 ± 0.116 in control (MRS broth without bile salts) to 6.39 ± 0.145, 5.19 ± 0.098 and 3.51 ± 0.064 at levels 0.3, 0.5 and 1.0, respectively, with reduction in viable count ranging between 3 and 6 log cfu ml^{-1}, in

comparison to LA-5, where the reduction in the viable count was between 1.78 and 2.48 log cfu ml^{-1}, at levels 0.3 and 1.0 respectively. The viable count of strain LcS in control was 8.60 ± 0.104 log cfu ml^{-1} which was reduced to 7.31 ± 0.113, 6.45 ± 0.063 and 5.59 ± 0.060 log cfu ml^{-1} at levels 0.3%, 0.5% and 1.0%, respectively, with reduction in viable count ranging between 1.29 and 3 log cfu ml^{-1} (Table 3.5). In this regards, Vernazza *et al*., (2006) found similar results when stated that the growth of probiotic strain BB12 at level 1% bile, was weak but exhibited good survival rates.

Table 3.5. Viable counts (log cfu ml^{-1}) of probiotic bacterial strains in the presence of bile salts at levels 0.0, 0.3, 0.5 and 1.0 % (w/v), after incubation 48-72 hours at 37°C, anaerobically for strain BB12 and at 5% CO_2 for strains LA-5 and LcS. (mean ± se) (n=3)

Probiotic bacterial Strains	**Bile salt (%)**	**Viable count^(log cfu ml^{-1}**
***Lb. acidophilus* LA-5**	0.0	9.90 ± 0.064[a]
	0.3	8.12 ± 0.080[b]
	0.5	7.72 ± 0.103[c]
	1.0	7.42 ± 0.116[c]
***B. animalis* subsp*. lactis* BB12**	0.0	9.89 ± 0.116[a]
	0.3	6.39 ± 0.145[b]
	0.5	5.19 ± 0.098[c]
	1.0	3.51 ± 0.064[d]
***Lb. casei* Shirota LcS**	0.0	8.60 ± 0.104[a]
	0.3	7.31 ± 0.113[b]
	0.5	6.45 ± 0.063[c]
	1.0	5.59 ± 0.060[d]

Mean values of viable count for each strain in the same column bearing the different superscripts differ significantly (P <0.05). **^** Viable counts of strains (log cfu ml^{-1}) in MRS broth with bile salts after incubation 24 hour at 37°C, which were enumerated on MRS agar.

Generally, the loss in the viable count of probiotic stain BB12, during exposure to the bile salts was higher than those observed in strains LA-5 and LcS, which differed in their resistance to bile salts. The results of Xanthopoulos *et al.* (2000) stated that the probiotic bacteria of species *Lactobacillus* differ in their abilities to resist bile salts, and found that their survival ranged between 10.3% - 57.4% at 0.15% bile salts. Begley *et al.* (2005), stated that there are differences between Gram-positive and Gram-negative bacteria regarding the mechanisms which contend with bile stress, and bile tolerance is a strain-specific attribute, where the tolerance of species cannot be generalized. In this regard, Charteris *et al.* (1998a) stated that the probiotic strains

of lactobacilli differ in their resistance to bile salts. On the other hand, Succi *et al.* (2005) mentioned that the bile are secreted in the small intestine and contributes to reduce the survival of bacteria through destroying their cell membranes, which their major components are lipids and fatty acids. These alterations may impact not only the cell permeability and viability, but also the interactions between the membrane and the environment. Generally, assessment the tolerance of probiotic bacterial strains to the influences of bile salts and acids, considered an essential matter before their application into food, such as cheese, where reflect the functional performance of probiotic strains during consumption of product (Collins *et al.*, 1998; Zavaglia *et al.*, 1998; Dunne *et al.*, 1999), as well as, the functional characteristics of probiotic bacterial strains, such as acid tolerance and bile resistant are considered the basic criteria during their selection for application into food (Mattila-Sandholm *et al.*, 1999a; Ouwehand *et al.*, 1999; Liu *et al.*, 2013).

The probiotic bacterial strains tested are different in their sensitivity to bile salt levels used. Considerable variations existed between the strains in their ability to grow in the presence of bile, this similar to that found in the study by Walker and Gilliland (1993), where it was stated that considerable resistance was found in cultures of *Lb. acidophilus* in their ability to grow in the presence of bile. The order of probiotic bacterial strains under studying, regarding to their resistance to bile salts, was LA-5, LcS and BB12, respectively. This is supported by many investigations in this respect by Liong and Shah (2005a) and Vinderola and Reinheimer (2003), who stated that the probiotic *Lb. acidophilus* was more resistant to bile salts when compared to *Bifidobacterium* spp.. Probiotic bacterial strains have good resistance to bile salts at 0.3%, and a similar trend of result was previously recorded by Charteris *et al.* (1998a). On the other hand, Antonio *et al*., (2012), found that the bile salt at 0.3% not affect the viable count of probiotic strain *B. animalis* subsp. *lactis*. In general terms,

the growth of strains in the presence bile salts was slower compared to growth in the absence of bile salts, and probiotic strain LA-5 was the strain that grew better at 1% (w/v) bile salt, followed by strain LcS and BB12. Similar results have been obtained by Zavaglia *et al.* (1998), Vinderola and Reinheimer (2003), who concluded that the probiotic strain *Lb. acidophilus* is the most interesting species because it showed high values of resistance to gastric juice and bile, and its resistance was higher than those obtained by probiotic strains of *Bifidobacterium* and *Lb. casei*. As well as the results of Schillinger *et al.* (2005), who found *in vitro* that the resistance of probiotic strains of *Lb. acidophilus* to bile salts was higher than those for strains of *Lb. casei.* Furthermore, the results of Sumeri *et al*. (2008) are similar to that obtained in the present study. The team found *in vitro* using single bioreactor gastrointestinal tract simulator that the resistance of *Lb. acidophilus* LA-5 to bile salts was higher than those for *Lb. casei* Shirota and similar to the results obtained by Sohail *et al.* (2011). The results obtained in this work showed that the probiotic bacterial strains attained the level 10^6 cfu ml^{-1} in the presence of bile salts at levels 0.3 % for strain BB12, 0.5 % for strain LcS and 1.0% for strain LA-5. The three strains have shown a good performance at this range of bile salts levels used in this work. In this respect, Goldin and Gorbach (1992), stated that the bile salts concentrations between 0.15% and 0.3% has been recommended as a suitable concentration for selecting probiotic bacteria for human use. All probiotic strains used in this study have a good survival in this range of bile salts levels. On the other hand, Huang *et al*., (2014) stated that although some LAB possessed probiotic properties beneficial to human health, the both gastric acid and bile salts affect the survival and viability of ingested probiotics. The guidelines of the evaluation of probiotic organisms, which reported by a joint FAO/WHO working group, two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile compounds, based on both survival and growth studies (Vizoso Pinto

et al., 2006). During the evaluation of bile tolerance by growth studies, the growth abilities of the examined strains in their culture media, containing different concentration of bile components can be assessed. These evaluations are obtained through the application of either of the following methods; a) by assessing the strain's ability in bringing about changes in optical density (Suskovic *et al*., 2000) and b) by assessing growth ability on solid culture media (Prasad *et al*., 1998; Chou and Weimer, 1999; Morelli, 2007). In this regards, Both *et al*., (2010) found that probiotic strain *Lb. casei* 01 showed high sensitivity to bile salts used (0.3 and 1% bile) *in vitro* than strain *Lb. acidophilus* LA-5.

With regards to the stability of probiotic strain *Lb. acidophilus* to bile salts and human health, Ren *et al*., (2014) confirmed that the most bile resistance strains are helpful on the reduction the symptoms of lactose intolerance, with a mechanism suggested by Noh and Gilliland, (1993) who concluded that the presence of bile increases the permeability of *Lb. acidophilus* cells, by allowing more substrate to enter the cells and thus increasing their β-galactosidase activity.

3.4.5 The tolerance of probiotic bacterial strains to simulated gastric juice

The ability of probiotic bacterial strains to survive the passage through the GIT is considered an important factor. Probiotics must survive in the acidic gastric environment if they are to reach the small intestine and colonize the host, thus imparting their benefits, and as reported by Charteris *et al.* (1998a), Chou and Weimer (1999) and Chung *et al.* (1999) that their abilities are variable, and they depend on the strains and the carrier food. In this respect, Stadler and Viernstein (2003) found that the best protective abilities against artificial gastric juice were observed when tablets were prepared from mixtures of LAB strains, hydroxypropylmethylcellulose acetate succinate and sodium alginate. A decrease of

viable cells was not more than one log unit, after 2 hours of incubation in low acidic conditions. On the other hand, Ranadheera *et al.*, (2010), found that the physicochemical characteristics of food carrier affected the growth and survival of probiotics during gastric transit. As well, gastric acid, resistance to juices and bile tolerance, adherence to epithelium of GIT and the production of acid by a probiotic, are also influenced by the food composition used in probiotic delivery. In this context, Madureira *et al.*, (2011), found that the Whey cheese matrices contributed to protect probiotic strains *Lb. acidophilus* L10, *Lb. casei* L26 and *B. animalis* B04 during transit throughout the simulated gastrointestinal system, thus, they are promising carriers of those probiotic bacteria. In addition, the conditions that encounter probiotic strains in the external environment or in the host before entry the small intestine, contributes to determine impact of bile on a strain (de Almeida Junior *et al.,* 2015).

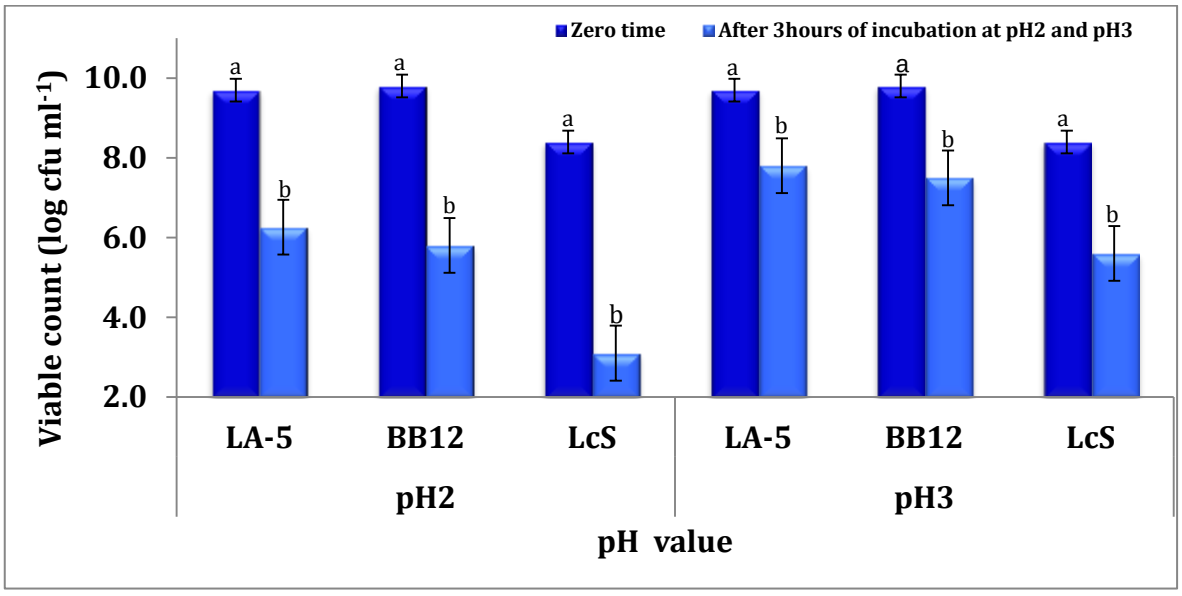

Figure 3.1. Viable counts (log cfu ml^{-1}) of each strain under all pH values before and after 3 hours of incubation on pH2 and pH3 at 37°C under anaerobic incubation for strain BB12 and at 5% CO_2 for strains LA-5 and LcS. Each strain enumerated on MRS agar (pH 6.5) before incubation and after 3 hours of incubation on MRS broth pH2 and pH3. (mean ± se) (n=3).

Data in Figure 3.1 shows that there were differences between probiotic bacterial strains regarding their resistance to the acidic environment. A probiotic strain LA-5 was more resistant than strains BB12 and LcS, where its viable count, after 3 hours of

incubation under pH2 and pH3 was 6.260 ± 0.114 and 7.810 ± 0.110 log cfu ml^{-1}, respectively. After 3 hours of incubation for strains BB12, LA-5 and LcS at 37°C at pH2, the decrease in their viable count was 4.10, 3.4 and 5.3 log cfu ml^{-1}, respectively. At pH3, the decrease was 2.3, 1.8 and 2.8 log cfu ml^{-1}, respectively.

The viable count of strains BB12 and LcS before incubation under pH2 and pH3 were 9.784 ± 0.129 and 8.378 ± 0.092 log cfu ml^{-1}, respectively. After 3 hours of incubation under pH2 and pH3 at 37°C, they were between 5.784 ± 0.084 and 7.484 ± 0.100 for BB12 and 3.10 ± 0.100 and 5.589 ± 0.140 log cfu ml^{-1} for LcS, respectively. The results obtained referred to the present differences between probiotic bacterial strains, in relation to their resistance to an acidic conditions. As illustrated in the report of Minelli *et al.* (2004), the pH value of gastric juices in a fasting individual is in a range between 1.0 and 2.0, but the value rises to 5.0 or more after food consumption. In the present work, the resistance to the simulated gastric juice was different between probiotic strains.

Depending on their resistance to gastric juice, the ordering of probiotic strains is LA-5, BB12 and LcS. These results are similar to that results obtained by Vinderola and Reinheimer (2003) who found *in vitro* that the reduced viable count of probiotic strains of *Lb. casei* under pH2 and pH3 was higher than those obtained by strains of *Bifidobacterium* and strains of *Lb. acidophilus* was lower than those obtained by both probiotic strains. With regards to the tolerance of strains tested to gastric juice, the results of this work are similar to that observed by Charteris *et al.* (1998a) who observed that the tolerance of strains *Lb. acidophilus* to a simulated gastric juice *in vitro* was higher than those by strains of *Bifidobacterium* and *Lb. casei.* In this respect, probiotic strains must be have an ability to survive high levels of lysozyme in human saliva. The compression of acid conditions and digestive enzymes (pepsin) of the stomach, and the bile in the upper intestine (Corzo and Gilliand, 1999; Marco *et al*.,

2006; Liu *et al*., 2013). Furthermore, Schillinger *et al.* (2005), found that the strains of the *Lb. acidophilus* group were more tolerant to the pH 2.0 than strains of *Lb. casei* which rapidly lost their viability after exposure to the simulated gastric juice containing pepsin *in vitro.* In addition, Antonio *et al.,* (2012) found that probiotic strains of *Bifidobacterium* exhibited sensitivity towards low pH (pH2.5). Probiotic bacterial strain LA-5 has a better survival than the other strains that tested. In this respect, tolerance to gastric acidity (pH 2 and pH 4) of probiotic bacterial strains *Lb. acidophilus* LA-5 and *Lb. casei* 01 were investigated *in vitro* by Both *et al*., (2010) and found that probiotic strain *Lb. casei* 01 showed high sensibility to gastric acidity than *Lb. acidophilus* LA-5. In general*, Bifidobacterium* cultures were more sensitive to acidic environment than *Lactobacillus* cultures and this is reflected through their poor tolerance to human gastric juice (Ross *et al*., 2005). On the other hand, Ranadheera *et al*., (2014), found that the probiotic strains *Lb. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB12 have a higher resistance to simulated gastric fluid when were applied as a combination in the goat milk.

3.4.6 Acidic tolerance of probiotic bacterial strains

Acid stress may strongly affect the viability of probiotic cultures (Shah, 2000). Consequently, dairy products, particularly cheese, are preferred for use as a carrier of probiotic strains for improving their viability in gastric juice may be through its buffering capacity and protective effect (Ross *et al.,* 2005). Holzapfel *et al*., (1998) stated that the low pH provides an effective barrier against the entry of bacteria into the intestinal tract and the pH of the stomach ranges from pH 2.5 to pH 3.5.

The threshold point to determine acid resistance was set at pH 3 and incubation period of 3 hours in this *in vitro* study as it stimulates the residence time in the stomach (Prasad *et al*., 1998; Haddadin *et al*., 2004). This is in accordance with similar findings obtained by Liong and Shah (2005a) which stated that resistance at

pH 3 is set as standards for acid tolerance of probiotic culture. Therefore, result in Figure 3.2 indicates the strong inhibition on the viable bacteria numbers at pH 2 was well supported. Figure 3.2 shows the tolerance of probiotic bacterial strains BB12, LA-5 and LcS to acidic conditions, when exposed to lower pH2 for 3 hours. The viable counts of all strains decreased after 3 hours of incubation. The decrease was at different levels, the viable count of probiotic strains at 37°C after 3 hours of incubation were 5.2 ± 0.072, 6.7 ± 0.047 and 3.6 ± 0.094 for BB12, LA5 and LcS respectively, the viable count of strains at zero time were 9.7 ± 0.047, 9.0 ± 0.100 and 7.5 ± 0.094 log cfu ml^{-1}, respectively. There were no significant differences between the viable count of strains at zero time and after 1 hour of incubation, but the significant differences in the viable count were noted after 2 and 3 hours of incubation. The greatest significant difference in the viable count of strains was after 3 hours of incubation at 37°C under pH2, in comparison with the viable count at zero time ($P < 0.05$).

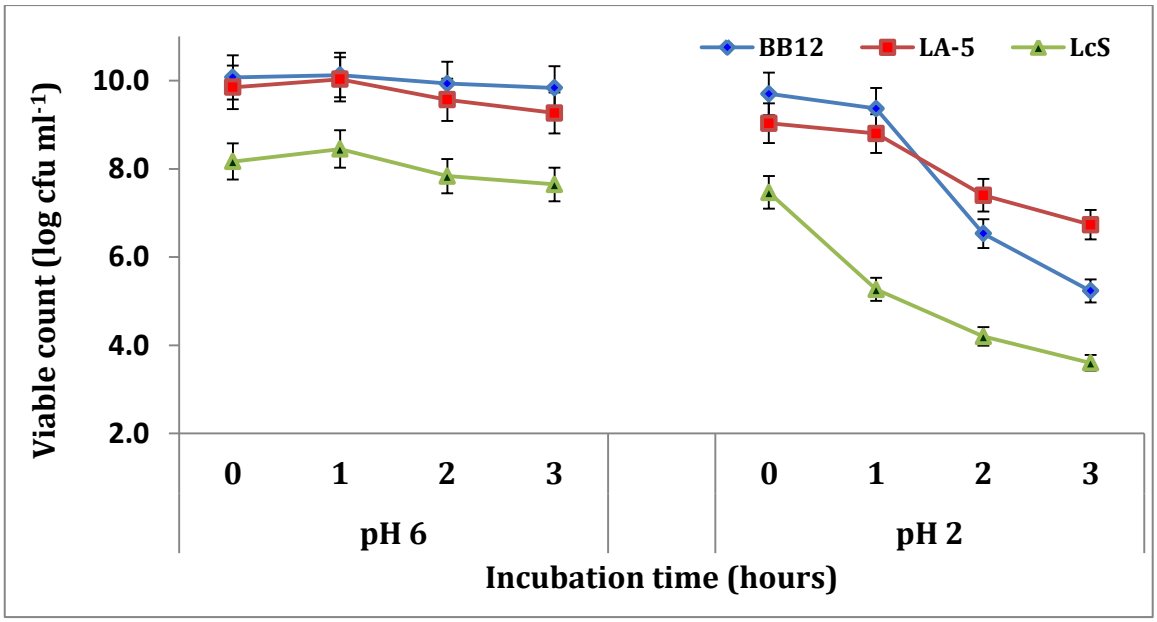

Figure 3.2. Viable counts (log cfu ml^{-1}) of probiotic strains inoculated on MRS agar after incubation at zero time and after 1, 2 and 3 hours under anaerobic incubation for strain BB12 and at 5% CO_2 for strains LA-5 and LcS at 37°C. (mean ± se) (n=3).

Huage *et al*., (2014) mentioned that most microbes have a low rate of survival and viability in acidic environments, like those occurring in the human stomach, where the pH value ranges from 1.5 to 3.5.Stability of a probiotic strain under an acidic environment could be a useful predictor for technological characteristic of probiotic strains in fermented dairy foods (Ross *et al*., 2005; Mättö *et al*., 2006).The viable count of probiotic strains at pH6 decreased slightly after 3 hours of incubation at 37°C. The viable count of strains at zero time (time of adding the strains) for strains BB12, LA-5 and LcS were 10.1 ± 0.081, 9.8 ± 0.147 and 8.17 ± 0.205 log cfu ml^{-1} respectively, and after 3 hours of the incubation, the viable count reached 9.8 ± 0.205, 9.3 ± 0.249 and 7.7 ± 0.107 log cfu ml^{-1} for strains BB12, LA-5 and LcS, respectively. *Lactobacillus* species are considered intrinsically resistant to acid. Although there are differences between species and strains, and they generally exhibit increased sensitivity at pH values less than 3.0, therefore, probiotic strains with proven beneficial effects may be consumed relatively high numbers in products (Holzapfel and Schillinger, 2002b; Tannock, 2004).

Exposure of the probiotic strains to pH2 for 3 hours caused significant decreases in the viable count of the strains tested, in comparison to the control. The decrease of the viable count was different for each strain. Probiotic strain LA-5 was more resistant to the low acidic conditions than LcS and BB12, strain LcS was more reduced by exposure to pH2 than LA-5 and BB12 after incubation for 3 hours at 37°C. It was found that the *Lactobacillus* species are considered to be basically resistant to acid, although there are differences between species and strains, where the organisms generally exhibit an increased sensitivity at pH values below 3.0. A similar trend in the result was previously recorded by Marteau *et al.* (1997), which found that the reductions in viability of probiotic strains *Lb. acidophilus* were significantly different between the bacterial species under low acidic conditions.

Regarding the survival of strains at pH6 and pH2, the viable count of strains at pH2 decreased significantly after 3 hours incubation at 37°C, in comparison with the viable counts at pH6, where there were no significant decreases in the viable counts, compared to the viable counts at zero time in both pH values. In this regard, the low pH value affect some probiotic strains of *Bifidobacterium,* such as *B. lactis* and *B. animalis*, through effects on the activity of enzyme H^+-ATPase, as stated by Matsumoto *el al*., (2004), who observed that the survival of these strains decreased after incubation at pH values between 3-5 for 3 hours. Also, low acid concentration affects the activity of ATPases in cell membrane of *Lb. acidophilus* (Lorca and de Valdez, 2001), and lower acidic conditions (pH 1.5) strongly affect the survival of probiotic strain *Lb. casei* NCDC-298 (Mandal *et al.* 2006). Cotter and Hill, (2003) stated that the mechanisms of acid tolerance used by probiotic bacterial strains includes changes in the cell membrane and regulatory mechanisms, alterations in different metabolic pathways, and amino acid decarboxylation, in addition to the release of protons via the enzymes F1Fo-ATPase. Survival studies are useful to predict performance and, therefore, cell enumeration of the tested strains were carried out before and after keeping cells under stressful conditions resembling the stressful environment of the stomach or the small intestine transit. Such conditions could be introduced either separately or sequentially, the latter to display possible interaction between these stresses (Mustapha *et al*., 1997; Charteris *et al*., 1998a; Alender *et al*., 1999; Succi *et al*., 2005; Martin *et al*., 2006; Vizoso pinto *et al*., 2006). The viable count of probiotic bacterial strain LA-5 was > 10^6 cfu ml^{-1}, which is generally recognized as the minimum therapeutic level for probiotics and a minimum level that was recommended by Guo, (2009), with regards to a live microorganism at a time of regular consumption from 400 to 500g of products per week.

3.4.7 Detection of interactions between probiotic strains with each other, and with starter strains

3.4.7.1 Well-diffusion agar assay

The dairy industry normally uses more than one strain in manufacturing dairy products, such as cheese and yoghurt, and therefore, assessment of the interactions between individual probiotic strains, and with starter culture strains, is considered an essential point before their application into cheese (Irlinger and Mounier, 2009). Such interactions may be direct, through physical contact, or via signaling molecules. Alternatively, indirect interactions may occur where the changes in the physicochemical properties of the environment is induced by one strain, and it causes a response in another strain (Fredrickson, 1977; Bull and Slater, 1982). The results of the assessment of inhibition interaction between probiotic bacterial strains BB12, LA-5 and LcS each other, and with starter culture strains (SC), are illustrated in Table 3.6.

Table3.6. Inhibition interaction between probiotic bacterial strains BB12, LA-5 and LcS with each other, and with starter culture strains SC grown on M17 agar, after 24 hours of incubation at 37°C under gas pack with CO_2 and H_2 atmosphere, using well-diffusion agar assay.

Probiotic and starter strains	**Probiotic bacterial strains**		
	LA-5	**BB12**	**LcS**
Lc. lactis* subsp. *lactis* and subsp. *cremoris	**–**	**–**	**–**
***Lb. acidophilus* LA-5**		**–**	**–**
***B. animalis* subsp. *lactis* BB12**	**–**		**–**
***Lb. casei* Shirota (LcS)**	**–**	**–**	

(–) = Negative (absence of inhibition interaction),

Table 3.7. Inhibition interaction between probiotic bacterial strains BB12, LA-5 and LcS with each other grown on MRS agar, after 24 hours of incubation at 37°C under gas pack with CO_2 and H_2 atmosphere, using well-diffusion agar assay.

Probiotic strains	**LA-5**	**LcS**	**BB12**
***Lb. acidophilus* LA-5**		**–**	**–**
***Lb. casei* Shirota (LcS)**	**–**		**–**
***B. animalis* subsp. *lactis* BB12**	**–**	**–**	

(–) = Negative (absence of interaction).

This indicates the absence of inhibitory activities between individual probiotic bacterial strains, and with starter strains (SC). Thus, the result of the test was considered to be a negative result as illustrated in Table 3.6.

As the probiotic bacteria must arrive alive into the intestinal tract, in order to confer their benefits, the study of inhibition interactions between probiotic bacterial strains and starter culture strains should be conducted (Rajagopal and Sandine, 1990). During incorporation of probiotics into fermented foods, such as cheese, several factors that may influence the capacity of the probiotics to survive in the cheese and after consumption by consumers should be considered. Possible interactions between those strains and starter culture strains may take place such as synergism or antagonism through bacteriocin production. Therefore, choosing the best combination of probiotic strains contributes to the improvement of their performance during the manufacture of cheese and their survival in the final product during the storage, as reported by Heller, (2001) and Sieuwerts *et al.,* (2008). The results were in line with those from previous studies by Vinderola *et al.* (2002b), Saccaro *et al*., (2009) and Casarotti *et al*., (2014) who explained possible inhibition interactions between strains for fermented dairy products. As shown in Table 3.7, there is no inhibition interaction between probiotic bacterial strains with each other when grown on MRS agar, after incubation 24 hours at 37°C using gas pack with CO_2 and H_2 atmosphere using well-diffusion agar assay. The use of MRS agar in this test for probiotic bacterial strains only, because it is considered a typical media for the growth and enumeration of *Lactobacilli*, on the other hand, it contains sodium acetate trihydrate ($CH_3COONa.3H_2O$), which may affect the growth of starter culture strains, which consequently may affect the results of test. In this respect, Uraipan and Hongpattarakere, (2015) found that the presence of probiotic bacterial strains *Lb. casei* and bifidobacteria in combination, showed synergistic interactions with each

other, and antagonistic interactions against pathogens, through antibacterial production. Therefore, these probiotic strains could be of great promise and possibility for the development of probiotic products to successfully prevent and control food-borne infection in humans.

3.4.7.2 T- cross inhibition test

The natural and industrial food fermentation processes are achieved by either simple, or complex populations of microorganisms. Obviously, these fermenting microbes will not only interact with the fermentable matrix, but also with each other. These interactions are complicated, but are assumed to be critical for obtaining the desired product properties (Smid and Lacroix, 2013).

The results of the T - cross test showed that there was growth in the cross zone between the streaks of probiotic bacterial strains BB12, LA-5, LcS and streaks of starter cultures, also in a cross zone between individual streaks of probiotic bacterial strains on M17 agar. This indicates there is no inhibitory activity between probiotic bacterial strains with each other, at the zone of the presence of strains together (each two strains together) and with starter culture strains. The test was carried out as illustrated in the schematic diagram in the Figure 3.3.

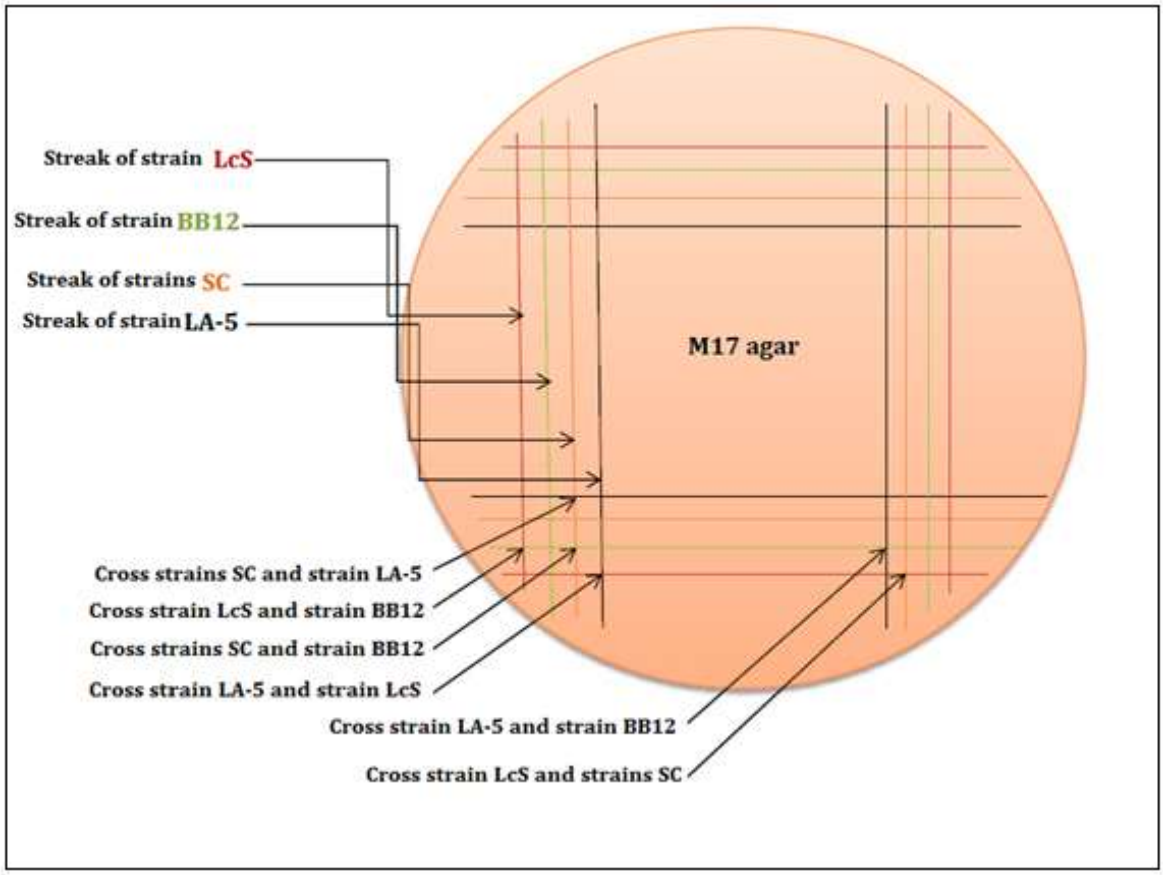

Figure 3.3. Schematic diagram of the possible inhibition interaction between individual probiotic bacterial strains LcS, LA-5 and BB12, and with starter strains (SC) after 24 hours of incubation using T-cross inhibition test. The test was carried out on M17 agar incubated at 37°C using gas pack with CO2 and H2 atmosphere.

The growth of probiotic bacterial strains, and starter culture strains at the crossing zone between probiotic strains BB12, LA-5 and LcS and each other, and with starter culture strains, was clear on M17 agar. This indicates the absence of inhibitory interactions between probiotic bacterial strains with each other, and with starter culture strains. The results of this test confirm the results of the detection of bacterial inhibition interactions, which was carried out using the well-diffusion agar assay. Therefore, according to the results, these methods, during soft cheese manufacturing, could use the starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* and probiotic bacterial strains *Lb. acidophilus* LA-5, *B. animalis* subsp*. lactis* BB12 and *Lb. casei* Shirota (LcS), together.

The report of Heller (2001) in this respect, was about the fact that the major problem which the production of probiotic fermented dairy products face is the interaction between probiotics and starter organisms. As well, the possible interactions of probiotics with the starter culture, e.g. bacteriocin production, antagonism, and synergism, may be even more concentrated when probiotics are used as a component of the starter culture. Furthermore, Sieuwerts *et al.* (2008) stated that most of the inhibition interactions that were identified in cheeses were related to LAB because of a knowledge of their physiology, as well as the recent publications of genome sequences of several species of LAB. The effect of such interactions on the appropriateness of the strains involved may either be positive, neutral, or negative. In this regards, Buriti *et al*., (2005b) found that there was separate synergistic interaction between probiotic strain *Lb. acidophilus* LA-5 and starter culture strains *Lc. lactis* subsp. *lactis* and/or *Lc*. *lactis* subsp. *cremoris*, which were incorporated together in Minas fresh cheese. As well as, Ranadheera *et al*., (2014) suggested that there is a synergistic interaction between probiotic strains *Lb. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB12, when were applied as a combination in the goat milk.

3.4.8 Enumeration of starter culture and probiotic strains on HHD agar

3.4.8.1 Inoculation of starter and probiotic strains into HHD broth

The change in the colour of the HHD broth from blue to yellow or green-yellowish indicates that the culture strains have an ability to ferment fructose in HHD broth and change the colour of broth from blue to yellow or green-yellowish. This change depends on the activity of the strain in the fermentation of fructose to lactic acid, by homofermentative strains, and to lactic acid and acetic acid by heterofermentative strains, like strain BB12 in the broth. The change in the colour of the HHD broth was by starter strains solely, and probiotic strain LcS was higher than those by strains

BB12 and LA-5. Changes in the colour of HHD broth, which was inoculated with starter culture strains and probiotic bacterial strains separately and together after incubation 24 hours at 37°C, is illustrated in Figure 3.4.

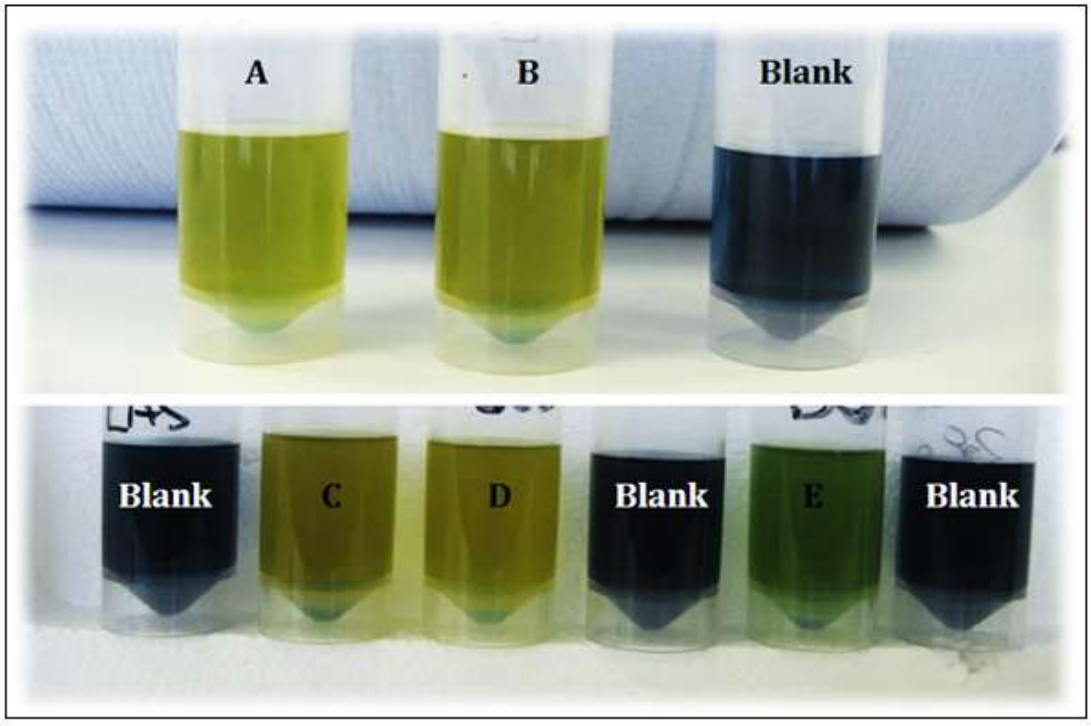

Figure 3.4. Fructose fermentation in HHD broth by starter culture strains (SC) (A) after 24 hours of aerobic incubation, and by probiotic strains LcS (C) and LA-5 (D) incubated separately at 5% CO_2, and by probiotic strain BB12 (E) under anaerobic incubation, and by a combination of starter culture strains and probiotic strains (B) using gas pack with CO_2 and H_2 atmosphere at 37°C.

3.4.8.1.2 Colony morphology on reference media (MRS and M17agar)

Images of probiotic bacterial strains and starter culture strains on plates of references agars (MRS and M17 agar), which were taken individually by using a digital camera of Spectro-photo3 microscope, are illustrated in Figure 3.5.

3.4.8.1.3 Colony morphology on HHD agar

Colonies of the starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* on HDD agar were circular, smooth, with a small central spot of green colour, diameter around 1.5 mm, surrounded by yellowish zone, resulted by the fructose fermentation to lactic acid. Colonies of probiotic strain LcS were white–cream,

with a green colour in the centre of the colony, which was bigger than those in SC and with a diameter 1.0 - 1.5 mm, surrounded by yellowish zone.

Colonies of LA-5 were of irregular shape, light brown, with central spot of green colour, with a diameter between 2.0 to 2.5 mm, and surrounded by yellowish green zone. Colonies of BB12 were translucent with diameter 1.5 to 2.0mm and surrounded by yellowish green zone. Images of probiotic bacterial strains and the starter culture strains individually, and together on HHD agar, which were taken by using a digital camera of Spectro-photo3 microscope, are illustrated in Figure 3.6 and Figure 3.7.

The images of combination of starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* and probiotic bacterial strains *Lb. casei* Shirota (LcS), *B. animalis* subsp. *lactis* BB12, and *Lb. acidophilus* LA-5 on HHD agar which were taken using a digital camera in the Spctro-photo3 microscope are illustrated in Figure 3.7.

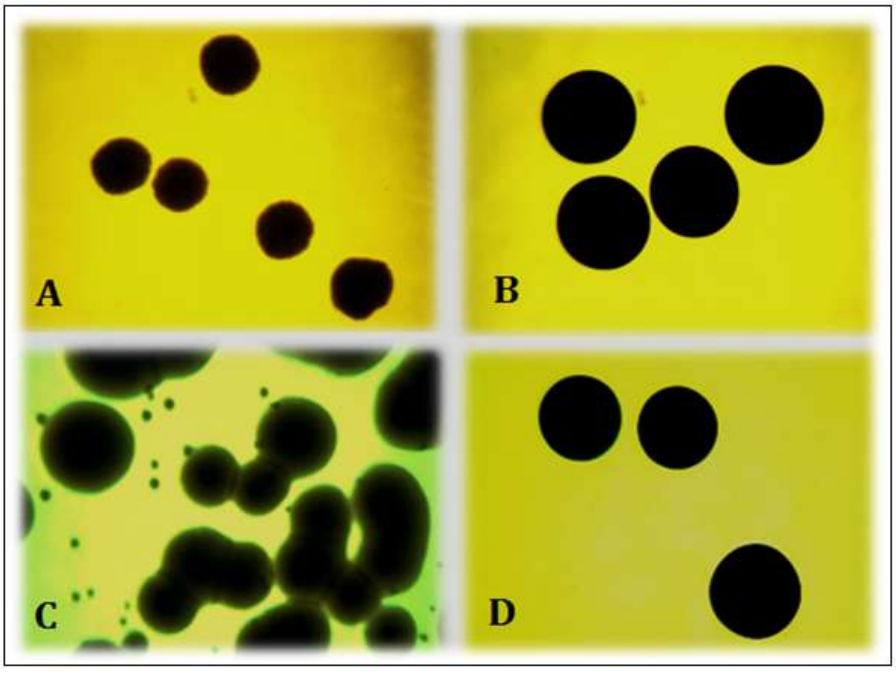

Figure 3.5. Images of starter culture and probiotic bacterial strains on references agar. (A) probiotic strain LA-5 on MRS agar after 48-72 hours of incubation at 5% CO_2 at 37°C, (B) starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (SC) on M17 agar after 48 hours of aerobic incubation at 30°C, (C) probiotic strain BB12 on MRS agar with 0.05% L-cysteine after 72 hours of anaerobic incubation at 37°C and (D) probiotic strain (LcS) on MRS agar after 48-72 hours of incubation at 5% CO_2 at 37°C. Images were taken individually using a digital camera of Spctro-photo 3 microscope.

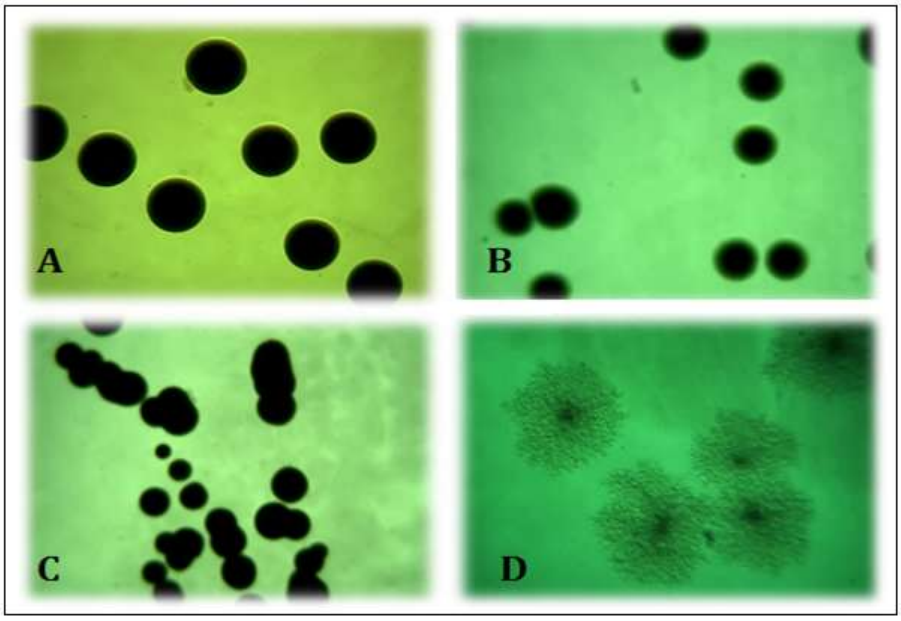

Figure 3.6. Individual images of starter culture and probiotic bacterial strains on HHD agar. (A) probiotic strain (LcS) after 48-72 hours of incubation at 5% CO_2 at 37°C, (B) Starter culture strains (SC) after 48 hours of aerobic incubation at 30°C, (C) probiotic strain BB12 after 72 hours of anaerobic incubation at 37°C and (D) probiotic strain LA-5 after 48-72 hours of incubation at 5% CO_2 at 37°C. Images were taken using a digital camera of Spctro-photo 3 microscope.

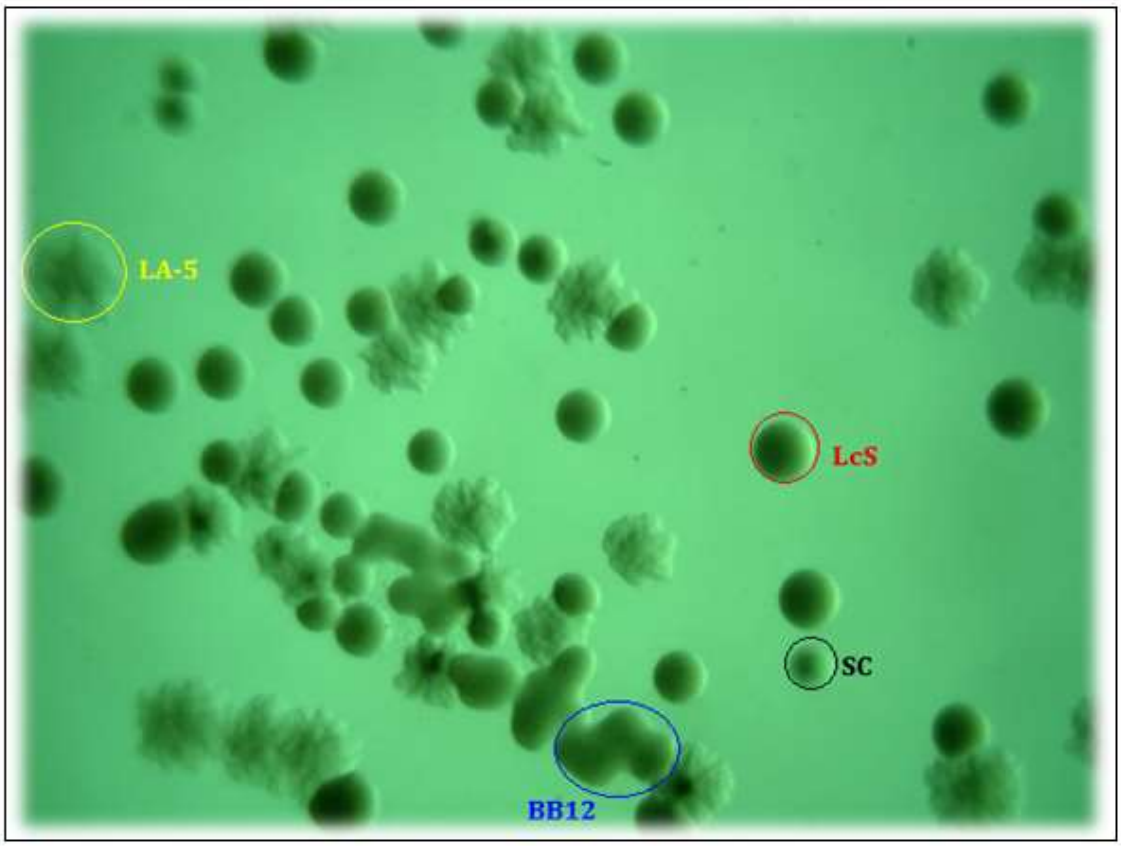

Figure 3.7. Images of a combination of starter culture strains (SC) and probiotic bacterial strains LcS, BB12 and LA-5 on HHD agar, after 72 hours of incubation at 37°C using gas pack with CO_2 and H_2 atmosphere. Images were taken using a digital camera of Spctro-photo3 microscope.

3.4.8.1.4 Enumeration of probiotic and starter strains together on HHD agar and separately on reference agars

Data in Table 3.8 shows that the viable count of starter culture strains SC and probiotic bacterial strains on reference agar and HHD agar. No significant differences ($P > 0.05$) in the viable count of probiotic strains BB12 and LA-5 on MRS (reference agar) and HHD agar and the viable count of starter strains SC on M17 agar (reference agar), and on HHD agar. Amongst the strains that tested, the numbers of probiotic strain LcS differed on MRS agar (reference agar) and HHD agar, where its viable count on HHD agar was 7.281 ± 0.127 and 8.610 ± 0.108 log cfu ml^{-1} on MRS agar ($P < 0.05$). In this respect, in a study by Tharmaraj and Shah (2003), some media were used as selective media for the enumeration of the combining strains of LAB, including, *Lb. delbrueckii* subsp*. bulgaricus, S. thermophilus, Lb casei, Lb. rhamnosus, Lb. acidophilus* and bifidobacteria. These media, including, MRS-bile agar, MRS-NaCl agar, MRS-lithium chloride agar, MRS-NNLP agar, sugar-based, such as maltose, galactose, sorbitol, manitol, esculin media, sodium lactate agar, arabinose agar, raffinose agar, xylose agar. The use of antibiotics and/or chemicals in these media adds an additional cost, in addition to some of these media need long incubation period, such as sodium lactate agar, which was applied into an enumeration of *Propionibacteria* under anaerobic incubation at 30°C for 7 to 9 days. Consequently, it is difficult to apply these media in the routine tests for differentiating microorganisms. Selective enumeration of the strains *Lb. casei* from probiotic products, based on the incubation at 25°C for 14 days, was studied by Champagne *et al.* (1997) and further developed by Ravula and Shah (1998), and it was known as LC agar, for selective enumeration of *Lb. casei* from yogurts and fermented milk drinks. This media was applied in this work for the enumeration of probiotic strain *Lb. casei* Shirota (LcS), but the results was invalid, and, in addition, the length of incubation period, which is

14 days at 25°C, which is considered impractical matter. The growth of starter culture (SC) and the probiotic bacterial strains BB12, LA-5 and LcS in this work is based on the use of a limited amount of fructose, which are the only source of carbohydrates and bromocresol green, as an indicator.

Fermentation of fructose in the agar and the production of lactic acid by homofermentative probiotic strains LA-5, LcS, and starter culture strains (SC), and with acetic acid by heterofermentative strain BB12 on the media, causes a change in the colour of bromocresol green to yellowish, yellowish green or blue colour, depending on the acid produced. In addition, bacterial strains were inoculated into HHD broth before pouring plates of the HHD agar, in order to monitor the change in broth colour, through the production of acid from the fermentation of fructose in broth media, by the activity of starter and probiotic strains. In this regard, Bifidobacteria are saccharolytic organisms that produce acetic acid and lactic acid without the generation of carbon dioxide, except during degradation of gluconate. As well as, *Bifidobacterium* spp. in some cases, have the ability to ferment complex carbohydrates. In addition, strains of *B. lactis* are considered to be oxygen-tolerant (De Vuyst, 2000). There are differences between starter strains and probiotic strains, in regards to their production of lactic acid only, and/or acetic acid, which was clear in the change of colour of broth by the inoculation of strains. The colour of zones which surround the strains differed in the intensity of yellowish green colour, which depends on the quantities of acids produced by strains. Table 3.7 shows no significant differences were found in relation to enumeration of BB12 and LA-5 on HHD agar and reference agar (MRS agar).

Table 3.8. Viable counts (log cfu ml^{-1}) of starter culture strains (SC) and probiotic bacterial strains BB12, LA-5 and LcS together on HHD agar, and separately on references agar. The incubation of HHD agar with strains was 72 hours at 37°C using gas pack with CO2 and H2 atmosphere. (mean ± se) (n=3)

Agar media	Bacterial strains	Viable count (log cfu ml^{-1})	Se
MRS agar	**BB12**	10.092^{A}	0.100
	LA-5	9.626^{ab}	0.061
	LcS	8.610$^{\wedge\wedge}$	0.108
M17 agar	**SC**	9.925*	0.120
HHD agar	**BB12**	9.984^{A}	0.089
	LA-5	9.758^{ab}	0.048
	LcS	7.281$^{\wedge}$	0.127
	SC	10.132*	0.109

Mean values in the same column bearing the different superscripts differ significantly (P <0.05). **(**SC) = Starter culture strains**,** MRS agar=Reference agar for probiotic bacterial strains LA-5, BB12 and (LcS); M17 agar=Reference agar for starter culture strains (SC).

The original colour of the media changed from blue to green, yellowish green, and yellow, depending on the acid produced from fermentation of fructose by the enzymes located in the cell wall and inside cell of strains, when grown separately or together on the HHD agar. It was observed that the morphology of the colonies was typical for each strain, and was easily for recognizable, when grown together on HHD agar. The results were in the line of the studies of McDonald *et al.* (1987) and Camaschella *et al.* (1998). The work of researchers was in regards to the differentiation between the strains of thermophilic LAB and bifidobacteria were used in a combination together. But, in this work, and in addition to the enumeration of strains, which were morphologically distinguishable on HHD agar, and the change in the colour of HHD agar around the bacterial strains grown on the media, were visible. Images were taken for the bacterial strains on the HHD agar by using a digital camera of Spectro-photo3 microscope (Osaka, Japan). Similar use of HHD agar was applied by Minelli *et al.* (2004) who stated that the use of HHD agar was useful to distinguish between colonies of probiotic bacterial strain *Lb. casei* and colonies of yoghurt culture strains, depending on morphological characteristics, and a selectively enumerated.

3.5 Conclusions

The results of testing the technological and functional characteristics of probiotic strains *B. animalis* subsp. *lactis* BB12, *Lb. acidophilus* LA-5 and *Lb. casei* Shirota LcS indicated that all strains have a tolerance to salt up to 4% (w/v), but the strains were sensitive to concentrations of more than 5%, with significant difference ($P<0.05$) in comparison with control (MRS broth and RSM without salt). In this sense, salt could be used in the manufacture of cheese at level up to 3% (w/w), in order to keeping the activity of probiotic bacterial strains during the manufacture and storage of products. In addition, the manufacture of cheese employing the new trends in regards to reduction the salt level in dairy products, such as cheese. Bile salts and acid have an influence on the survival of probiotic strains in different levels. Probiotic bacterial strain LA-5 was retained at level more than 10^6 cfu ml^{-1}.

All probiotic bacterial strains tested have the ability to grow at temperatures ranging between 20°C and 42°C, and the optimum temperature is 37°C. Also, fermentation of milk is possible in this range, and the optimum environment is at 37°C, which is considered suitable temperature for adding probiotic strains to milk during the manufacture of cheese. No inhibition interactions between the probiotic bacterial strains with starter strains were detected. Therefore, during the manufacture of cheese, a combination of these strains can be applied. Generally, from the results obtained could be concluded that the probiotic bacterial strains tested differ in some of technological and functional characteristics.

The bacterial strains tested were recognizable from their different colours, the shape, appearance, and the size of the colony. There was no significant difference between HHD and references agar M17 and MRS agar, with regards to the viable count of probiotic strains and starter strains, when grown separately, and together on HHD agar, but probiotic strain LcS differed significantly. Moreover, the use of one plate in

the test was easier than the use of many plates, in addition to saving media. Use of bromocresol green as an indicator contributed to facilitate the determination of the strains on the HHD agar. The change in the colour of the HHD broth depended on the activity of strain.

Chapter 4

Improvement of the quality and sensory properties of non-starter soft cheese using starter culture solely and with probiotic strains *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* Shirota (LcS) and *Bifidobacterium animalis* subsp. *lactis* BB12

4.1 Introduction

The word cheese refers to a group of food products based on milk fermentation, produced in several flavours and forms, in a wide range around the world (Fox and McSweeney, 2004a). Cheese starter culture is the acid-producing microbial culture which is added during cheese manufacturing, and is inoculated deliberately in the milk in order to impost the specific attributes in the cheese (Johnson and Law, 1999). Holzapfel (2002), reported that the selection criteria of starter cultures should focus on the substrate used, the technological characteristics of starter strains, the necessities of food safety, and the quality prospects. Acid production by the starter culture used combined with heat and the stirring of curd and whey mixture contributes to syneresis of curd, and that eject the whey from coagulum, in order to produce cheese with lower water content (drop from 87% to 35-60%), as well as the decrease of pH value (6.6 down to 4.6 – 5.2). Besides gel syneresis, acid production affect the activity, denaturation and the retention of coagulant in the product, the curd strength produced, as well as, affect the dissolution of colloidal calcium phosphate . In this respect, it was stated that the pH and moisture of Feta cheese manufactured with 0.75% starter were lower than those manufactured at levels 0.2 and 0.5% starter. Lower protein contents and yield were obtained with a low levels of starter (Cogan and Hill, 1993; Pappas *et al.*, 1996). Mesophilic cheese starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are the main and essential

blend of LAB, which are commonly and mostly used in the manufacture of various types of cheese (Crow *et al.*, 1993; Broome and Limsowtin, 1998).

Lactococci are the most important group of LAB used as a starter culture in the manufacture of several fermented foods, including cheese (Ayad, 2009). The important point for cheese producers is to use a suitable starter culture, in order to produce cheese with constant high quality. Therefore, it is important to study the starter culture characteristics rigorously, to ensure that it has consistent performance (Ayad *et al.*, 2001b). Parente and Cogan (2004b) stated that cheese cannot be manufactured without using the specific strains of LAB. But the same researchers on the same occasion stated that some varieties of the Spanish and Italian cheeses are manufactured without adding starter cultures, and the cheeses are manufactured depending on the environmental contamination of cheese milk.

The functions of starter cultures are the production of lactic acid from the fermentation of lactose in cheese milk during the manufacture, and to cause biochemical changes during storage and ripening, which contribute to develop the flavours which attribute to the final product. In addition, the early acidification contribute to minimize the chance of the pathogens to be introduced to the product, which consequently leads to a high quality and safe product (Abd El-Salam and Benjamin, 2003). Vinderola *et al.* (2000b) stated that the soft cheese can be used as an appropriate carrier for adequate combination of the three probiotic bacteria of strains bifidobacteria, *Lb. acidophilus* and *Lb. casei*. Some factors affects the microbiological properties of soft cheese, such as, the quality of milk used, type of heat treatment used, in addition to level of cheese contamination during manufacture and storage. According to Cardello (1995), food quality is a consumer-based perceptual/evaluative concept, that is relative to person, place, and time, and is also

subject to the same impacts of context and expectations as are other perceptual/evaluative phenomena.

The term "consumer acceptability" is the assessment concept that comes closest to being an adequate index of food quality. On the other hand, the definition of cheese quality by Peri (2006) was the degree of acceptability of the product to the consumers. Quality criteria involve different types of characteristics, including sensory properties, physical characteristic, cooking, compositional/nutritional, chemical and safety attributes. Measurement of consumer preferences cannot be achieved through using the objective tests, which depends on the numerical score instead of a person's intuition. It is a test that has right or wrong answers and so can be marked objectively. Cheese attributes, such as the appearance, flavour, and texture, result originally from a blend of microbiological, biochemical, and technological parameters, which directly or indirectly affect the microstructure of cheese. Consequently, it affects the consumer's acceptance of the product. Human understanding of the sensory properties of food correlates strongly with the status of the consumer at the moment of selection, rather than the data built using any type of analytical tools. And, the success of any food products depend on the consumer's acceptability of that product (Adhikari *et al.*, 2003).

Iraq has a very long cheese production. Soft cheese have been manufactured by local farmers on a small scale for decades, mostly using cow's milk or buffalo's milk or a mixture of both, and traditional techniques are passed down from generation to generation, and are now manufactured on the industrial scale from pasteurized milk by adding rennet only, and is commonly consumed by most of the population of Iraq. Commercial starter cultures are not used in the production of soft cheese. The product contain 15 – 20 % fat, salt is added at level up to 3% (w/w), its pH value 5.9

± 0.1, total solids 45 – 50 %. The cheese matrix should be without holes and food additives non-permissible.

Cheese manufacture without starter culture strains contributes to the decrease in the shelf life of the product, and it increases the risks of foodborne diseases. Therefore, maintaining the quality, safety, and the functionality, as well as extending the shelf life of the product, and decreasing its risks are important matters. Iraq enjoys a hot, dry climate, which is not suitable for the storage of hard cheese. Therefore, the most popular type of cheese produced is an unripened soft cheese without starter culture, locally known as white cheese (Arab cheese). It is generally consumed within the shelf-life period, which is lower than those for semi-hard and hard cheeses. Buriti *et al.,* (2005 a,b) and Buriti *et al.* (2007a) mentioned that the application of selected LAB as a single strain and a combination of cultures in cheeses, and through improving their viability, can increase the acidification, development of flavour, and the sensory characteristics of the product during storage. This is considered to be a good topic for work, and it encourages the application of starter culture and probiotic bacterial strains that are used in this study to non-starter unripened soft cheese, and increase its functionality, in particular, when there is a lack of detailed information regarding the incorporation of probiotic strains into soft cheese, particularly in Middle Eastern countries like Iraq, and the impact of probiotics on the quality parameters of the cheese during storage. Regarding of these aspects, this study was performed with the main aim for assessing the microbiological, physicochemical, and sensory characteristics of non-starter unripened soft cheese, manufactured with starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* solely, and in a combination with probiotic bacterial strains *Lb. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB12 and *Lb. casei* Shirota (LcS), during cold storage. Selecting a suitable

combination to positively affect the cheese quality and give the best and highest consistent quality during the manufacture and storage of the product was a priority.

4.2 Materials and methods

4.2.1 Cultures

Cheese starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (Chr. Hansen's Lab, Horsholm, Denmark) were used as starter cultures in the manufacture of starter soft cheese (SCS), and in combination with probiotic strains in the manufacture of probiotic soft cheese (PSC). Probiotic bacterial strains LA-5. BB12 and LcS was inoculated into MRS broth (De Man-Rogosa and Sharp-Oxoid, Basingstoke, England), and incubated between 18 - 20 hours at 37°C .

4.2.2 Manufacture of soft cheeses

Ninety litres of pasteurized semi skimmed milk from the same batch were purchased from Tesco supermarket, collected, and divided into three equal parts (30 liters each) and poured into three sterilized stainless steel vats. Three cheeses, including non - starter soft cheese (NSC) as a control, starter soft cheese (SCS), and probiotic soft cheese (PSC), were manufactured by applying the procedure employed in dairy factories in Iraq for the manufacture of soft cheese. Non - starter soft cheese (NSC) was manufactured as a control by heating 30 Kg of cheese milk to 36 ± 1°C in a stainless steel tank. Food-grade calcium chloride $CaCl_2$ (C/1280/53, Fisher Scientific, UK) was added at level 2.5 g $10L^{-1}$ cheese milk. Liquid Chymosin rennet (Chr. Hansen's Laboratory, Copenhagen, Denmark) was added at level 2.5 ml $10L^{-1}$ milk, which completed coagulation of milk in time between 45-60 min. Milk for SCS was inoculated with starter culture strains at level 1% before adding rennet. Milk of probiotic soft cheese (PSC) was inoculated with freeze-dried probiotic strains *Lb. acidophilus* LA-5 (10^{10} – 10^{12} cfu $ml^{-1)}$ and *B. animalis* subsp. *lactis* BB12 (10^{10}-10^{12} cfu ml^{-1}), and freeze strain *Lb. casei* Shirota (10^{7}- 10^{9} cfu ml^{-1}), in addition to starter

culture strains, which were inoculated at level 1%. Starter culture strains were added solely and a combination of starter strains and probiotic strains were added to the milk of SCS and PSC respectively, at the same time, before adding the rennet. Cutting the curd into 2 x 2 x 2 cm^3 using sterilized cutting knives, and the curd left 15 minutes for resting. After draining the whey salt was added at level 25g Kg^{-1} curd. After mixing the curd with the salt, it was transferred to a cheesecloth which was sterilized using a standard solution of hypochlorite, prepared, by dissolving one tablet in 5 litres tap water, and after that by using boiling water and warm water to avoid any contamination. The draining of the whey was then completed. After that, the curd was pressed by using weight 8.0 Kg per 20 Kg cheese milk, and leaving the curd in cheese cloths under pressure overnight at room temperature (20 - 22°C). The next day, the cheeses were sampled for microbiological and physicochemical analysis before packaging. Figure 4.1 is a schematic diagram for the manufacture of three soft cheeses.

4.2.3 Cheese packaging and storage

Cutting the cheeses into cubic shape (7 X 5 X 3 cm^3). Cheese samples were packaged in sterilized sealed plastic bags, and then stored under refrigerator condition 2-5°C for 7 days. After 7 days and under the same storage conditions, the cheeses were transferred into sterilized brine 3% (w/v) for 14 days, using sterilize polystyrene containers.

4.2.4 Cheese sampling

Cheese samples were taken for the microbiological and physicochemical analysis after one day of manufacture, and after 7, 14 and 21 days of storage. The sensory properties evaluation was carried out on the final products after 21 days of storage. Colour measurement of cheese was tested after one day of manufacture and after 21 days of storage. The steps of cheeses manufacture are illustrated in Figure 4.1

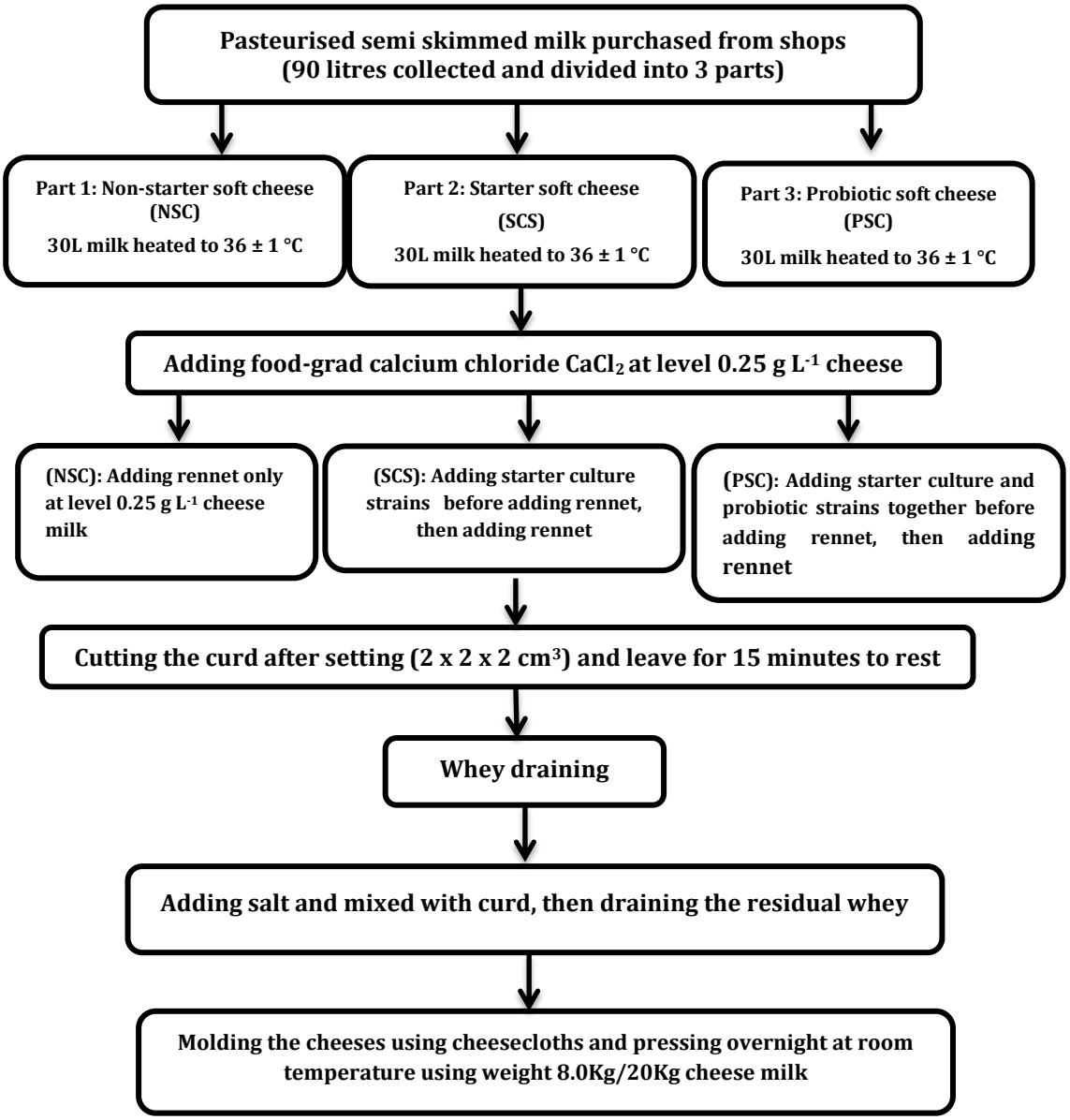

Figure 4.1. A schematic diagram for the manufacture of NSC, SCS and PSC

4.2.5 Preparation of maximum recovery diluent (MRD)

Maximum recovery diluent (MRD CM0733, Oxoid Ltd., Basingstoke, Hampshire, UK) was prepared, according to the method of Health Protection Agency (2004a), by dissolving 8.5g NaCl and 1.0g peptone in 1litre of distilled water, pH 7.0 ± 0.2 at 25°C, dispensed into the glass containers, and autoclaved.

4.2.6 Microbiological analysis of cheeses

Microbiological analysis were carried out on cheese samples after one day of manufacture (before packaging), and after 7, 14, and 21 days of the storage at 2-5°C. From each cheese, samples were taken aseptically for microbiological analysis. A composite sample of 10g was formed with portions of at least 3 areas of surface and depths of cheese, and then homogenised with 90 ml sterile 1.5% peptone water, in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 2 min, following the method of the Health Protection Agency (2004a). Serial dilutions of MRD, with culture, were made and plated out, following the national standard methods for enumeration of microbial groups in cheeses. The following microbial groups were enumerated in cheeses:-

4.2.6.1 Mesophilic aerobic bacteria count (MABC)

MABC was achieved on plate count agar (CM0325, Oxoid Ltd., Basingstoke, Hampshire, UK), using aerobic plate count (surface plate method) of the Health Protection Agency (2004b). One hundred µl of each dilution was spread onto the centre of a dried plate count agar plate was made. Leaving the plates on the bench for approximately 15 minutes to allow absorption of the inoculum into the agar was commenced. The inverted plates were incubated aerobically at 30°C for 48 ± 2 hours. APC per g was calculated as follows:

$$\mathrm{APC} = \frac{\mathrm{C}}{\mathrm{v(n1 + 0.1n2)d}}$$

Where: - C is the sum of colonies on all plates counted, v is the volume applied to each plate, n1 is the number of plates counted at the first dilution, n2 is the number of plates counted at the second dilution, and d is the dilution from which the first count was obtained.

4.2.6.2 Lactic acid bacteria count

LAB count were determined on the MRS agar (CM0361, Oxoid Ltd., Basingstoke, Hampshire, UK) following the method of Turkoglu *et al.* (2003). One hundred µl of each dilution spread onto the centre of a dried MRS plate. The plates were left on the bench for approximately 15 minutes, to allow absorption of the inoculum into the agar. Inverted plates were incubated 48-72 hours at 5% CO_2 at 37°C. Colonies were enumerated for plate which contained between 30-300 colonies using the colony counter.

4.2.6.3 Enterobacteriaceae count

Enterobacteriaceae were enumerated on Violet Red Bile Glucose Agar (VRBGA) following the method of the Health Protection Agency (2004c). One ml of each decimal dilution was transferred to a sterile Petri dish. About 15 ml of molten violet red bile glucose agar (CM 1082, Oxoid Ltd., Basingstoke, Hampshire, UK), tempered in a 45°C water bath, was poured into each Petri dish. The time passing between the end of the preparation of the initial suspension, and the time when the medium was poured, did not exceed 15 minutes. The inoculum with the medium was carefully mixed and allowed to solidify. Ten to Fifteen ml of VRBGA media which was tempered at 45°C, was poured onto the surface of the inoculated medium and allowed left to solidify. After that, plates were incubated for 24 hours at 37 ± 1°C. Colonies of *Enterobacteriaceae* produced purple, red colonies, with a diameter of 0.5mm or greater, and sometimes were surrounded by a red zone of precipitated bile. Counting and recording the characteristic *Enterobacteriaceae* colonies on each plate, the results showed that they contained not more than 150 colonies. Confirmatory tests were achieved by selectively at least five suspect *Enterobacteriaceae* colonies from the highest dilution, showing 15 to 150 colonies, and then the subculture was placed onto a segment of a nutrient agar plate (BO0336, Oxoid Ltd., Basingstoke, Hampshire, UK).

Plates were incubated for 24 ± 2 hours at 37°C. Using the growth obtained for biochemical confirmation the following was confirmed through tests.

Oxidase test: A fresh solution of the reagent for each time of use was prepared. Immerse a swab in oxidase reagent and touch lightly on the surface of the colony to be tested. The immediate appearance of a dark purple colour at the point of contact denotes a positive reaction, but no colour change, or a purplish colour which develops later were both negative reactions. Alternatively, moisten a piece of filter paper a Petri dish with 2 – 3 drops of oxidase reagent. Using a stick, glass rod or platinum (not nichrome) loop transfer a colony of the test organism to the filter paper, and rub it on the moistened area. A positive reaction is indicated by the appearance of a dark purple colour within 10 seconds. Positive control: *Pseudomonas aeruginosa* NCTC 10662, Negative control: *E. coli* NCTC 9001. Fermentation test: The fermentation test on oxidase negative subcultures was performed using a deep stab inoculation of tubes of glucose agar, incubated for 24 ± 2 hours at 37°C. *Enterobacteriaceae* produces a yellow colour throughout the medium. Counts were calculated using the dilutions, giving 15 to 150 colonies on the plate. Calculate the count of *Enterobacteriaceae* per gram of cheese with the formula as follows:

$$\text{Count per g} = \frac{\text{No. of colonies confirmed}}{\text{No. of colonies tested}} \text{X} \frac{\text{No. of colonies counted}}{\text{Volume tested} \times \text{Dilution}}$$

2.4.6.2 Moulds and yeasts count

Moulds and yeasts count were determined on Potato Dextrose Agar (PDA) (CM0139, Oxoid Ltd., Basingstoke, Hampshire, UK), acidified with solution of 10% tartaric acid (Merck, Darmstadt, Germany) to be (pH 3.5). One hundred µl of each dilution spread onto the centre of a dried Potato Dextrose Agar plate. The plates were left on the bench for approximately 15 minutes, to allow absorption of the inoculum into the

agar. Inverted plates were incubated for 5 days under aerobic incubation at 25°C, following the method of Vassiliadis *et al.* (2009).

4.2.7 Physicochemical analysis

4.2.7.1 Measurement the pH value of cheese

The pH value of cheese samples were measured by submerging an electrode of a pH meter (Hanna pH 213, Portugal) into a cylinder filled with grated cheese, while recording the pH value within 30 to 45 seconds (Saldamli and Topçu, 2006).

4.2.7.2 Measurement of total solids in cheese

Measurement of the total solids content of cheese samples was carried out using the method of Vassiliadis *et al.* (2009) by drying 5.0 g of cheese in an oven (Thermo Scientific, Germany) at 105°C, to achieve constant weight. The difference in the weight of the cheese samples and dish before and after 24 hours of drying represents the moisture loss. The following equation was used for calculating the percentage of total solids: Total Solids % = 100 – moisture %.

4.2.7.3 Measurement of fat content in cheese

The fat content was determined using the Gerber method, following the British Standards (1969) as follows:- 1- The ground cheese sample was mixed thoroughly and 3 ± 0.001g was weighted using the counterbalance weighting funnel with stopper. 2- Ten ml of sulphuric acid were dispensed into the butyrometer 3-Warm distilled water was added to form a layer about 6 mm deep on top of the acid, to allow for water to flow down the side of the bulb. The neck of the funnel containing the 3 g of cheese was inserted into the neck of the butyrometer, and all of the cheese was transferred to the butyrometer. 4 - One ml of amyl alcohol was added into the butyrometer. 5- Warm distilled water was added to fill the butyrometer to about 5 mm below the shoulder. The butyrometer was closed firmly with a stopper without disturbing the contents. 6- Samples in butyrometer were shaken in a protected stand,

until all the particles were visible homogeneous. Incubation in water bath for 3 and 10 minutes. 7-Butyrometers were centrifuged immediately at 1100 rpm for 4-5 minutes. 8- Butyrometers were removed from the centrifuge to adjust the stopper to bring the fat column on the scale, and then placed in the water bath for between 3 and 10 minutes, before contents were examined. 9- Reading was conducted immediately recording where the sharp line between acid and the fat, and where the acid was clear. Before reading the fat level, the position of the fat column was adjusted to bring the lower end of the column on to the main graduation mark.

The scale readings corresponding to the lowest point of the fat meniscus, and to the interface of fat and acid was observed; the difference between the two readings corresponds to the percentage of fat in the cheese to the nearest 0.3%.

4.2.7.4 Measurement of salt content in cheese

4.2.7.4.1 Preparation of chemicals and reagents

The chemicals and reagents for the measurement of salt in cheese were prepared as follows: 1-1N $AgNO_3$ was prepared by dissolving 4.5g $AgNO_3$ in 100ml, volumetrically, with distilled water. 2- Preparation of 5% K_2CrO_4 indicator was made by dissolving 1.0g of K_2CrO_4 (Sigma-Aldrich Ltd, UK) in 20ml, volumetrically, with distilled water. Drops of 1N $AgNO_3$ (Sigma-Aldrich Ltd, UK) was added until the first permanent red precipitate was formed. The solution was filtered and diluted to 100ml, volumetrically. 3- 0.02N $AgNO_3$ solution was prepared by dissolving 3.4g $AgNO_3$ in 1liter, volumetrically, with distilled water. 4-Preparation of 0.02N NaCl (Fisher Scientific Ltd, UK) for standardizing $AgNO_3$ was made by dissolving 1.168g of oven-dried overnight NaCl, in 1liter, volumetrically, with distilled water. 5-Preparation of chloride standard solution (1g L^{-1} as Cl^{-2}) was made by dissolving 0.824g oven dried NaCl, and in 500ml, volumetrically, with distilled water. The solution was used in

checking the standard solutions 25, 50, 100, 200 mg per litre. Salt content in cheese was detected by using the Mohr titrated method as follows: - an accurately weighted 25g sample of cheese (Sartorius, Germany) was placed in a 250ml clinical flask after grinding in a mortar. Fifty ml of distilled water was added, and the mixture was stirred. The sample was boiled for 2 minutes, and then immediately cooled. The sample was then transferred to a 100ml volumetric flask, and any residual contents were rinsed into the volumetric flask, and it made up to the mark with distilled water. The solution was then filtered through filter paper. Five ml of the filtrate was transferred by pipette into a small conical flask. Three drops of K_2CrO_4 were added, and titrated with 0.1M $AgNO_3$ to a reddish-brown endpoint.

The NaCl (%) was calculated as follows:-

$$\text{Salt\%} = \frac{\text{T X 0.585 X 20}}{\text{W}}$$

T =Titration Value (ml of $AgNO_3$), W= weight of original sample used (g)

4.2.8 Sensory properties evaluation of cheeses

The sensory evaluation of cheeses were carried out by applying the methods of British Standard Institution (1986) (British Standard BS 5929, Methods for sensory analysis of food, Part 1, 1986, ISO 6658-1985). The sensorial properties of cheese samples were achieved with the scoring test, by participation of 32 panellists from the Food and Nutrition Department's staff, postgraduate, and undergraduate students. Panellists were asked to evaluate the intensity of cheese attributes, which includes flavour, hardness, acidity, colour, and crumbliness, and for their preference of cheese attribute, including appearance, flavour, colour, texture, and overall acceptance. Cheese samples as blocks of NSC, SCS and PSC were presented to the panellists in a disposable dish, with a plastic knife and a cup of water for cleaning their palates between samples. A coded sample was written on the surface of the dish in the

standard panel room, using white fluorescent light and a temperature of 21 ± 2°C. Before participation, each panellist was briefed, and they signed on a consent form. The process took place in two sessions of 90 minutes each. All ballot sheets were collected, and the data analysed statistically.

4.2.9 Measurement the colour differences of cheeses

Measurement of the colour of cheeses was carried out using spectrophotometer CM 2600d (Konica Minolta Sensing. Inc., Japan). Changes in the surface colour of cheese were measured, using Commission International d'Eclairage (CIE) L* a* b* colour values (CIE 1976 L* a* b* Colour Space). The test was on the surface of packaged cheese slices after one day of manufacture (before packaging), and after 21 days of storage at 2-5°C. The results were expressed as L* (lightness), a* (redness), and b* (yellowness). The measure of L* values representing black to white, a* values representing green to red, and b* values representing blue to yellow. Colour differences (CD) were represented by the differences in L*, a* and b* values of cheeses, after one day of manufacture (before packaging), and after 21 days of storage at 2-5°C, and between control cheese (NSC), SCS, and PSC, and between SCS and PSC themselves, using the following equation:

$$\mathbf{\Delta}E_{ab}^{*} = \sqrt{(\Delta \mathbf{L}^{*})^{2} + (\Delta \mathbf{a}^{*})^{2} + (\Delta \mathbf{b}^{*})^{2}}$$

Where ΔL*, Δa*, Δb* are differences in the values of L^*, a^*, and b^* for (Control) NSC, SCS, and PSC, and between SCS and PSC themselves, after 21 days of the storage. Colour difference ΔE_{ab}^* in the L^* a^* b^* colour space, indicates the degree of colour difference, but not the direction. Before each series of measurements, the spectrophotometer was standardized by using a white standard plate. The results were reported to be averages of five measurements on each cheese sample.

4.3 Statistical analysis

Statistical analysis was performed using Minitab 16, England, using a balanced ANOVA with a main effect of treatments. Least significance difference (L.S.D) was used for detecting the difference between means at $P < 0.05$. Chi Square was used in statistical analysis of the scores obtained by the panellists using low, moderate, and high intensity of cheese attributes, where level significance was ($P < 0.05$). Also, the differences between the cheese attributes were achieved, using paired (T) test. One-way variance analysis was used for analysis of the means of colour parameters.

4.4 Results and discussion

4.4.1 Microbiological analysis

The soft cheese manufactured without starter culture (NSC) had a high level of MAB, in comparison with other cheeses manufactured with starter culture solely (SCS), and with probiotic bacteria (PSC). The viable count of these microorganisms ranged between 6.025 ± 0.164 and 8.059 ± 0.219 log cfu g^{-1} in NSC. The initial viable count of these microorganisms were 6.025 ± 0.164, 5.651 ± 0.20 and 5.492 ± 0.199 log cfu g^{-1} in NSC, SCS, and PSC, respectively. In the final products, the numbers were 8.059 ± 0.219, 6.392 ± 0.357 and 6.318 ± 0.389 log cfu g^{-1}. The significant difference ($P < 0.05$) between NSC, SCS and PSC were detected, but there were no significant differences between SCS and PSC (Figure 4.2). The viable count of these microbes in starter cheese and probiotic cheese showed a slight increase during storage period, in comparison to the control cheese.

The milk used in this work was purchased from local shops, which did not state which method was applied into the pasteurization the milk. But mostly, the temperatures in pasteurization for milk used in the manufacture of cheese contributes to destruction of pathogens, coliform, and a partial elimination of the MAB, as mentioned by Grappin and Beuvier (1997), which stated that besides the destruction of pathogenic bacteria,

it causes significant changes in cheese milk, including a partial elimination of the milk microorganisms, which may grow in cheese during ripening. As well, researchers stated that a partial or total inhibition or inactivation of lipoprotein lipase and alkaline phosphatase was observed. Furthermore, it was mentioned presence enzymes that produced by psychrotrophic bacteria, with little or no modification of the cheese making properties, such as coagulation and acidification by LAB. Therefore, these microbes were found in all cheeses, after one day of cheese manufacture, with differences in initial viable count, which depended on the nature of the cheese, and the protocol conditions used in each cheese, which plays an important role in the contamination of cheese in these microorganisms. in this regards, Gobbetti *et al.* (1999b) who reported that the differences in the protocol of the production of cheeses, and between the types of milk used, causes differences in the viable count of MAB and specific microbial groups. As mentioned in the report of the Health Protection Agency (2010c), the viable count of these microbes are considered an indicator of quality of ready-to eat food. On the other hand, Bongo *et al*., (2008) found that the poor milk handling conditions, contributes to increasing total mesophilic aerobic bacteria in cheese.

The present results are in line with the report of Beresford *et al.* (2001), which mentioned that one of main roles of starter bacteria, is to make of the environment unsuitable for the growth of undesirable microorganisms, with regards to redox potential, the pH value and the moisture content of cheese, which affect the enzymatic activity of rennet and starter cultures. Similar findings have been obtained by Briggiler-Marcó *et al.* (2007), where it was found that the production of lactic acid by LAB in cheese, with the subsequent decrease in pH, affected a number of aspects of the cheese manufacturing process, and ultimately the cheese composition and its quality. In addition, the lactic acid which is considered an antimicrobial compound

and may be produced by the lactic starter strains, and the probiotic bacterial strains in SCS and PSC. Similar results have been reported by Adesokan *et al.* (2009), which stated that the use of starter culture strains and pasteurized fresh milk in the manufacture of unripened "Soft Cheese Wara" contributed to the decrease in the levels of unwanted microbes, in comparison to those cheese samples that were manufactured from raw milk. Meurman, (2005) stated that LAB and probiotic bacteria, particularly the strains of *Lactobacillus* and *Bifidobacterium,* have the ability to produce different antimicrobial components, such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, and bacteriocins. On the other hand, Van De Guchte *et al.* (2001), found that the cheese made from raw milk represents an important proportion of the traditional cheeses.

The pH value of the control cheese was more than 5.0, which was mainly produced by the activity of the spontaneous LAB present in the control cheese. Thus, the status of this cheese, that have high moisture, low salt content, in addition to an essential elements for the growth of these microbes, such as fat and proteins, enhance the growth of these groups of microorganisms. Furthermore, the contamination of cheese may occur during the manufacture and storage stages. This needs to be applied in strict measures, in order to minimize the contamination, in particular in the case of NSC. Microbes are certainly introduced during slicing, packaging, portioning, and other manipulations, but this should be minimised by good hygiene, both of the personnel and of the equipment. It was suggested that the measurement of these microbes may not serve as a predictor of other bacterial presence, including some pathogenic organisms (Health Protection Agency, 2010c).

The MABC in the cheeses were lower than those found in fresh, or short-time-ripened Cebreiro cheese and Tetilla soft cheese, Centeno *et al.* (1996b), Montasto soft cheese

(Marino *et al.*, 2003) and whey Myzithra cheese (Dermiki *et al.*, 2008), and in the range of their numbers in semi soft Port Salut cheese produced with starter culture (Iurlina and Fritz, 2004) and in Turkish soft cheese (Turkoglu *et al.*, 2003). But the MABC in NSC were higher than those observed in Sudanese soft cheese, which was manufactured from the salted milk 6% (w/v), after keeping in storage for 21 days under refrigerated temperature. It was reported that the viable count at room temperature was higher than those determined in cheese under cold conditions (El Owni and Sana, 2009b; Idris and Alhassan, 2010).

The MABC in the present study was in the range observed in the Non - starter white cheese manufactured from pasteurized milk (65°C for 30 minutes) content 8% (w/v) salt (Kheir *et al.*, 2011). As well as, the MABC in Sikma cheese were higher than those observed in SCS and PSC, which is closer to the range of those observed in control cheese (NSC) which was between 5.92 and 8.72 log cfu g^{-1} (Ceylan *et al.*, 2003). The same observation was with Karin Kaymagi cheese, manufactured from raw milk, Beyaz cheese and Civil cheese (Özdemir *et al.*, 2010). The viable count in the present cheeses were slightly lower than those detected in traditional Turkish cheese, where the researchers stated that the pH value of the samples varied between 4.53 and 6.32, with a mean of 5.24. The microbiological findings showed the presence of high counts of microorganisms investigated, and the poor hygienic quality of the product (Aygun *et al.*, 2005).

Not complying with hygienic rules and presenting cheese in fresh form for consumption without maturation are among the most important causes that affect MABC (Hayaloglu and Kirbag, 2007). Alper and Nesrin, (2013) found that the samples of fresh white cheese contained MAB at the interval of 5.2 $x10^4$ and 5.68 x 10^{11} cfu g^{-1} . The differences between the analysis results of fresh white cheese samples in this work and the values reported by other researchers may be either due to the

variations in the manufacture of cheese, storage conditions and durations of the examined samples or due to the difference in the methods applied. Soft cheese is usually produced under non-mechanized conditions. Thus, several types of microbes may enter the cheese during the manufacture and subsequent handling of the product.

The initial viable count of LAB in NSC was lower than those enumerated on SCS and PSC. The viable count of LAB in the cheese samples that manufactured with starter cultures (SCS), was significantly higher than those samples manufactured without starter culture NSC (control cheese). As well, cheese samples that were produced by a combination of starter cultures and probiotic bacterial strains, *Lb. acidophilus* LA-5, *Lb. casei* Shirota LcS and *B. animalis* subsp. *lactis* BB12, had the highest viable count of LAB in the final product (>9.0 log cfu g^{-1}), while the cheese samples that were produced without probiotic strains had the lowest (< 8.0 log cfu g^{-1}) at the end of storage (Figure 4.3).

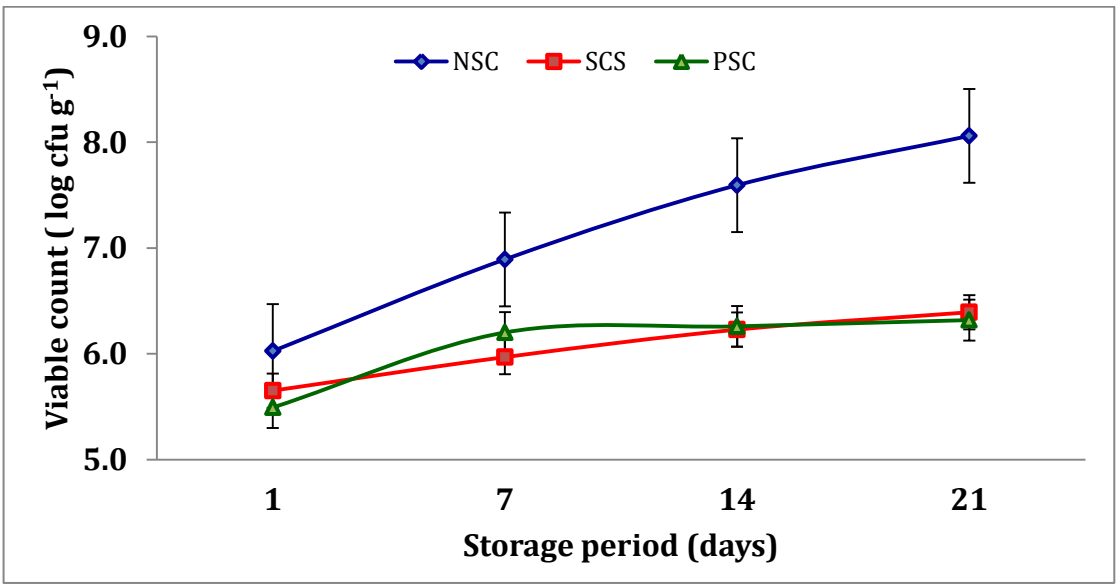

Figure 4.2. Viable counts of MAB (log cfu g^{-1}) in NSC, SCS and PSC one day after manufacturing, and after 7, 14 and 21 days of the storage at 2 – 5 °C. (mean ± se)(n=3). Results are average of three replicates of each cheese of three cheeses, which were manufactured at the same time.

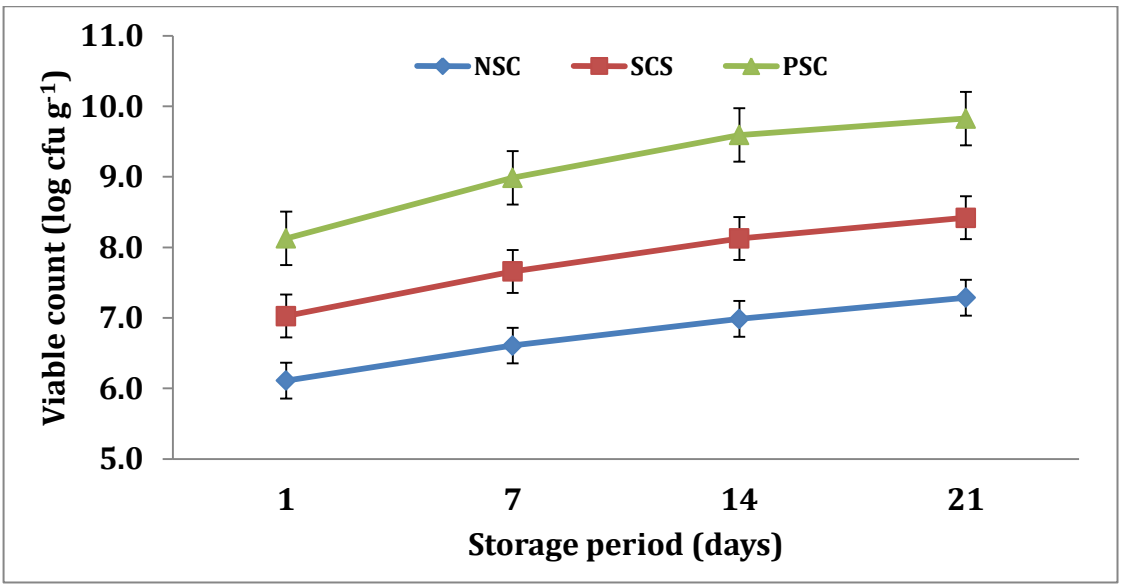

Figure 4.3. Viable counts of LAB (log cfu g^{-1}) in NSC, SCS and PSC one day after manufacturing, and after 7, 14 and 21 days of the storage at 2 – 5 °C. (mean ± se)(n=3). Results are average of three replicates of each cheese of three cheeses, which were manufactured at the same time.

The LAB group occupies a central role in the fermentation process during cheese making, and has a long and safe history of application and consumption, in the production of fermented foods and beverages (Ray, 1992; Holzapfel and Wood, 1995 ; Caplice and Fitzgerald, 1999). LAB are considered to be part of the normal microbial flora of raw milk, and the importance of this group is linked to the lactic fermentation, which is a spontaneous or promoted process that results in improved dairy preservation, when linked to traditional cheese-making (Kongo *et al.,* 2007; Kongo, 2013). This group of microbes cause rapid acidification of milk through the production of organic acids, mainly lactic acid from lactose. Additionally, the probiotic and starter strains may produce acetic acid, ethanol, aroma compounds, and several important enzymes, in addition to antibacterial bacteriocins, which could play a role on preservation of foods (Ross *et al.*, 2002b). In this respect, the combination of microbial groups which may have an interdependent activity when they are active together in the same ecosystem, and thus their physiological characteristics may change as a result of disruptions of the diversity. It has been shown that certain

microbes in combination contribute to a protective effect against pathogens such as *Listeria* spp. (Montel, 2010).

These activities contribute to enhance the shelf life and microbial safety, to improve texture, and contribute to the sensory properties of the final product. The viable count of LAB in cheeses, were high in SCS and PSC treatments, in comparison to NSC. These results were consistent with those by Adesokan *et al.* (2009), who found that the viable count of LAB in non-starter soft cheese Wara, was lower than those found in cheeses manufactured with starter cultures only, and with starter cultures and probiotic bacterial strains *Lb. bulgaricus* and *Lb. plantarum*. Also similar, were the results of Oliveira *et al.* (2012), where the high counts of LAB, including probiotic strains *Lb. acidophilus* and *Lb. paracasei* and *B. lactis*, were found in Brazilian goat cheese stored for 21 days at 10°C. But in contrast, Ortigosa *et al.* (1999), reported that the viable count of LAB in ewe's cheese, which was manufactured without a starter culture, was higher than those in cheese with starter culture. The environment of NSC, SCS, and PSC are considered suitable for the growth of LAB, including, probiotic strains that are incorporated into the product through the availability of the essential elements needed for their survival and multiplication, such as lipids, proteins, vitamins and minerals.

The pH value of cheeses were between 4.99 and 4.65, which are considered favourable for the growth of LAB and probiotic strains, in addition, the salt concentration in brine used in the present study do not affect negatively the growth of these groups of microorganisms. Tzanetakis and Litopoulou-Tzanetaki (1992), found that the pH value of Feta cheese ranged between 5.19 and 4.56, and for Teleme cheese was between 4.85 and 4.57. As well, the brine concentration between 3.0 % and 6.0 %, was more suitable for the growth of LAB in Feta cheese with viable count between 4.59 and 7.95 log cfu g^{-1}, and in Teleme cheese was between 5.66 to 8.14 log

cfu g^{-1}. In this matter, the use of brine in this percentage of salt is to guarantee the survival of starter cultures and probiotic bacterial strains in cheese, as they have the ability to grow and survive at this level of salt. In addition, it acts as a preservative, contributes directly to flavour, and is a source of dietary sodium, which related to consumer health. Together with the desired pH, water activity, and redox potential, salt plays an important role in the preservation of cheese quality, through reducing spoilage and slowing the growth rate of some pathogens (Guinee, 2004). In this regards, the report of Gomes *et al.* (2011) stated that the development of food products, such as cheese, that may contribute to reduce problems linked to public health in a constructive way is a challenge for the dairy industry, because of the negative effects of high levels of salt on human health, such as an increase in the blood pressure and decrease in calcium absorption. Furthermore, the study mentioned that further knowledge is required regarding the quality of cheese and levels of salt that are acceptable in the manufacture of cheeses with reducing the sodium content. Menéndez *et al.* (2001) found that the mean count of LAB in 24 samples of soft Tetilla cheese, which were produced in the North of Spain from raw cow's milk, were higher than those present in all the cheeses in this study and was >10 log cfu g^{-1}. During the storage period, the viable count of this microbial group showed a slight increase in the control cheese, in comparison to the starter cheese and probiotic cheese, when the increases were 1.175, 1.397 and 1.7 log cfu g^{-1} in NSC, SCS and PSC, respectively. Considering that cheese consumption is increasing worldwide, importance should be given to dropping the salt as a sodium-carrier, without affecting its consumption. With regards to the salt level in the brine used, and its effect on viable and activity of LAB, there is a variance between the strains of LAB in their resistances to the level of salt in foods (Fortina *et al.*, 2003), and it has been noted that the salt concentration in cheese may affect the quality of cheese and the survival of LAB, including probiotic

strains, as well as the low levels of salt in brine used in all cheeses. Consequently, the viable count of LAB was increased in cheeses SCS and PSC, and in low levels in NSC. In this respect, Yilmaztekin *et al.* (2004) found that the colony count of probiotic strain *Lb. acidophilus* LA-5, which was incorporated in white-brined cheese, declined during the storage 90 days from 10^9 to 10^6 cfu g^{-1}. In another study by Özer *et al.* (2009) found that the viable count of probiotic strain *Lb. acidophilus* LA-5, which was incorporated in white-brined cheese, decreased around 3 log cfu g^{-1}. Therefore, the use of 3% (w/v) brine was considered to be the best environment for the growth of the essential microbial group in cheese manufacturing. The present results similar to that obtained by Gobbetti *et al.* (1998) which reported that the viability of probiotic bacteria strongly reduced in cheese, when salt concentration is above 4%. Gobbettia *et al.* (1999a), found that the use of salt at level higher than 3.7% during the cheese manufacture inhibit the enzymes produced by the starter culture, *Lc. lactis* subsp. *lactis*. These enzymes include endopeptidase, which breaks peptide bonds of nonterminal amino acids and alanine aminopeptidase, in addition, aminopeptidase N, which plays a role in the final digestion of peptides that produced from hydrolysis of proteins, by gastric and pancreatic proteases. Therefore, cheeses which naturally present high salt content should have their processing optimized to incorporate the functional status, in order to be probiotic bacteria carriers.

There were significant differences in the count of *Enterobacteriaceae* between the cheeses under the same conditions of storage, and there was a high count of these microorganisms in NSC, in comparison with SCS and PSC. The initial viable count of the microorganisms in cheeses NSC were 3.175 ± 0.024 log cfu g^{-1}, which insignificantly increased after 21 days of storage, where it reached 3.247 ± 0.012 log cfu g^{-1}. In SCS and PSC, the viable count decreased significantly after 7 days of storage, where it reached (< 1.0), and remained until the end of storage in the both cheeses.

The viable count of bacteria in cheeses after 21 days of storage was 3.247 ± 0.012 log cfu g^{-1}, < 1.0 and < 1.0 in NSC, SCS, and NSC, respectively (Figure 4.4). In this regards, the heat treatment used in pasteurization of milk used for the cheese manufacture, could be enough for destroying these microbes, and should be readily removed from the equipment and surfaces using appropriate cleaning procedures. The use of sanitising solutions may reduce these organisms, but unlikely to remove them totally (Health Protection Agency, 2009). In this regards, Bongo *et al.*, (2008) concluded that the application of poor conditions into milk production at the farm, contributes to increasing the numbers of *Enterobacteriaceae* in cheese.

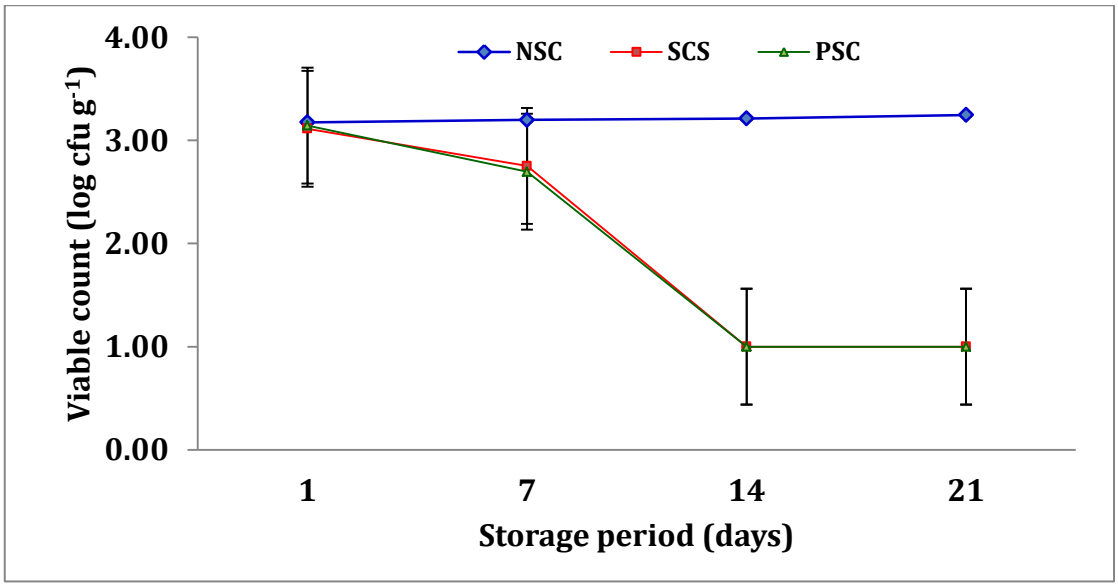

Figure 4.4. Viable counts of Enterobacteriaceae (log cfu g^{-1}) in NSC, SCS and PSC one day after manufacturing, and after 7, 14 and 21 days of the storage at 2 – 5°C. (mean ± se) (n=3). Results are average of three replicates of each cheese of three cheeses, which were manufactured at the same time.

The viable count of *Enterobacteriaceae* in SCS and PSC was lower than those observed in NSC, because of the unsuitability of the environments of SCS and PSC for growth of this microbial group, through the decrease in the pH value for these cheeses that resulted from the activity of starter culture, and the probiotic bacterial strains. Similar findings have been obtained by Medina *et al.* (1991), who found that more

acidic conditions in La Serena cheese made with starter culture, causes in the reduction of *Enterobacteriaceae* count during storage of 60 days, and was lower than those found in cheese, which was manufactured without starter culture. As well as, the results similar to that obtained by Macedo *et al.* (2004), which reported that the viable count of these microbes were high in the Serra da Estrela soft cheese, which was manufactured without starter cultures in comparison to those manufactured with starter cultures. In this respect, report of Centeno *et al.* (1996b) mentioned that the manufacture of Cebreiro soft cheese, contributed to reproduce the typical characteristics of the traditional raw milk cheese through reduction in the count of these microorganisms in the product. In this study, the viability of *Enterobacteriaceae* found in all cheeses were lower than those observed in different Mediterranean cheeses, which were manufactured using ewe's milk and goat's milk after 30 days of ripening, such as Turkish Carra cheese (Aygun *et al.*, 2005), was 5.60×10^5 cfu g^{-1} cheese, traditional Greek cheese manufactured from goat milk and starter culture strains *Lc. lactis* subsp. *lactis* (Psoni *et al.*, 2003) and La Serena soft cheese (Sanchez-Rey *et al.*, 1993). The viable count obtained in this work were in the range of that obtained in Serra da Estrela soft cheese (Dahl *et al.*, 2000) and soft white cheese Anevato (Hatzikamari *et al.*, 1999), where it was reported that the viability in cheese manufactured from refrigerated milk were lower than those found in cheese manufactured from non-refrigerated milk. Although these microorganisms are usually present in low levels in the final product, *Enterobacteriaceae* could release decarboxylases in the early stages of cheese manufacturing. The growth of *Enterobacteriaceae* usually contributes to decarboxylation of amino acids into biogenic amines (BA). Histamine, one of these compounds produced by histidine decarboxylase activity in certain conditions occurring during the manufacture of

cheese and or during storage under inadequate temperatures (Bover-Cid *et al.*, 2001; Health Protection Agency, 2009).

In this regard, Martuscelli *et al.* (2005) found that high levels of BA, produced by the strains of *Enterobacteriaceae* and LAB, during the manufacturing and storage of Pecorino Abruzzese cheese. Differences on manufacture from sheep's milk without starter culture, and from pasteurized milk with a starter culture, resulted in BA levels of 697 and 1086 mg Kg^{-1} cheese, respectively. On the other hand, the starter culture strains used in the manufacture of cheese have not produced BA. Additionally, starter cheeses had higher scores of acceptability, which indicates that the presence of BA produced by some of these microbes, would negatively affect the flavour of product. High levels of these microbes have been attributed to microbial contamination, linked to inappropriate manipulation, and have been used as an indicator of microbiological quality, and hence of hygiene practices (Kongo *et al.*, 2008). This indicates that the application of good hygiene practices in relation to milk collection during the cheese manufacture, and the handling of it, and /or the equipment used in the process, affect the quality of the final product. The results of the present study indicate that the application of appropriate hygienic quality contributed to reduce the growth of these microbes during cheese manufacturing. From aforementioned results, it could be seen that there was an application of a good sanitation procedure during cheese manufacture. However, the contamination probability should also be considered during the manufacture of cheese, in addition, all precautions should be taken during cheese manufacture, including the handling of milk used in manufacture, storage conditions, and sampling, in order to avoid and prevent any contamination, as well as maintaining the quality of the final product. As mentioned in the report of the guideline on the interpretation of results for hygiene indicator organisms in ready-to-eat foods by a Health Protection Agency (2009) and a Health Protection Agency

(2010c) in regards to the viability of *Enterobacteriaceae* in cheese, as a ready-to-eat food, NSC is considered in the borderline of satisfaction, and SCS and PSC are considered satisfactory. Therefore, the use of starter culture strains in the manufacture of Iraqi soft cheese could contribute to changing the microbiological characteristics and decrease its risks. But, as mentioned in the Guideline levels for *Enterobacteriaceae* in ready-to eat foods of New South Wales Food Authority (2009), NSC is considered an acceptable product (10^2 - $<10^4$ cfu g^{-1}), and SCS and PSC are considered good products ($< 10^2$ cfu g^{-1}). The initial viable count of moulds and yeasts in all cheeses was more than 3 log cfu g^{-1}, and in the final products after 21 days of storage were in a range between 2.201 ± 0.14 and 3.27 ± 0.021 log cfu g^{-1}. During the storage period, the viable count of microbes did not decreased in NSC, but did significantly decrease in SCS and PSC. Significant differences ($P < 0.05$) were detected between NSC, and both SCS and PSC cheeses.

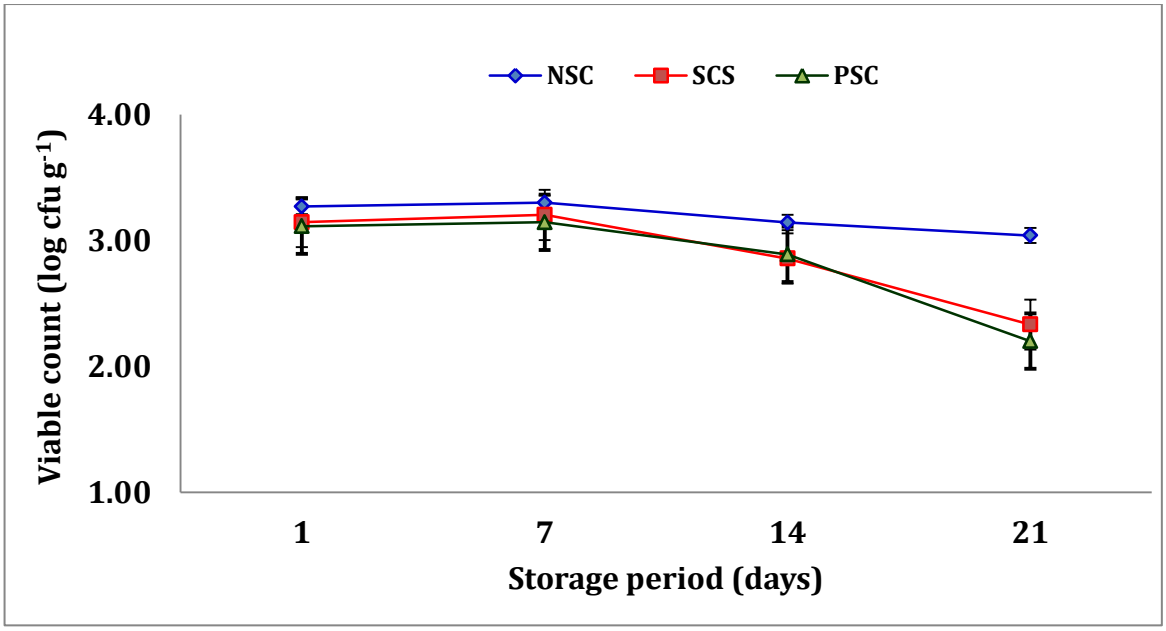

Figure 4.5. Viable counts of M&Y (log cfu g^{-1}) in NSC, SCS and PSC one day after manufacturing, and after 7, 14 and 21 days of the storage at 2 - 5°C. (mean ± se) (n=3). Results are average of three replicates of each cheese of three cheeses, which were manufactured at the same time.

Figure 4.5 shows that the viable count of M&Y in NSC, SCS and PSC, were higher than 3.0 log cfu g^{-1}, one day after manufacture, and after 14 days of the storage, the viable

count decreased gradually to reach less than 3.0 log cfu g^{-1} in SCS and PSC, and remained higher than 3.0 log cfu g^{-1} in NSC, until the end of the storage period. Some of these microorganisms have resistance to heat, and they remain in milk after pasteurization, which were determined in cheese, through their abilities to grow in the curd of cheese under storage condition. But, the contamination probability during the manufacture process should be put in mind, which explains the high initial viable count of the microorganisms in all cheeses, which decreases gradually during storage, due to oxido-reduction potential and metabolites existing in the matrix (Kiliç *et al.*, 2004). These microorganisms have the ability to grow in the environment of cheese manufacturing and in storage, which could greatly affect the characteristics of traditional cheese, as stated by Lopes *et al.* (1999) and Marino *et al.* (2003).

The viable count of M&Y in the present cheeses was lower than those found in some soft cheeses, such as Sudanese white cheese (Abdalla *et al.*, 2012), where an increase in the viable count of these microbes in the product was because of the drop the pH of cheese, and in whey cheese ''Myzithra Kalathaki'', the count was < 10^6 cfu g^{-1} during the storage of the product for 45 days at 4°C (Dermiki *et al.*, 2008). Vasek *et al.* (2008) mentioned that the high count of these microbes in Argentinean Corrientes soft cheese was probably because the contamination of raw materials. The viability of these microorganisms in Sikma soft cheese was > 10^7 cfu g^{-1} (Ceylan *et al.*, 2003). Pereira-Dias *et al.* (2000), found that the viable count in Portuguese ewes' cheese ranged between 2.7 and 6.4 log cfu g^{-1}. The viability of M&Y in SCS and PSC were in the range that was found in Slovenian cheese which were between 2.1 and 2.5 log cfu g^{-1} (Torkar and Vengušt, 2008) and the viable count in semi-soft Montasio cheese was higher than those found in cheeses of the present study, which was 4.46 log cfu g^{-1} after 60 days of storage (Marino *et al.*, 2003).

The initial viable count of M&Y in the NSC was more than those in the SCS and PSC, but the count started to decline after 14 days of storage, when the cheeses were transferred from sterilized bags to the brine. This decrease probably happened because the growth of these microorganisms on the surface of the cheeses, which were directly exposed to brine, whereby the brine affected the activity of the microorganisms, and caused them to minimize their count. Moreover, use of sterilized salt (Autoclaved), distilled water, and, in addition, use of sterilized containers in the preparation of the brine and the storage of cheeses under control low temperatures, may affect these microbes. As it has been proposed that the yeasts in cheese are primarily from the brine used and from the use unpasteurized brine, it contributed to the accumulation of salt resistance yeasts (Hansen and Jakobsen, 2001; Hansen and Jakobsen, 2004). In the same trend Coppola *et al*., (2008) stated that the brines mostly are considered a source of cheese contamination by salt-tolerant microbes, including, yeasts, such as *Debaryomyces hansenii* and its imperfect form *Candida famata*, bacteria such as staphylococci, micrococci, enterococci, corynebacteria and some LAB. In addition, consumption the oxygen in the brine by M&Y, may have influence on their growth and activity. Besides, the storage of cheese under controlled hygienic conditions could also play a role in reducing the growth of M&Y in cheese, which affect the flavour and texture of the final product (Ayad *et al.*, 2004). Furthermore, the presence of starter cultures and probiotic bacterial strains may be contributing to decreasing the viable count these microbes. In this respect, similar findings have been reported by Osman and Abbas (2001), which observed the absence of M&Y in probiotic cheese throughout the storage periods. However, Shehata *et al.* (2004) found that M&Y significantly decreased along the storage period of the same cheese, and they also found that the viable count of these microbes in cheese samples containing bifidobacteria was lowest during the storage period. On

the other hand, the yeasts that were found, may contribute to improve the organoleptic characteristics of NSC, SCS and PSC, as some investigations have shown that some lipolytic and proteolytic enzymes, produced by these microbes, contributes to the development of the compounds of flavour and aroma in the food products (Marino *et al.*, 2003).

4.4.2 Physicochemical analysis

4.4.2.1 Physicochemical analysis of cheeses

The quality of cheeses may differ from the application the differing manufacture processes, including the addition of starter culture strains during manufacture of SCS, and with probiotic bacterial strains into PSC. Through the effect on the pH value for these cheeses in comparison to non-starter soft cheese which was manufactured with rennet only. As shown in Figure 4.6, the average of the total solids in cheeses ranged between 40.140 ± 0.101 and 41.108 ± 0.108. No significant differences were found between cheeses. The percentage of fat in cheeses ranged between 12.417 ± 0.133% and 13.586 ± 0.146% (Figure 4.7). There were no significant differences between the cheeses. With regards to the salt level in the cheeses, it was seen that there is no significant differences between the cheeses, but the storage period significantly affected the salt content of cheeses.

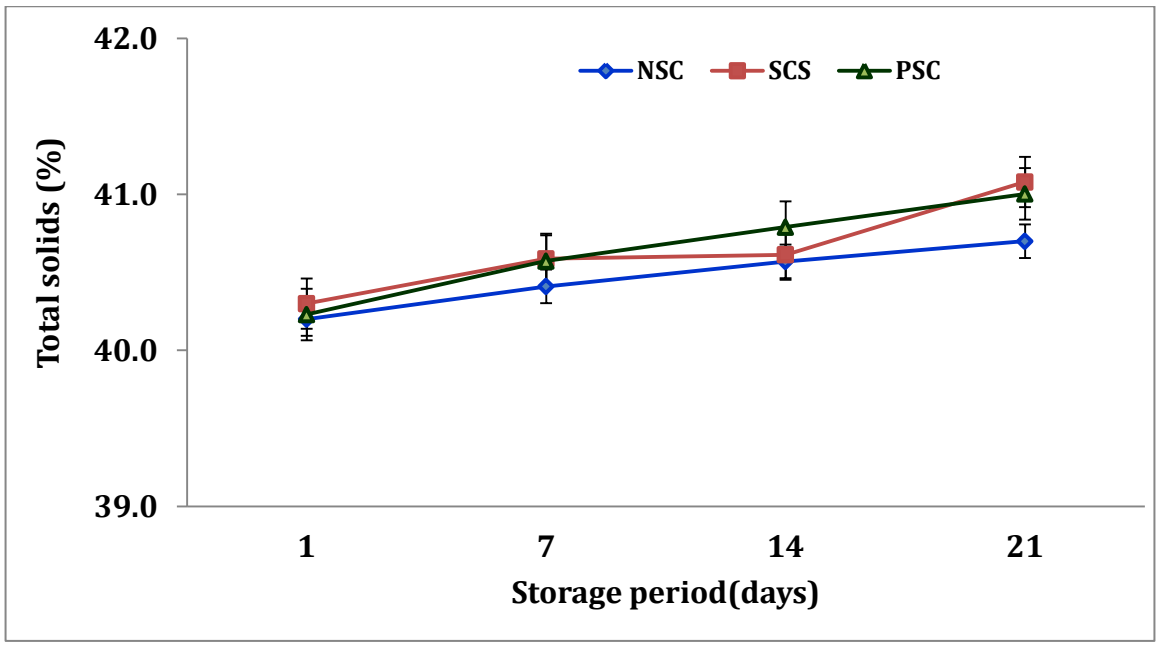

Figure 4.6. Average of total solids (%) of NSC, SCS and PSC after one day of manufacturing, and after 7, 14 and 21 days of storage at 2-5°C. (mean ± se)(n=3).

The salt concentration in the cheeses ranged from 2.154 ± 0.005 % to 2.309 ± 0.046% in NSC, from 2.162 ± 0.012% to 2.297 ± 0.072% in SCS, and from 2.156 ± 0.001% to 2.312 ± 0.033% in PSC (Figure 4.8). Bachmann *et al.* (2009) stated that cheese manufacturing is a process of enzymatic coagulation of milk, followed by the separation of protein, removal of carbohydrates, through its conversion to lactic acid by starter cultures, and an extended bacterial fermentation. The number of variables in this complex process affects the cheese quality, and the changes, determine the ingredients, processing, and the characteristics of the cheese. Furthermore, the steps of cheese manufacturing, such as whey draining, salting, and the type of method used in cheese pressing (e.g. Using a small amount of mass at the end of the handle, or applying the hydraulic presses method), may have has an effect. Data obtained from the analysis of physicochemical properties of cheese indicates that no significant differences were found between the three cheeses with regards to total solids, fat, and salt contents.

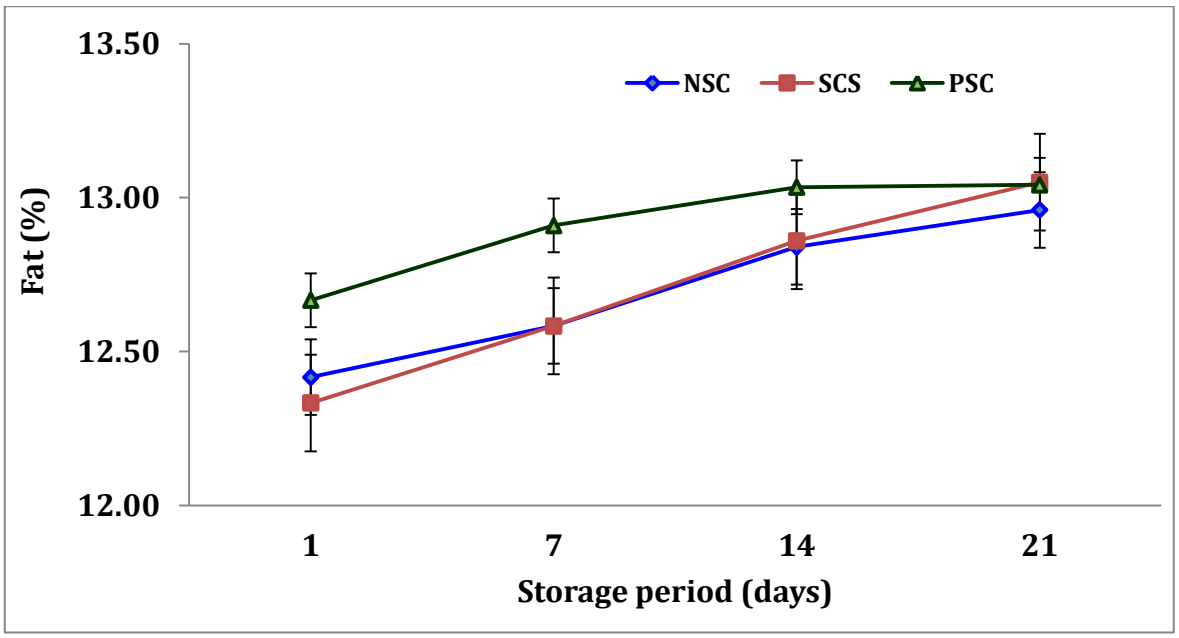

Figure 4.7. Average of fat content (%) of NSC, SCS and PSC after one day of manufacturing, and after 7, 14 and 21 days of storage at 2-5°C.(mean ± se)(n=3).

In this regards, Gomes *et al.* (2009) stated that the changes between the probiotic products and traditional products should be at a minimum. For all cheeses, the levels of TS, fat and salt content, which depends on the quality of raw materials used in cheese manufacturing, were lower than those determined in some soft cheeses studied by numerous researchers in some Mediterranean countries, such as White cheese by El Owni and Hamid (2007), which stated that the levels of TS, fat and salt in the product were from 45.17% to 58.17 %, from 19.27% to23.83%, and from 2.27% to 8.77 %, respectively. As well, TS and fat levels of present cheeses were lower than those determined in Spanish Corrientes soft cheese, as stated by Vasek *et al.* (2008), where the means of TS and fat content were 46.71 ± 4.53%, and 24.43 ± 2.13% respectively, with salt at level 1.28 ± 0.58%, and the pH value 5.24 ± 0.70.

The level of salt in NSC, SCS and PSC, is in line with the report of Fox and McSweeney (2004a), which stated that the level of salt and method of salting used during cheese manufacture affect the change in the pH value of cheese strongly, and a suitable concentration of salt, that usually used in cheese manufacture, is between 0.7 and 4.0 %, with regards to the activity of starter cultures. The high level of salt (i.e. 10%

salt in moisture phase) is adequate to inhibit the growth of starter cultures. In relation to adding the salt to the curd of cheese, Fox *et al.* (2000b), stated that during the use of dry-salting milled curd, the determination of the amount of salt that should have been added would be easy. And similar results have been obtained by Aly (1995), which stated that the production of Feta-type cheese with good quality, could produce using low level of salt (0.74 g 100 g^{-1}). The differences were observed after 14 days of storage, and no differences in the period between 14 and 21 days of storage, when the cheeses were transferred to the sterilized brine (3%), after the first week of storage. The increase in the diffusion of salt in cheeses depends on several factors, such as storage temperature, concentration of brine, and the surface of the cheese (Fox *et al.*, 2000b). On the other hand, the report of Melilli *et al.* (2003), show the diffusion of salt through the cheese placed in brine, 3% (w/v) slower than those placed in brine with concentration between 5.0% and 25% (w/v). In addition, the low temperature of cheese storage, up to 5 °C, plays an important role in decreasing both the diffusion rate and the quantity of salt absorbed in the cheese. Furthermore, the temperature of brine influenced the viscosity of the aqueous phase of the cheese (Turhan and KaletunÇ, 1992; Melilli *et al.*, 2003), and this is similar to our observation during the storage period of cheese at 2-5°C, where there was an increase in the viscosity of the brine.

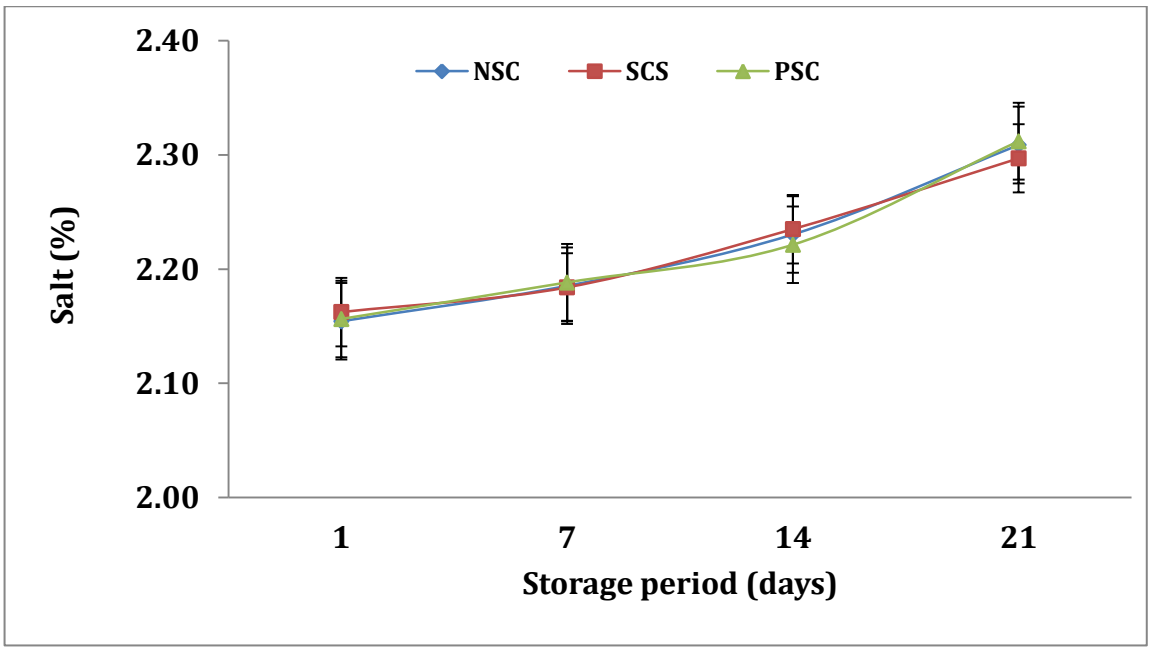

Figure 4.8. Average of salt content (%) in NSC, SCS and PSC after one day of manufacture and after 7, 14 and 21 of storage days at 2-5°C (mean ± se) (n=3).

The manufacture of present cheeses with low levels of salt was compatible with the new trend for the manufacture of cheese, with low levels of salt without affecting the quality and the sensory properties of the product, and keeping the health of consumers, particularly their blood pressure. In this regards, the report of Blood Pressure UK (2014) mentioned that there are advices given to the consumers regarding to the consumption of food, including cheese that contain low levels of salt, which are as follows: Low = 0.3 g salt or less per 100g of food (eat plenty of product), Medium = 0.3 - 1.5 g per 100 g of food (eat small amounts occasionally) and High = 1.5 g or more per 100 g of food (avoid and limit of these products).

Although, the use of salt in the manufacture of cheeses in this work was low, but according to the guidelines of Blood Pressure UK (2014), the level of salt that was used was considered high. Therefore,, the cheeses in this work are considered healthier than Feta and Domiati cheese, where mostly, the final product have high levels of salt, which are added to cheese milk at high level. These cheeses are

consumed widely in the Middle Eastern countries like Iraq. Thus, their content of salt has a harmful effect on the health of the consumers, in regards to the blood pressure. The pH value of the cheeses differed significantly during storage for 21 days. The drop in the pH value of the SCS and PSC was faster than those observed in NSC. SCS and PSC significantly differed with NSC ($P < 0.05$), but this not observed between SCS and PSC ($P > 0.05$) (Figure 4.9). The pH value of SCS and PSC differed significantly with NSC, because the use of the starter culture strains in the manufacture of these cheeses contributed to the drop in the pH value for these cheeses, through the fermentation of lactose. Thus, these affect the safety and characteristics of the product (Carminati *et al.*, 2010). On the other hand, starter culture should be added during cheese making, in regards to cheese yield determination. Cheese yield can be expressed in various ways, but the most common way is the mass of cheese in kg obtained per 100 kg of milk (Walstra *et al.*, 2005). In this respect, cheese yield obtained for NSC, SCS and PSC was 9.17% (2.75 kg), 9.5% (2.85 kg) and 9.9% (2.97 kg), respectively.

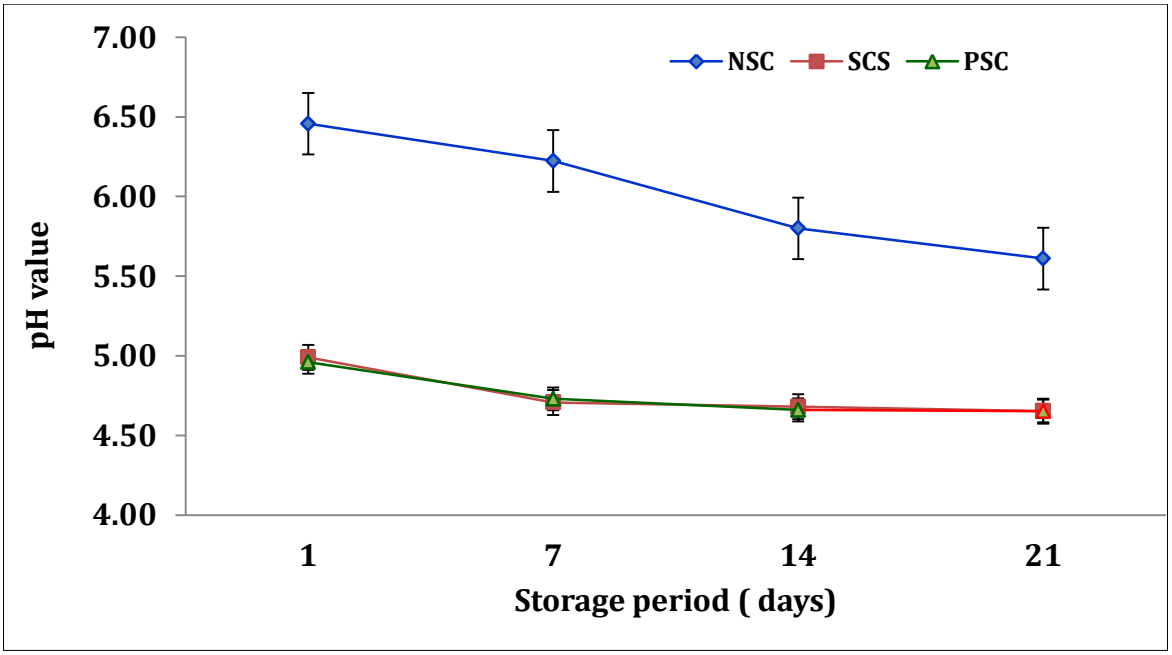

Figure 4.9. pH value of NSC, SCS and PSC after one day of manufacturing, and after 7, 14 and 21 days of storage at 2-5°C. (mean ± se) (n=3).

The value of pH in the final products was less than 5.0 in SCS and PSC, which is considered to be the safe levels for the quality and safety of the cheese, and more than 5.0 in the NSC, which is considered to be near to the optimal pH value for growth of pathogens in cheese, and, it is as stated by Beresford *et al.* (2001).

The viable count of LAB which is considered responsible for the change in the pH value of NSC was slightly increased with increasing the storage period, when compared to the SCS and PSC, and therefore, the pH value of NSC slightly decreased, when compared to the SCS and PSC that contained starter cultures (Figure 4.9). These results similar to that obtained by Adesokan *et al.* (2009), which stated that the pH value of unripened soft cheese, "Wara", decreased during the storage period, which might be a result of fermentation of the product during storage. As well as, the cheese samples manufactured with starter cultures had lower microbial loads than those manufactured by natural fermentation.

According to the report of Food and Drug Administration (2013), with regards to the evaluation and definition of potentially hazardous foods factors that influence microbial growth, the pH value of SCS and PSC was lower than values which considered as minimum pH values, for the growth of some pathogens and the production of toxin, including, *Vibrio parahaemolyticus, Shigella* spp., *Campylobacter* spp., *Bacillus cereus, Vibrio vulnificus* and *Clostridium perfringens.*

4.4.3 Sensory evaluation of soft cheeses

4.4.3.1 Sensory evaluation using intensity of soft cheese attributes

The sensory evaluation of foods is considered the final measurement of the product quality. Determining to what extent the consumers like or dislike the food product could be achieved through sensory tests, which are included in the quality control of foods. Most often hedonic scales, structured by 5, 7, or 9 points, are used by food manufacturers, and also by researchers (Drake, 2007). According to Urala and

Lähteenmäki (2004), the consumer's taste plays important role in the development of functional foods, in regards the consumer's attitudes towards the factors that affect the development of these foods. Different scores were obtained by NSC (control), SCS, and PSC, in their sensory evaluation, achieved by the panellists after 21 days of storage under 2-5°C. The frequencies of scores (%) obtained for cheeses NSC, SCS and PSC, to the intensity of the cheese attributes, are present in Table 4.1.

The results present the intensity of cheese attributes of flavour, hardness, colour, acidity and crumbliness. The attributes of flavour, hardness, and acidity for NSC presented high frequencies in the score 4, where they ranged between 31% for acidity and 50% for flavour. Data showed that the score for the intensity of attributes were at a high level, between score 3 and score 5, but the crumbliness attribute showed the high frequencies between score 5 and score 7. This indicates that all cheeses are deemed acceptable. In this respect, several researchers showed that probiotic cultures have not affected the sensory quality of cheeses, to the side of health benefits to consumers. These studies were with regards to the sensory evaluation, in order to observe the behaviour of different types of cheeses after the addition of probiotic cultures, and the properties of these products with regards to their attributes, like flavour, taste and/or texture, during their shelf life, and verification of the acceptability by the consumers (Gardiner *et al.*, 1998; Gobbetti *et al.*, 1998; Ryhänen *et al.*, 2001). In other studies by Katsiari *et al.* (2002a) and Menéndez *et al.* (2001), their main aims were to improve the sensory features of cheeses, with lower emphasis on functional properties of these cultures.

Flavour attribute of probiotic soft cheese was recorded at a high level, with score 4 (moderate intensity). With regards to the intensity of colour attribute, the highest score that was obtained is score 5, which obtained 41% and 31% for a control, and probiotic soft cheese, respectively, whilst for SCS it was 31% in score 4. The sensory

evaluation of cheese was attributed to the flavour and textures, which are considered the important attributes and plays an important role in the determination of its quality. In addition, it is important to understand that the texture is a sensory property, which gives proper trend to the plans of texture research, and the intensity of flavour in food is the main indicator for food selection (Szczesniak, 2002; Urala and Lähteenmäki, 2004). On the hand, in order to develop the new probiotic products, it is important to compare probiotic food products with non-probiotic controls, through a sensory evaluation, for both the products are mentioned by De Souza *et al.* (2008), and therefore, the comparison between the scores, obtained from the sensory properties evaluation for NSC, SCS, and PSC, is considered to be an important occasion for the development of probiotic soft cheese, which is one of the aims of this study. Although, no significant differences were found in the intensity of attribute of flavour between control cheese and SCS and PSC, but the mean scores obtained for SCS and PSC were higher than those obtained in control cheese. Similar results were obtained in the preference test for the attributes of flavour and texture (Table 4.4 and Table 4.5). This is in line with the report of Katsiari *et al.* (2002b), which stated that the use of *Lb. casei* subsp. *rhamnosus* in co-culture with two strains of *Lc. lactis* subsp. *Lactis,* and one strain of *Lc. lactis* subsp. *cremoris* affected positively the texture and flavour of low-fat Kefalograviera-type cheese, after 90 and 180 days of ripening, in comparison with non-starter cheese. With regards to the attribute of crumbliness, the cheeses were recorded as high frequencies in score 6, which was to 34%, 47% and 28 % in NSC, SCS and PSC, respectively (Table 4.1). Low (score 1 + 2), moderate (scores 3-5) and high (scores 6 + 7), scores were observed, and they were used independently for evaluating the intensity of cheese attribute by the assessors (Table 4.2).

Table 4.1. Score (%) obtained for NSC, SCS and PSC in the sensory properties evaluation according to the intensity of attributes, performed after storage 21 days at 2-5°C.(n=32)

Cheeses	Attributes	Scores (%)						
		1	2	3	4	5	6	7
NSC	Flavour	9	6	13	50	9	9	3
	Hardness	6	13	16	34	19	6	6
	Colour	0	0	3	13	41	34	9
	Acidity	13	32	31	31	3	0	0
	Crumbliness	6	13	6	13	22	34	6
SCS	Flavour	6	6	19	28	19	19	3
	Hardness	13	22	16	22	22	6	0
	Colour	0	9	16	31	22	22	0
	Acidity	16	13	25	25	16	3	0
	Crumbliness	0	6	9	3	22	47	13
PSC	Flavour	0	16	3	47	19	9	6
	Hardness	13	9	31	22	19	3	3
	Colour	0	3	19	28	31	19	0
	Acidity	6	34	16	22	13	6	3
	Crumbliness	6	3	6	13	13	28	22

Score 1 = Low intensity, 4 = Moderate intensity, 7 = High intensity, NSC **=** Non-starter cheese, SCS **=** Starter culture cheese, **PSC =** Probiotic soft cheese.

Statistical analysis, using Chi-squared test of the panellists frequencies, using low, moderate and high intensity of attribute, did not show significant differences ($P > 0.05$) in the attributes intensity of flavour, hardness, and acidity of cheeses, but significant differences were detected in the colour and the crumbliness attributes (Table 4.2).

Table 4.2. Score of cheeses NSC, SCS and PSC obtained in the sensory properties evaluation according to low, moderate and high intensity of attributes, performed after storage 21 days at 2-5°C. (n=32)

Attributes	Score	NSC	SCS	PSC
Hardness	Low	22	31	21
	Moderate	63	63	72
	High	16	6	6
Flavour	Low	16	13	16
	Moderate	75	66	69
	High	9	22	15
Colour	Low	3^{b}	25^{a}	28^{a}
	Moderate	53	53	53
	High	44^{a}	22^{b}	19^{b}
Acidity	Low	19	19	41
	Moderate	66	58	50
	High	16	23	9
Crumbliness	Low	22	13	9
	Moderate	56^{a}	31^{b}	31^{b}
	High	22^{b}	56^{a}	59^{a}

Mean values in the same row bearing the different superscripts differ significantly ($P < 0.05$). Low = low intensity of cheese attribute (score 1 + 2), Moderate = moderate intensity of cheese attribute (score 3-5), High = high intensity of cheese attribute (score6 + 7). NSC = Non-starter cheese, SCS = Starter culture cheese, PSC = Probiotic soft cheese.

No significant differences were found between the intensity of hardness, flavour, and acidity attribute, using t test (Paired). With regards to the intensity of cheese attributes, and as shown in Table 4.5, the mean scores were 2.906 ± 0.192 for acidity attribute of NSC and 5.531 ± 0.262 for the crumbliness attribute of SCS. There were significant differences between cheeses in cheese attributes of colour and crumbliness. This was similar to the results obtained in the sensory properties evaluation, using low, moderate, and high intensity of attributes (Table 4.2). Cheese attributes were acceptable for the consumers, although there were differences between the cheeses in the intensity of some of cheese attributes, such as attributes of colour and crumbliness.

4.4.3.2 Sensory evaluation using the preference of soft cheese attributes

An evaluation of the preferred attributes of the cheeses by the panellists was performed according to the recorded scores, in showing preferences for certain attributes of cheese, using a 7- point hedonic scale, from score 1 (like extremely) to score 7 (dislike extremely). The scores obtained were based on the preference test for the cheese attributes, including, appearance, flavour, texture, colour, and overall acceptance, were between score 1 and score 3 (Table 4.3). All cheeses showed good results in the acceptance, with regards to the preference of attributes. The high levels of frequencies for control cheese were obtained in score 1, and ranged between 28% for the flavour attribute, and 38% for the colour attribute. For SCS, the high frequencies were obtained in score 2 and score 3, where they reached 44% in score2 for flavour attribute, and 41% in score 3 for appearance attribute. On the other hand, for PSC, the higher frequencies were obtained in score 3 for the overall acceptance attribute, which reached 38%. These results referred to the acceptability of the cheeses by assessors, through a high record between score 1(like extremely) and

score 3 (like moderately), and comparisons between score 5 and score 7. But higher frequencies recorded were in score 4, with regards to the attributes of appearance, flavour, texture, colour, and overall acceptance. Significant differences were found between the colour of NSC and both SCS and PSC, but the colour of SCS and PSC did not differ significantly.

As shown in Table 4.4, the sensory evaluation for an appearance attribute have not differed significantly between cheeses, and this is in line with the results of Escobar *et al.* (2012), who found that the manufacture of fresh-style Panela cheese with probiotic strains *Lb. rhamnosus* only, *B. breve* only or with both strains, had no effect on taste or appearance of the final product, in comparison to the cheese without probiotic supplementation after storage for 4 weeks at 4°C. Too, is in line with the results of Marcatti *et al.* (2009), who manufactured Minas-type fresh cheese from buffalo milk without a starter culture, and by direct acidification with lactic acid plus *Lb. acidophilus* LAC4 (0.7% w/v).

Table 4.3. Scores (%) obtained for cheeses NSC, SCS and PSC in the sensory properties evaluation according to the preference of attributes, performed after storage 21 days in 2-5°C. (n=32)

Cheeses	Attributes	Scores (%)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
NSC	Appearance	31	25	22	9	9	3	0
	Flavour	28	25	22	9	9	3	3
	Texture	16	31	28	3	3	9	6
	Colour	34	38	16	6	0	6	0
	Overall Acceptance	31	19	25	6	19	0	0
SCS	Appearance	9	19	41	13	13	6	0
	Flavour	3	44	16	19	6	6	6
	Texture	6	44	25	6	13	6	0
	Colour	9	31	31	16	6	6	0
	Overall Acceptance	6	38	19	22	6	6	3
PSC	Appearance	22	19	34	9	13	3	0
	Flavour	9	34	25	6	13	9	3
	Texture	9	31	34	9	9	6	0
	Colour	16	25	28	9	6	6	0
	Overall Acceptance	13	25	38	3	3	16	2

Scored according to a hedonic scale ranging form score 1 to 7 (1= like extremely, 2 = like a lot , 3 =like moderately, 4 = neither like or dislike, 5 = dislike moderately, 6= dislike a lot and 7= dislike extremely). NSC = Non-starter cheese, SCS = Starter culture cheese, PSC = Probiotic soft cheese.

The results indicated that there were no differences between the cheeses, on attributes of texture, taste, and overall acceptance, although the viable count of probiotic strains into the final product was more than log 10^6 cfu g^{-1} after 28 days of storage.

Colour attribute of NSC differed with SCS and PSC in the test of preference of attributes and intensity of attributes, similar findings have been reported by Albenzio *et al.* (2013), which found that there were differences in colour between Scamorza cheese that was manufactured from ewe's milk, without and with a mix of probiotic strains *B. longum* 46 and *B. lactis* BB12, and with *Lb. acidophilus* LA-5 solely, and control cheese. And, a differentiation in the attributes of texture and appearance of probiotic bacteria cheeses and control cheese could be made easily.

Table 4.4. Mean scores of preference of cheese attribute, obtained from the sensory properties evaluation of NSC, SCS and PSC cheeses after 21 days of storage at 2-5°C. (mean ± se)(n=32)

Attributes	NSC	SCS	PSC
Appearance	2.375 ± 0.219	3.188 ± 0.248	2.813 ± 0.248
Flavour	2.594 ± 0.257	3.094 ± 0.275	3.188 ± 0.289
Texture	2.813 ± 0.285	3.063 ± 0.258	2.969 ± 0.235
Colour	2.219 ± 0.232^{b}	3.000 ± 0.242^{a}	2.938 ± 0.246^{a}
Overall Acceptance	2.531 ± 0.250	3.156 ± 0.262	3.188 ± 0.299

Scored according to a hedonic scale ranging form score 1 to 7 (score 1= like extremely, 2= like a lot, 3 =like moderately, 4 = neither like or dislike, 5 = dislike moderately, 6= dislike a lot and 7= dislike extremely). Mean values in the same row bearing the different superscripts differ significantly ($P < 0.05$). NSC = Non-starter cheese, SCS = Starter culture cheese, PSC = Probiotic soft cheese.

Table 4.5. Mean scores of the intensity of cheese attribute, obtained from the sensory properties evaluation of NSC, SCS and PSC cheeses after 21 days of storage at 2-5°C. (mean ± se)(n=32)

Attribute	NSC	SCS	PSC
Flavour	3.750 ± 0.250	4.125 ± 0.268	4.219 ± 0.237
Hardness	3.906 ± 0.278	3.406 ± 0.265	3.469 ± 0.262
Colour	5.313 ± 0.170^{a}	4.313 ± 0.222^{b}	4.375 ± 0.205^{b}
Acidity	2.906 ± 0.192	3.250 ± 0.246	3.313 ± 0.271
Crumbliness	4.344 ± 0.316^{b}	5.531 ± 0.262^{a}	5.219 ± 0.304^{a}

Scored according to intensity of cheese attributes ranging from score 1 =low intensity and 7= high intensity. Mean values in the same row bearing the different superscripts differ significantly ($P < 0.05$). NSC = Non-starter cheese, SCS = Starter culture cheese, PSC =Probiotic soft cheese.

As shown in Table 4.4, the mean scores of cheeses were obtained between score 1 (like extremely) and score 3 (like moderately), in relation to the preference of attributes, appearance, flavour, colour, texture, and the overall acceptance without significant differences between SCS and PSC. The mean scores ranged between 2.219 ± 0.232 for colour attribute of NSC, and 3.188 ± 0.248 for appearance attribute of SCS. This result agreed to some extent with the results of Oliveira *et al.* (2012), which showed the manufacture of Brazilian goat semi-hard cheese, with starter cultures *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* only, separately with probiotic strains *Lb. acidophilus* LA-5, *Lb. casei-01*, *B. lactis* (BB12), and with a combination of *Lb. acidophilus*, *Lb. paracasei* and *B. lactis*. Cheeses manufactured with probiotic strains record high scores, but the cheese with starter culture obtained lowest value without a significant difference in cheese attributes appearance, colour or flavour, which were found between probiotic cheeses during a storage period of 21 days at 10°C.

In this respect, storage of cheese with starter culture at 10°C contributed to the acceleration of the fermentation of available carbon sources into lactic acid, and converted sugars into other organic compounds, such as formate, acetate, and ethanol, negatively influencing the sensory properties of unripened cheeses, such as Coalho cheese (Even *et al.*, 1999). The different profiles were found in cheeses manufactured with some strains of probiotic lactic acid bacteria could cause a satisfactory influences on the sensory characteristics of these cheeses, particularly in their flavour, in comparison with cheeses manufactured without the addition of these strains (Oliveira *et al*., 2012). In similar occasions, Vinderola *et al.* (2009) found that the effect of probiotic strains *B. bifidum* A1 and *Lb. acidophilus* A3, which were applied to an Argentinian soft cheese and stored at 12°C for 60 days, was negative when compared to the effect of those stored at 5°C for the same period. This explains that the storage conditions of probiotic cheese affect the sensory characteristics of the final product, in relation to the acceleration of biochemical reactions that create an effect in the sensory properties of the product. On the other hand, Kourkoutas *et al.* (2006) reported that there were no differences in the flavour attribute of Feta cheese that was manufactured with rennet only, and with probiotic strain *Lb. casei* ATCC 393 ripened at 4 - 6°C, and so, this agrees with the results of this study with regards to the sensory evaluation of flavour attribute. In addition, both salted and unsalted Feta cheeses were acceptable by the sensory panellists. The results are in line with the results of Pereira *et al.* (2010), which stated that there were no significant differences between the evaluated attributes of flavour, texture and overall acceptability for Petit-suisse cheese that were manufactured with starter culture *S. thermophilus* TA040 and with starter culture and a combination of probiotic strains *Lb. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BL04.

Probiotic cheese has high levels of probiotic strains during the storage, and excellent sensory acceptance after 28 days of storage at 4 ± 1°C, in comparison to the cheeses which were separately manufactured with *Lb. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BL04. On the other hand, De Souza *et al.* (2008) found that the application of probiotic strain *Lb. acidophilus,* either solely, or in co-culture with starter culture *S. thermophilus* into Minas fresh cheese, resulted in a good acceptance of the product after storage of 14 days at 5°C, when compared to the non-starter cheese, that was significantly different with cheeses. But, Medina *et al.* (1991) obtained a different results during the manufacture of La Serena soft cheese with starter culture *Lc. lactis* subsp. *lactis* at level 1%, and found that the quality and intensity of cheese flavour were significantly weak by using *Lc. lactis*. On the other hand, similar results have been obtained by Albenzio *et al.* (2010), with regards to studying the effect of probiotic strains on cheese attributes of colour, smell, taste, and texture of Ovine cheese, which are manufactured without starter cultures (control), with rennet and *Lb. acidophilus* LA-5, with rennet and a combination of *B. lactis* BB12 and *B. longum* BB-46 after 30 days of storage. The results indicated that the use of these probiotic strains did not adversely affect the preference or acceptability of the cheese. The assessors rated probiotic cheeses higher than those which were obtained in the traditional cheese, although not significantly. Undesirable effects on the sensory characteristics SCS and PSC were not noted by the panellists from the application of the starter culture strains, and probiotic bacterial strains to cheese, with regards to the intensity and preference tests of the cheese attributes. Adesokan *et al.* (2009) found that the sensory properties of cheese attributes, appearance, taste, texture, colour, aroma, and acceptability of unripened soft cheese Wara, manufactured with a combination of probiotic bacterial strains, were better than those recorded by the cheese manufactured through spontaneous fermentation. This could be due to the

formation of flavour compounds, such as diacetyl and acetoin, produced by the lactic starter cultures. As well as, for NSC although it was without starter culture strains and probiotic bacterial strains, but, no negative comments were recorded on the ballot sheet of this product. Beside the health benefits of probiotic bacterial strains to the consumer, their survival in the product during the manufacture and storage period, they should contribute to maintaining and /or improvement the sensory properties of the final product (Champagne *et al.*, 2011; Vasiljevic and Shah, 2008).

4.4.4 Measurement the differences in cheese colour

The data of colour test using one way analysis of variance showed that there were significant differences ($P < 0.05$) in the lightness and yellowness of NSC, SCS and PSC cheeses. ΔE_{ab}* the difference between colours of cheeses between NSC and SCS was 4.861, 3.468 between NSC and PSC, and 1.603 between SCS and PSC.

Table 4.6. Colour parameters L*, a* and b*of cheeses NSC, SCS and PSC after 21 days of storage at 2-5°C. (mean ± se)(n=5)

Cheeses	Colour parameters		
	L^*	a^*	b^*
NSC	19.08 ± 0.073[a]	0.710 ± 0.051[c]	12.42 ± 0.082[c]
SCS	15.50 ± 0.058[b]	0.956 ± 0.036[a]	16.00 ± 0.052[a]
PSC	16.58 ± 0.060[b]	0.824 ± 0.081[b]	14.82 ± 0.071[b]

Mean values in the same column bearing the different superscripts differ significantly ($P < 0.05$). NSC = Non-starter cheese, SCS = Starter culture cheese, PSC = Probiotic soft cheese. L^*= lightness, a^*= redness, b^*= yellowness.

Data in Table 4.6indicate that there was a close correlation between the attribute of colour for SCS and PSC, and correlation between NSC with SCS and PSC was weak. Furthermore, it shows that the lightness of the NSC compared to SCS and PSC, but the SCS and PSC are closer to yellowness than the NSC. The results are compatible with the results of the preference test for attribute of colour for NSC, where the average score for colour attribute was less than 2.5, when compared with the scores of SCS and PSC, which were between 2.5 and 3.0. Moreover, the results of colour measurement were compatible with the sensory properties of the intensity of colour

attribute for SCS and PSC. The average of the scores was between 4.0 and 4.5, without difference between each other, but they differed from NSC, where the average of the score was more than 5.0.

4.5 Conclusions

This study was of three soft cheeses, which were manufactured without a starter culture, with starter culture solely, and with starter culture and probiotic bacteria strains. The cheeses were different in some of their chemical characteristics, and there were microbiological properties which affected the quality and safety of soft cheese. Application of the starter culture solely and with probiotic bacterial strains, contributed to improving the microbiological characteristics of Middle Eastern style soft cheese. The latter is mostly manufactured without starter culture, and is considered the optimal substrate for the growth of spoilage microbes, since it contained all the requirements for their growth. Changing the cheese nature by the use of starter culture strains played an important role in decreasing the spoilage indicators and some species of undesirable microbes, which are considered the main reasons in deteriorating the soft cheese, through changes in the environment of cheese, to harsh conditions responsible for the growth of these microbes. The decrease in the pH value of cheese, resulting from the fermentation of lactose in milk, which is considered to be an important ingredient for the growth of these microorganisms to lactic acid during the manufacture of cheese, in addition, may produce antimicrobial substances for fighting the unwanted microbes. Consequently, prevention and /or delay the occurrence of undesirable biochemical changes, by their enzymatic activities.

The application of selected starter culture and probiotic bacterial strains into a non-starter cheese contributed to maintaining the quality, safety, and the acceptability of the cheeses, which were assessed by the panellists by using the tests for the

preference attributes, and the intensity of the cheese attributes. The non-starter cheese, the starter culture cheese, and the probiotic soft cheese obtained the highest scores for all cheese.

The application of starter culture and probiotic bacterial strains to Iraqi non-starter soft cheese, could contribute to maintaining its microbiological properties, which are mostly associated with the health problems for consumers, through fighting unwanted microorganisms, as well as the spoilage indicators, in order to be safer, through the manufacture of the product under hygienic conditions. In addition, the improvement of its sensory properties is encouraging to the consumers for buying this popular product.

Chapter 5

Evaluation of modified atmosphere packaging in extending the shelf life of starter soft cheese

5.1 Introduction

Dairy products are an important food group with excellent nutritional value. Therefore, extending the shelf life of dairy products and keeping them fresh for a longer period of time is considered an important matter. In this regard, keeping cheese fresh for a longer period of time, and delaying unwanted changes in the product requires an effective method. Packaging provides the consumer with information about the product, such as weight, volume, nutritional value, ingredients and the manufacturer's details. The main roles of the food packaging are to contain protection/maintaining food, and inform the consumers. Consequently, it may contribute to decrease the losses and keeping the health of the consumers. Modified atmosphere packaging is one of the techniques used, and it works by changing the natural gas around the product in the package, in order to delay unwanted changes in the product (Coles *et al.*, 2003; Khoshgozaran *et al.*, 2012).

Extending the shelf life of cheese by the application of MAP and suggestions for using mixtures and compositions of gases for several types of foods, including cheese, have been examined in several other studies (Alves *et al.*, 1996; Eliot *et al.*, 1998; Floros *et al.*, 2000; Gonzalez-Fandos *et al.*, 2000; Pintado and Malcata, 2000; Farber *et al.*, 2003; Alam and Goyal, 2007). The effect of the gases used in MAP is affected by some factors, including permeability of package materials, light exposure, and storage temperatures (Juric *et al.*, 2003; Trobetas *et al.*, 2008; Rodriguez-Aguilera *et al.*, 2011). Some factors affect the microbiological characteristics of cheese, such as milk quality, survival of heat – tolerance of the microbes during cheese manufacture, and post–processing microbial contamination (Del Nobile *et al.*, 2009). The inhibitory effect of

CO_2 on microbial spoilage in cheese under MAP, as mentioned in the report of Farber (1991), is to increase the lag phase, and then decrease the growth rate over a logarithmic phase, and therefore, the microorganisms will have to adapt to the new atmospheric conditions (Olarte *et al.*, 2002). On the other hand, numerous researchers stated that the LAB in cheeses were not affected under MAP, because these microorganisms are facultative, and are able to grow under MAP, including Cottage cheese (Maniar *et al.*, 1994), Mozzarella cheese (Eliot *et al.*, 1998), Greek whey cheese (Papaioannou *et al.*, 2007) and Apulian fresh cheeses (Gammariello *et al.*, 2009a). Alves *et al.* (1996) and Eliot *et al.* (1998), stated that the growth of psychrotrophic bacteria was prevented in some cheeses which were stored under MAP, such as Sliced Mozzarella Cheese and Shredded Mozzarella Cheese, where it was found that the growth of these bacteria was slower at 100% CO_2 atmospheres, in comparison with those observed at levels 50% and 75% CO_2, which were stored at 7-10°C. The physicochemical properties of cheese are affected by the presence of CO_2 in the package, and according to Farber (1991) and Christopher and Hotchkiss (2002), the pH value of cheese drop in the presence of CO_2 through the formation of carbonic acid and amino acidic acids. Some factors affect the shelf life of cheese packaged under MAP, including the cheese type, use of starter culture strains in cheese manufacture, the storage conditions, and the initial load of the microbial contaminant (Floros *et al.*, 2000). MAP play an important role in extending the shelf life of the product, through decreasing the unwanted changes, which affect its sensory properties (Romani *et al.*, 2002). With regards to the effect of gas mixtures on cheese moisture or weight loss when packaged under modified atmospheres, several studies have indicated that different gas mixtures did not significantly affect the moisture content or weight loss (Rodriguez-Aguilera *et al.*, 2011). Other studies indicated a slight effect, such as Greek whey cheese (Papaioannou *et al.*, 2007) and Provolone

cheese (Favati *et al.*, 2007). Controlling the storage temperature during MAP is considered to be important for effective use of this method, it increases the activity of carbon dioxide, which is greatly enhanced in low temperatures, and with MAP, it will become a hurdle in the growth of pathogens in packaged cheese (Moir *et al.*, 1993; Alves *et al.*, 1996; Eliot *et al.*, 1998; Gonzalez-Fandos *et al.*, 2000; Gould, 2000). The total volatile basic nitrogen (TVB-N) test, works through the measurement of all the volatile nitrogen compounds present in the samples. It is one of important indexes of the microbiological quality and sensory properties of food. There are good correlation between the changes in the value of trimethylamine (TMA) and bacterial count, storage period, storage temperature, and sensory tests of food (Gray, 1978; Shi *et al.*, 2012). Another form of food spoilage is via a lipid oxidation, which are considered the important factors associated with reduced quality of foodstuffs (Irwin *et al.*, 2004). Thiobarbituric acid (TBA) has been used in the detection of oxidative rancidity development in foods, and its reaction with malondialdehyde is considered an active means of assessing the extent of auto-oxidation (Jardine *et al*., 2002).

The California Milk Advisory Board, (2009) described soft unripened cheese, as a young cheese that has not been allowed to age. They have a shorter shelf life than aged cheeses and are freshness dated. They are typically soft and their flavour close to that of fresh whole milk. Soft cheese must be kept at low temperatures, after tightly wrapped under the conditions to prevention absorbing flavour from other foods. Soft white cheese is cheese with high moisture content and limited shelf life (British Cheese Board, 2014). In this study, soft cheese was manufactured with starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* and packaged under four packaging methods. These methods include air packaging (AP) as control, modified atmosphere packaging (MAP) (gas mixtures composed of 30% CO_2: 70% N_2), vacuum packaging (VP), and brine 3% (w/v) (BP), for investigating the effect of these

methods on shelf life and quality characteristics of the product, by monitoring the microbiological, physicochemical, and sensorial changes. In addition, determining the shelf life for product, through measuring the microbiological characteristics, the TBA value and an odour attribute of product during the storage period for 50 days at 2-5°C.

5.2 Materials and methods

5.2.1 Soft cheese manufacture

Cheese was manufactured following a procedure of manufacture of Iraqi unripened soft cheese, which was employed in dairy factories (non-starter cheese) with starter cultures, as applied in the manufacture of starter soft cheese (SCS), in chapter 4 (section 4.2.2). In soft cheese manufactured in this chapter, salt was added at level 20 g Kg^{-1} curd. The steps of the manufacture of soft cheese illustrated in Figure 5.1.

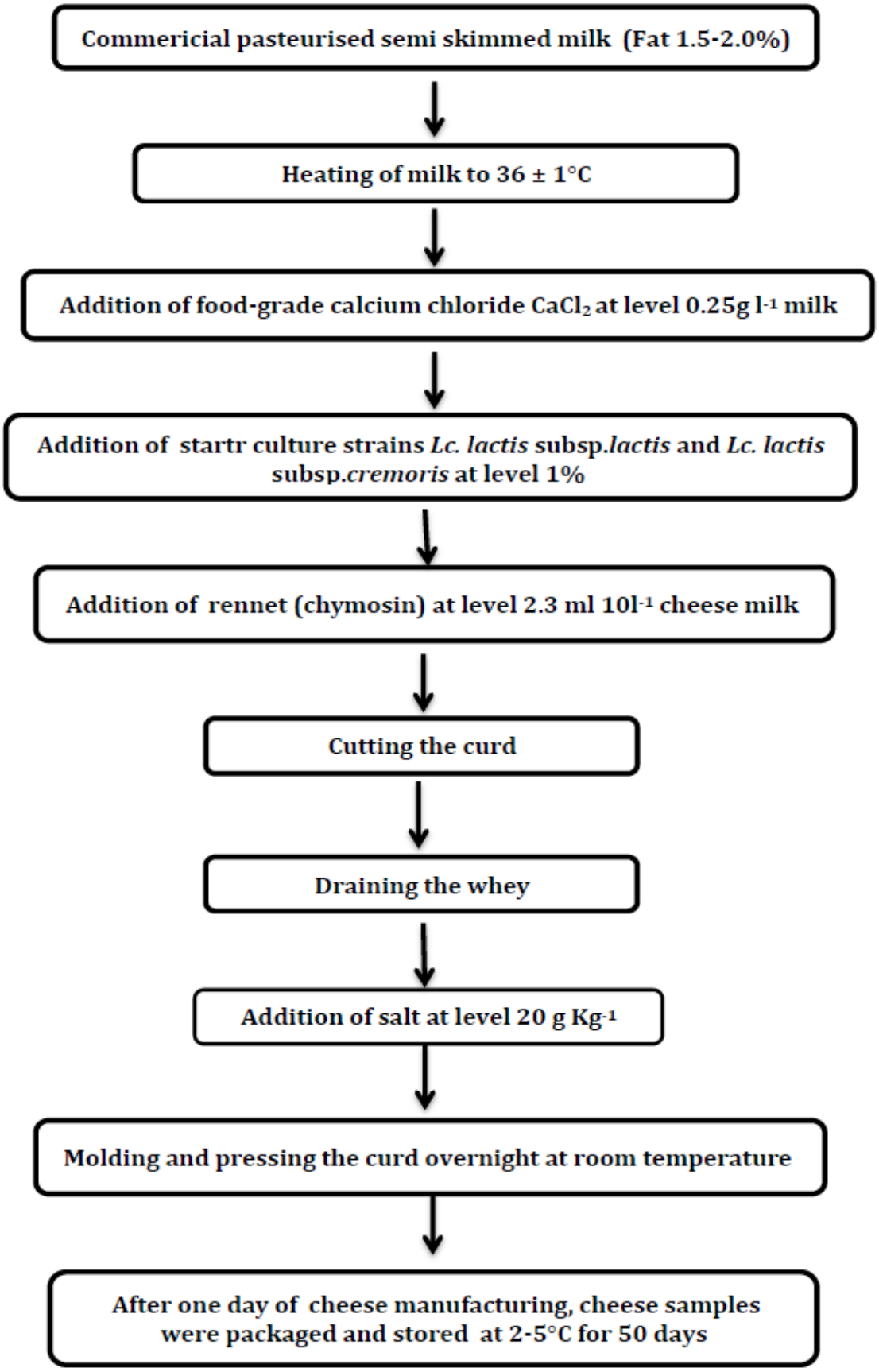

Figure 5.1. A schematic diagram of the manufacture and packaging of starter soft cheese

5.2.2 Packaging of cheese samples

After one day of manufacture (before packaging), the cheese samples were taken for the microbiological, physicochemical, and sensorial analysis, then the cheese samples were packaged under MAP (a mixture of carbon dioxide/nitrogen was flushed into the pouches at level 30% CO_2:70% N_2), vacuum packaging (VP), brine packaging (PB) and samples packaged in air (control samples) (AP). Approximately 200g (12 x 6 x 1 cm^3) of cheese for each packaging method were placed into plastic pouches (20 x 30 cm^2) which were composed of co-extruded polyamides and a polyethylene sealing layer, 70µm in thickness with ± 10% tolerance, with an oxygen permeability 50 cm^3 m^{-2} day^{-1} ATM^{-1} at 75% (RH) relative humidity at 23°C. Pumping a mixture of gas (70% N_2 and 30% CO_2) into the cheese samples under MAP was accomplished using the gas mixer (Boc, Gases, UK). The bags were sealed using Multivac MU 61, Germany for VP and MAP respectively. The same bags were used for control samples (AP) and brine packaging (BP), where after placing the samples of the latter in the sterilized brine 3% (w/v), where both salt and distilled water were autoclaved separately, then the brine was prepared under aseptic conditions. After that, the bags were sealed by using food sealer (Calor 24.03, France). The packaged cheese samples for all packaging were stored at 2-5°C in a cool room (Williams Refrigeration and air-conditioning, installation of general refrigeration, Limited). Periodically after 10, 20, 30, 40, and 50 days of storage, duplicate cheese samples from each treatment were taken for microbiological, physicochemical, and sensory analysis.

5.2.3 Microbiological analysis

The microbiological analysis was carried out on cheese samples after one day of manufacture (before packaging), and during the storage period of 50 days at 2-5°C. From each packaged cheese, samples were taken aseptically for microbiological analysis. Preparation of cheese samples for microbiological analysis, and the count of

MAB, LAB, *Enterobacteriaceae* and M&Y, was carried out as described in chapter 4 (section 4.2.6). The viable count of PB was carried out following the pour plate method on standard plate agar (SPA). Inoculating 100µl of each dilution onto the centre of SPA was made. The plates were left on the bench for approximately 15 minutes, to allow absorption of the inoculum into the agar. Inverting the plates to incubate aerobically for 10 days at 5-7°C, following the method of Hatzikamari *et al.* (1999), was commenced. Colonies were counted and expressed as log cfu g^{-1}.

5.2.4 Physicochemical analysis

5.2.4.1 Lipid oxidation

The oxidation of lipids was estimated using the thiobarbituric acid (TBA) method of Tarladgis *et al.* (1960), with minor modifications (distillation method), using a Vapodest 40 - Programmable distillation unit for Kjeldahl digestion from Gerhardt, Dublin). A 10g sample was stomached, with 80 ml distilled water for 1 min, and then transferred to a distillation flask. The bag used for blending was washed with additional 17.5 ml distilled water, which was added to the same distillation flask with 2.5 ml 4N HCl, and a few drops of the anti-foam agent silicone o/w (Fisher Scientific, Loughborough, UK). The mixture was distilled, and 50 ml distillate was collected. Five ml of 0.02 M 2-thiobarbituric acid in 90% acetic acid (TBA reagent) was added to a vial containing 5 ml of the distillate and mixed well. The vials were capped and heated in a boiling water bath (Grant, England) for 30 minutes for colour development, and cooled to room temperature for 10 min. Then, 3ml of the mixer was dispensed into a cuvette, to measure the absorbance at 532 and 538 nm using a spectrophotometer (Unicom Hexois, spectrophotometer). A blank was prepared, with 5ml distilled water and 5ml TBA-reagent. Thiobarbituric acid-reactive substances (TBARS) were calculated from a standard curve of malondaldehyde (MA) which was freshly prepared through acidify of 1, 1, 3, 3-tetraethoxy propane (TEP) (Sigma-Aldrich,

Poole, UK). The numbers of TBA were expressed as mg malondaldehyde Kg^{-1} cheese sample.

5.2.4.1.1 Preparation of the standard curve

Sixty Six mg of 1, 1, 3, 3-Tetraethoxypropane (TEP) was weighed into a 1L volumetric flask, and diluted to volume with distilled water, in order to produce a 3 10^{-4} M stock solution. This solution was refrigerated at 2-4°C. A 3 10^{-5} M working solution was then prepared by diluting 10ml of the stock solution to 100ml with distilled water. Aliquots of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 ml of working solution were accurately pipetted into 10-ml volumetric flasks, and distilled water were added to the volume. Five ml was pipetted into screw-capped test tubes containing 5 ml of TBA reagent (0.02 M TBA in 10 % glacial acetic acid), and the tubes were placed in boiling water for 15 minutes, and subsequently cooled using water to room temperature immediately. The absorbance of the standards was determined by the wavelength at 532nm and 538nm using spectrophotometer (Unicom Hexois, spectrophotometer), and the first derivative analysis was carried out. A calibration curve was constructed, by plotting the distance between peak and trough of the first derivative spectrum, against the concentration of each of the working standard solutions. A thiobarbituric acid-reactive substance (TBARS) was determined by using the slope and intercept data of the computed least-squares fit of the calibration curve, and expressed as mg malondaldehyde Kg^{-1} cheese.

5.2.4.1.2 Total volatile basic nitrogen (TVB-N) assay by the TCA-extract steam distillation method

Assay of TVB-N was by the method of Malle and Tao (1987). 100g of cheese sample were mixed with 200 ml of 7.5% (v/v) aqueous trichloroacetic acid (TCA) solution. and the mixture was homogenised for 1 min using homogenizer. The homogenized solution was centrifuged using centrifuge (Centaur 2, UK) until clear, and the

supernatant was filtered using paper Whatman no.1. 25 ml of filtrate was transferred into the distillation tube followed by 5ml of 10% NaOH, using a micro Kjeldahl distiller (Gerhard, Vapodest 40, Germany). A mixture of 10ml 4% boric acid, 0.04ml of methyl red, and bromocresol green indicator (0.1% bromocresol green + 0.02% methyl red in methanol) (Sigma-Aldrich, UK), for the titration of ammonia, was placed at the end of condenser. Starting the distillation and steam dispersed continued until a final volume of 50ml was obtained in the beaker (40ml of distillate). The boric acid solution (Sigma-Aldrich, UK) turned green when alkalinised by the distilled TVB-N. which was titrated with aqueous 0.1N sulphuric acid solution (Sigma-Aldrich, UK), using 0.01ml graduated micro burette. Pink colour was obtained when a further drop of sulphuric acid was added. The quantity of TVB-N in mg was determined from the volume of sulphuric acid (Nml) multiplied by a factor 16.8 mg of nitrogen $100g^{-1}$.

5.2.4.1.3 Test of trimethylamine (TMA) using TCA-extract steam distillation method

TCA-extract by steam distillation method was used for the determination of TMA, as carried out in determination of TVB-N. The only difference was the addition of 5ml of 37% of formaldehyde (Sigma-Aldrich, UK) to 24ml of filtrate (Malle and Tao, 1987). Steam distillation was performed as for TVB-N determination. The amount of volatile amines was measured and the results expressed in mg of nitrogen per 100 mg cheese.

5.2.4.1.4 Other physicochemical analysis

The pH value, total solids, fat content and salt content of cheese samples under packaging methods during storage period of 50 days at 2-5°C were detected, as described in chapter 4 (section 4.2.7). Water activity for soft cheese samples were determined each for 10 days, for soft cheese stored under MAP, VP, BP and AP in duplicate after adjusting a Novasina Thermoconstanter, Novasina Aw Sprint TH-500 (Novasina, Switzerland) at 21°C. Cheese samples were placed in a small plastic

container designed for the machine. The water activity of the cheese sample was recorded, when a digital number on the screen of the machine being constant, which needed to be between 45 and 60 minutes of starting the reading.

5.2.5 Sensory evaluation of the odour attribute of cheese

On each day of sampling, sensory evaluation was carried out. according to the International Dairy Federation (1995) standards, as applied by Dermiki *et al.* (2008). A panel of 6 experienced assessors, members of the laboratory of nutrition food microbiology, and postgraduate students, evaluated the soft cheese quality on the basis of odour, using a 0–5 point scale (0 unfit for human consumption, 5 very good, where a score of 4–5 corresponds to quality class I, (no off-flavour), 3.5–3.99 corresponds to class II (initial off-flavour but not spoiled) and a score < 3.5 corresponds to quality class III, (spoiled sample unfit for human consumption). Sensory evaluation was performed on the cheeses before packaging, and during storage shelf life for 50 days at 2-5°C.

5.2.6 Measurement the difference of cheese colour

Measurement of cheese colour was achieved using spectrophotometer CM 2600d (Konica Minolta Sensing. Inc., Japan). Changes in the surface colour of cheese were measured using Commission International d'Eclairage (CIE) L* a* b* colour values (CIE 1976 L* a* b* Colour Space). The test was on the surface of packaged cheese slices after one day of manufacture (before packaging), and after 50 days of storage at 2-5°C. Results were expressed as L* (lightness), a* (redness) and b* (yellowness). Colour differences (CD) represented the differences in L*, a* and b* values of cheese samples after one day of manufacture (before packaging) and after 50 days of storage at 2-5°C under packaging VP, MAP, BP and AP using the following equation

$$\Delta E_{ab^*} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Where ΔL*, Δa*, Δb* are differences in values of *L**, *a**, and *b** of cheese samples after one day of manufacture, and after 50 days at 2-5°C under VP, MAP, BP and AP (Control). Colour difference ΔE_{ab}^* in the *L** *a** *b** colour space, indicated the degree of colour difference, but not the direction. Before each series of measurements, the chromometer was standardized using a white standard plate.

5.3 Statistical analysis

Experimental data were statistically tested by the analysis of variation, by using the Minitab Version 16 (English) software program. One way analysis was applied for variation in colour and odour attributes. Differences between the treatment and the storage means were compared at the 5% level of significance, by using Least Significant Difference (L.S.D). Three replicate samples were analysed for each packaging treatment in each test (n=3).

5.4 Results and discussion

5.4.1 Microbiological analysis

Results for mesophilic aerobic bacteria count are illustrated in Figure 5.2. Control samples, as well as the samples packaged under brine, reached log 7.0 cfu g^{-1} after 30 days of storage, while samples packaged under MAP and VP reached this limit after 50 days. A comparison between these results with the results for Dermiki *et al.* (2008) for cheese ''Myzithra Kalathaki'', stored at 4 ± 0.5°C, these numbers of MAB (log cfu g^{-1})were after 10 days for control and vacuum, and after 24 and 31 days for composed gas 40% CO_2:60% N_2 and 60% CO_2: 40% N_2, respectively. As well as, for results of Papaioannou *et al.* (2007) for Greek Whey Cheese "Anthotyros", stored at 4 °C, the viable count of MAB reached 7 log cfu g^{-1} after 12, 22, and 32 days under VP and MAP, with the gas mixture 30% CO_2:70% N_2 and 70% CO_2:30% N_2, respectively. These effects for MAP have been observed by many of researchers, such as Olarte *et al.* (2002) for Cameros Cheese, stored at 4°C for 28 days, Del Nobile *et al.* (2009) for

Ricotta Cheese, stored at 4°C for 8 days, and Temiz (2010) for sliced Kashar Cheese, where it was stated that the presence of CO_2 in MAP contributed to efficiently decrease these microorganisms in packaged cheeses, and the highest efficiently of CO_2 was at level 100%. PB showed similar growth patterns with MAB. The viable count of theses microbes in cheese samples under AP and BP reached 7.0 log cfu g^{-1} after 30 days of storage, and after 50 days of storage under VP and MAP. Similar results have been reported by some other studies, which used MAP in packaging of various types of cheese, including, Temiz *et al.* (2009) in Turkish whey cheese, where the results showed that MAP delayed the growth of these microbes, when compared with air and VP samples. Too, the results that obtained by Gonzalez-Fandos *et al.* (2000) and Olarte *et al.* (2002) on Cameros soft cheese stored at 3 – 4 °C. It was stated that the presence of CO_2 reduced the growth rate of PB in cheese samples under MAP, and a concentration of 100% CO_2 showed the lowest microbial count in comparison to the gas mixtures 20% CO_2: 80% N_2 and 40% CO_2 : 60% N_2. But, Irkin (2011) found the contrast, which stated that the use of CO_2 at levels 80% CO_2 : 20% N_2, and 60% CO_2 : 40% N_2 in MAP during packaging of unsalted and light Turkish whey cheese "Lor", stored at 4°C for 25 days, did not affect the growth of PB. Furthermore, Maniar *et al.* (1994), stated that the viable count of PB remained in Cottage Cheese with unchanged under MAP with CO_2 at levels 100% CO_2, and with 75% CO_2: 25% N_2. The results, aforementioned, could be explained that the nature of cheese affects the microbiological quality, where the results showed that the viable count of MAB and PB in the cheese of this study were lower than those found in "Myzithra Kalahari" and "Anthotyros" cheeses, although they have been packaged using level of CO_2 higher than those applied in this work without O_2. This is because of the low pH value of cheese under study (pH < 5.0), when compared to the pH value of "Myzithra Kalahari" and "Anthotyros" cheeses (pH > 5.0), through the fermentation of

lactose to lactic acid, by the starter culture, which is considered inhibitory to MAB and PB. The environment of cheese is unsuitable for the growth of MAB and PB, as mentioned by Beresford *et al.* (2001). In addition, the presence of CO_2, contributed to the prevention or delaying their growth (Farber, 1991; Christopher and Hotchkiss, 2002).

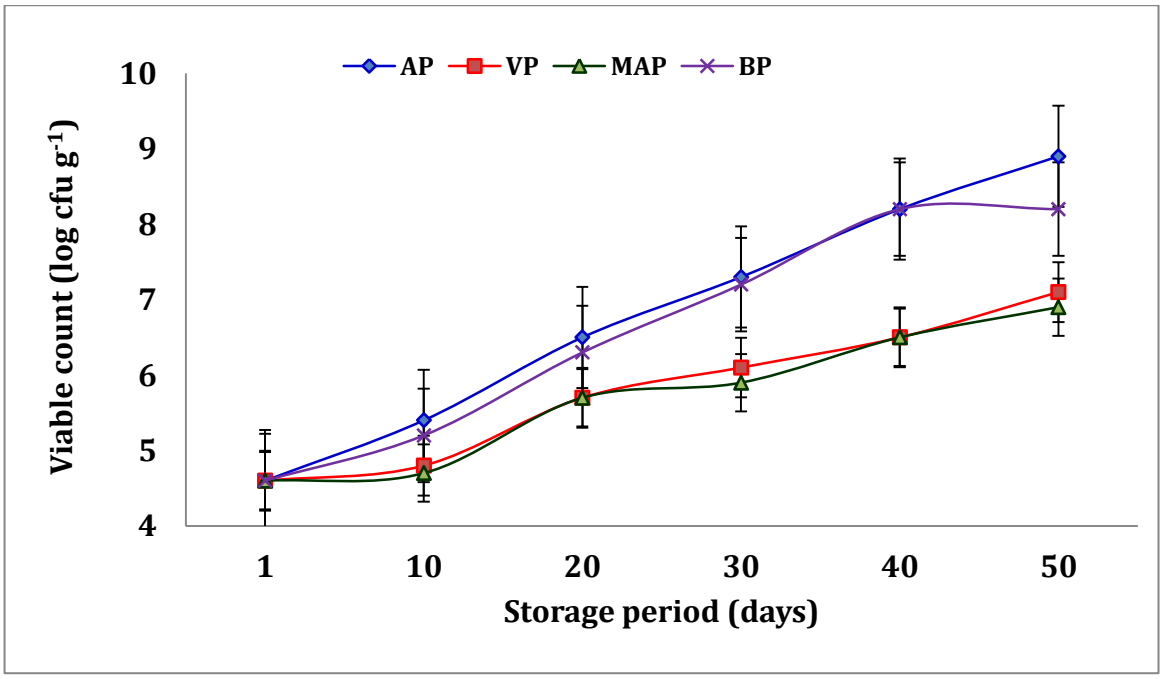

Figure 5.2. Viable counts of MAB (log cfu g^{-1}) in soft cheese samples, after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

Furthermore, it can be explained that the storage of cheese at low temperature increases the influence of CO_2 on aerobic population in cheese samples, as stated by Farber (1991). Through its ability to soluble in water and lipid, the inhibitory effect of CO_2 on the growth of aerobic Gram-negative bacteria could be explained by prolonging the lag phase of growth, and the decrease in the growth rate through the logarithmic phase. The detailed mechanisms on how CO_2 inhibiting the microorganisms, are not fully understood. There are few hypotheses, including, that of Löwenadler, (1994) who stated that the contact of CO_2 with membrane proteins having free amino groups on their surfaces, changes ionic charges from positive to

negative and vice versa. The new charge can cut the transport of specific ions required for maintaining the balance in the cytoplasm such as the exchange of Na+ and K+ ions. In regard to the mechanism of CO_2 and metabolism, CO_2 permeates the membrane and reacts with water in the cytoplasm to form bicarbonate and hydrogen ions. The hydrogen ions acidify the inside of the cell and the organism requires cellular energy to pump the protons back out. The additional energetic needs produces a load on the cells, thus inhibiting their growth. All of the theorized CO_2 mechanisms vary among different bacterial species because species react differently to specific growth requirements in the medium, environmental parameters and substrates. In most cases, the growth rate and lag phase of the spoilage bacteria correlate well with CO_2, while Gram-negative bacteria are more sensitive to CO_2 than Gram positive bacteria are (Devlieghere and Debevere, 2000). Moreover, some results of studies supports the results of the present study, including the results of the study which is carried out by Papaioannou *et al.* (2007) on Anthotyros cheese, which state that the total viable count was higher in cheese samples stored at 12°C, when compared to those stored at 4°C. In this respect, storage of cheese under low and controlled temperature, contributes to increasing the solubility of CO_2, thus increasing its inhibitory activity and preventing or delaying the growth of undesirable microorganisms. At the lower temperatures, the higher CO_2 solubility leads to a greater inhibitory effect of CO_2 (Moir *et al*., 1993; Alves *et al*., 1996; Eliot *et al*., 1998; Gonzalez-Fandos *et al*., 2000; Dermiki *et al*., 2008). Also, a study of Chen and Hotchkiss (1991) on cottage cheese found that the viable count of PB in cheese samples stored at 7 °C was higher than those in samples that were stored at 4°C. Generally, the viable count of MAP and PB under VP and MAP methods were lower than those found in some cheeses, such as Cameros cheese (fresh goat cheese) (Olarte *et al.,* 2002) which were packaged under the levels of CO_2 higher than those used in

this work. In this regards, Parry (2003) mentioned that the gas mixture of 30% CO_2 and 70% N_2 was recommended to its application into MAP during packaging soft cheese.

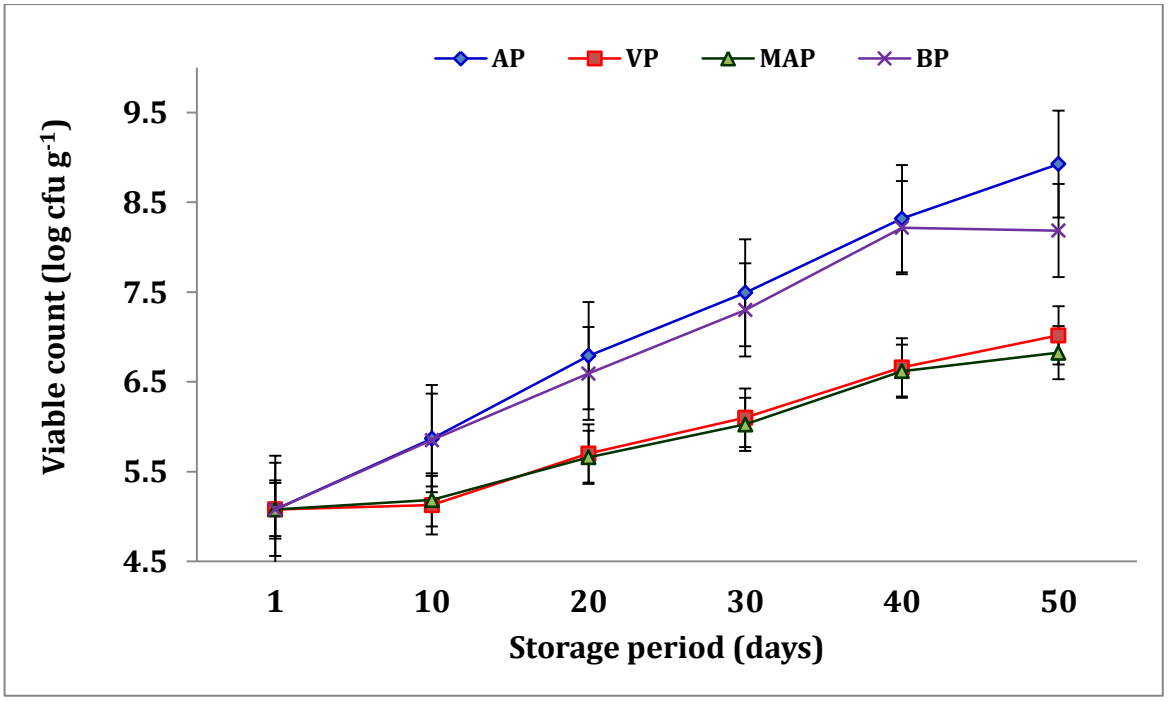

Figure 5.3. Viable counts of PB (log cfu g^{-1}) in soft cheese samples after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

The low pH value of packaged cheese affects the growth of MAB and PB, as reported by Caridi *et al.*, (2003) who stated that the use of selected mesophilic lactococci as starter cultures would control or restrain the growth of unwanted microorganisms. In addition to the absence of oxygen under VP, and the presence of CO_2 under MAP, this has a bacteriostatic effect, especially on Gram-negative aerobic bacteria. Moreover, the controlled low temperature of storage is considered a hurdle with MAP for the growth of these types of microbes. Similar findings have been reported by Moir *et al.* (1993), that found that the CO_2 activity in the inhibition of microorganisms was greater at 5°C, than those at 15°C, and greater at the surface of creamed-style cottage cheese, than those in the depth of the cheese.

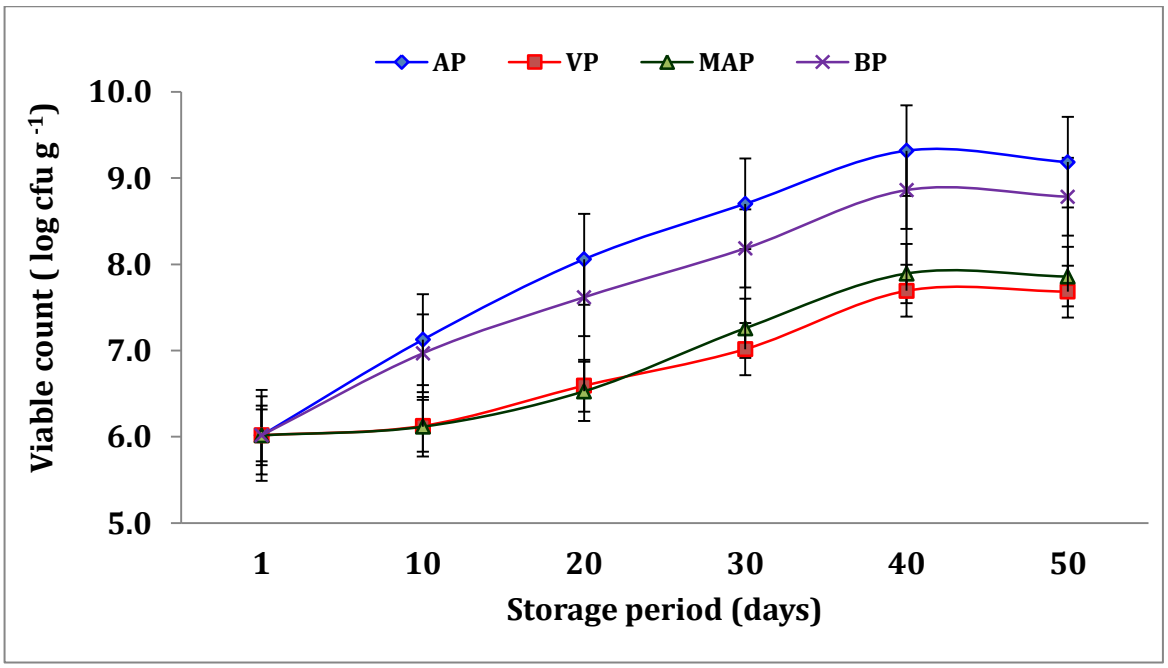

Figure 5.4. Viable counts of LAB (log cfu g^{-1}) in soft cheese samples, after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

In this respect, taking the cheese sample different places of cheese during the microbiological analysis is considered important, with regards to the effect of CO_2 under MAP on the cheese characteristics. and with results of Gonzalez-Fandos *et al.* (2000), which stated that the low temperatures in the storage conditions contributed to the enhancement of CO_2 for inhibiting microorganisms in Cameros soft cheese when stored at 3-4°C. On the other hand, during the storage of a traditional Greek whey cheese at 4°C for 28 days, Lioliou *et al*., (2001) found that the microorganisms developed better on the surfaces than in the interior.

With regards to the count of LAB (Figure 5.4), the viable counts of these microorganisms in cheese samples under AP and BP had increased by 3.17 and 2.79 log cfu g^{-1}, respectively, in comparison with the VP and MAP. The viable counts were increased by 1.66 and 1.84 log cfu g^{-1}, after 50 days of storage, respectively, without any significant differences between AP and BP, and between VP and MAP. In this respect, Coppola *et al.* (1995), stated that the use of MAP did not inactivate these beneficial dairy bacteria, suggesting the use of this as part of the marketing of these

dairy products the existence of "preservative-free and rich in viable lactic acid bacteria". Similar results by Papaioannou *et al.* (2007) for Anthotyros cheese and by Dermiki *et al.* (2008) for Myzithra Kalathaki cheese, show that viable counts of LAB did not differ significantly under MAP during storage for 45 days at 4±0.5°C when comparison to levels found in VP cheese. These facultative anaerobic microbes have the ability to grow under VP and MAP. Gammariello *et al.,* (2009a), mentioned that the storage of Apulian fresh cheeses using MAP conditions did not affect the growth LAB during the storage period 8 days at 8°C, while Maniar *et al*., (1994) found that the viable count of LAB in Cottage cheese, packaged under MAP using 100% CO_2, 75%CO_2:25%N_2 and 100% N_2, remained unchanged during the storage period 28 days at refrigerated conditions. The present results are similar to those by Conte *et al*., (2009), who reported that the viable count of LAB in Fior di Latte soft cheese not affected by the MAP method, and suggested that the selection of MAP conditions (30% CO_2, 5% O_2, and 65% N_2) for packaging soft cheese do not impact the growth of typical dairy microorganisms.

The viable count of *Enterobacteriaceae* in cheese samples under all packaging conditions was (< 1.0), after 10 days of storage, and remained at this limit until end of storage after 50 days (Figure 5.5). The viable count of microbes in this work was lower than those found in some cheeses, such as Greek whey cheese (Papaioannou *et al.*, 2007), when the range of these microbes after 36 days of storage at 4°C under air packaging and under MAP with CO_2:N_2 30%:70% and CO_2:N_2 70%:30%, were between 4.0 and 7.0 log cfu g^{-1}, whey cheese (Dermiki *et al.*, 2008) were between 5.3 and 5.6 log cfu g^{-1} in all treatments used, and Ricotta cheese (Del Nobile *et al.*, 2009) were between 4.0 and 7.0 log cfu g^{-1}. The results reflects the application of good manufacturing practices and the conditions during the manufacture and storage of this product, as mentioned in the report of a Health Protection Agency (2009).

On the other hand, Gonzalez-Fandos *et al.* (2000), observed that the samples of the Fresh Cameros cheese under vacuum packaging have a higher count of *Enterobacteriaceae* than those observed in samples that were packaged under CO_2, and Gammariello *et al.* (2009a) found that the viable count of these microbes in fresh cheeses Giuncata and Primosale significantly decreased under the combination made up of CO_2 and N_2 75%:25%.

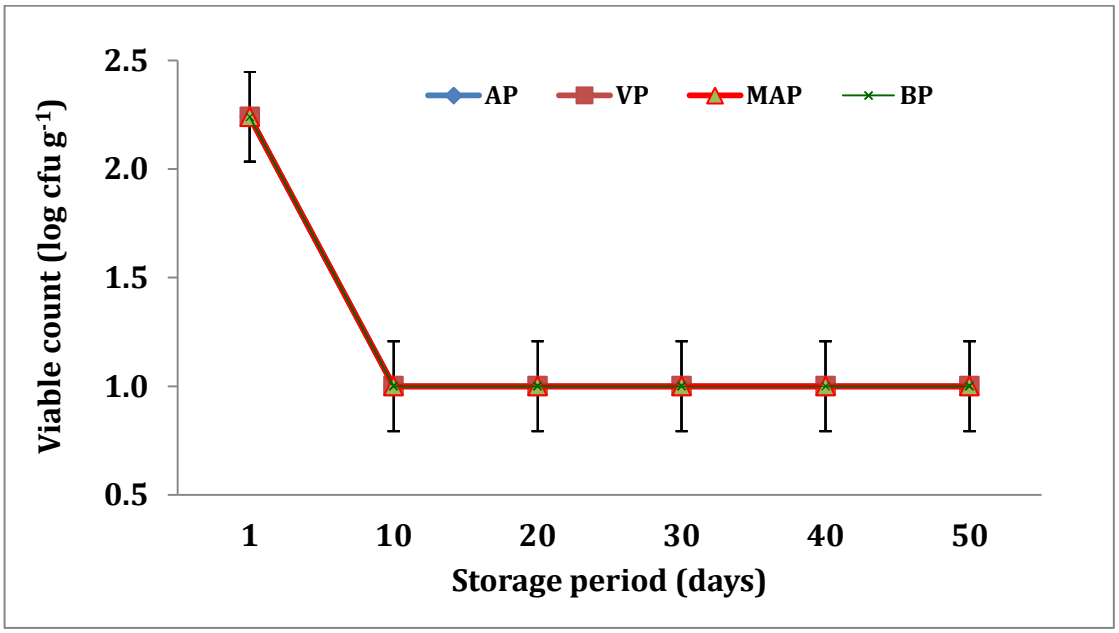

Figure 5.5. Viable counts of *Enterobacteriaceae* (log cfu g^{-1}) in soft cheese after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se)(n=3).

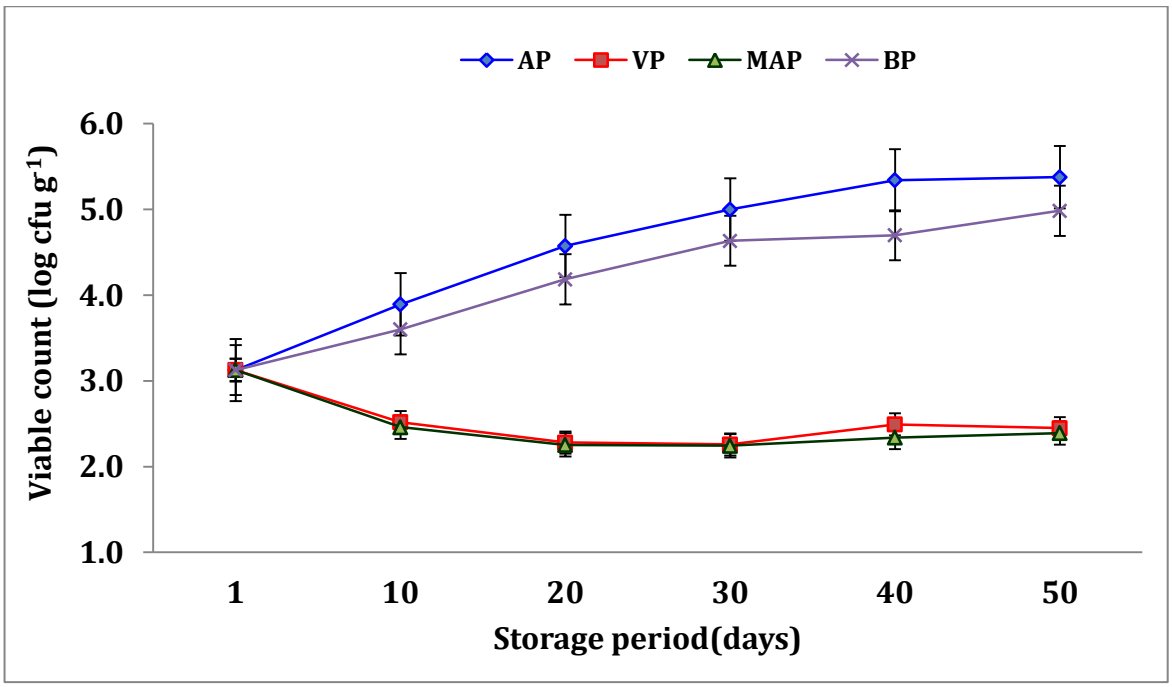

Figure 5.6. Viable counts of moulds and yeasts (log cfu g^{-1}) in soft cheese samples after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

With regards to moulds and yeasts group, which are considered to be important microorganism contaminants in dairy products (Beresford *et al.*, 2001), the viable M&Y count is shown in Figure 5.6. Data show that the count of M&Y in cheese samples before packaging was 3.1, and after 50 days of storage had reached 5 log cfu g^{-1}, and the average during the storage period was > 4.0 log cfu g^{-1} in AP and BP cheese samples (Figure 5.6), whilst in cheese samples under VP and MAP, the viable count decreased to below 3 log cfu g^{-1} after 10 days of storage, and remained at this level until the end of storage with average less than 3.0 log cfu g^{-1}.

The viable count of M&Y in cheese samples under AP and BP was higher than those found in cheese samples under VP and MAP. The suitable environment under AP and BP was compared with those under VP and MAP, with regards to the availability of O_2, which is considered one of essential requirements for the growth of these microbes. These results are similar to the findings of some researchers in this regards, including, Sarais *et al.* (1996) in Stracchino soft cheese, Gammariello *et al.* (2009a) in fresh

cheeses like Giuncata and Primosale, Temiz (2010) in sliced Kashar cheese, Irkin (2011) in unsalted and light Turkish whey cheese "Lor", and Kirkin *et al.* (2013) in pre-cut white cheese, where they found that under different temperatures and periods, inhibition of these microbes under MAP took place by using CO_2 at levels ranging between 20% and 100% with N_2, as well under VP. The use of MAP with 30% CO_2:70% N_2 gas mixture and VP, was efficient in inhibiting the growth of M&Y, and the major effect of CO_2 on cheeses samples with thickness up to 1.0 cm under MAP is the inhibition of surface mould growth. Also, the viable counts of M&Y in BP were at lower level than in AP, possibly because the exposure of these microbes to salt, which is expected to occur in the surface. Linked to this, Kizilirmak *et al.* (2009), found that for samples of goat cheese (Crottin de Chavignol) kept for 15 weeks on storage at 4°C at VP and MAP (20% CO_2 : %80 N_2 gas mixture) mould viable counts increased by 3.8 log cfu g^{-1} and 2.3 log cfu g^{-1}, respectively.

5.4.2 Physicochemical analysis of cheese

The average total solids (%) of cheese increased after the storage 50 days at 2-5°C in cheese samples under all packaging methods, in comparison to the cheese samples before packaging (TS% was measured before packaging and after 50 days of storage under VP, MAP and AP). Total solids in cheese samples under MAP differed significantly with the AP samples (Figure 5.7). Similar findings have been obtained by Gonzalez-Fandos *et al.* (2000) in Cameros cheese and Temiz *et al.* (2009) for Turkish whey cheese.. There were no differences in salt content for these cheese samples under AP and MAP, where the concentration in cheese sample before packaging was 1.27± 0.03%, and it increased after 50 days in samples under AP, BP, VP and MAP to 1.571 ± 0.04%, 2.004 ± 0.02%, 1.636 ± 0.04% and 1.642 ± 0.03%, respectively.

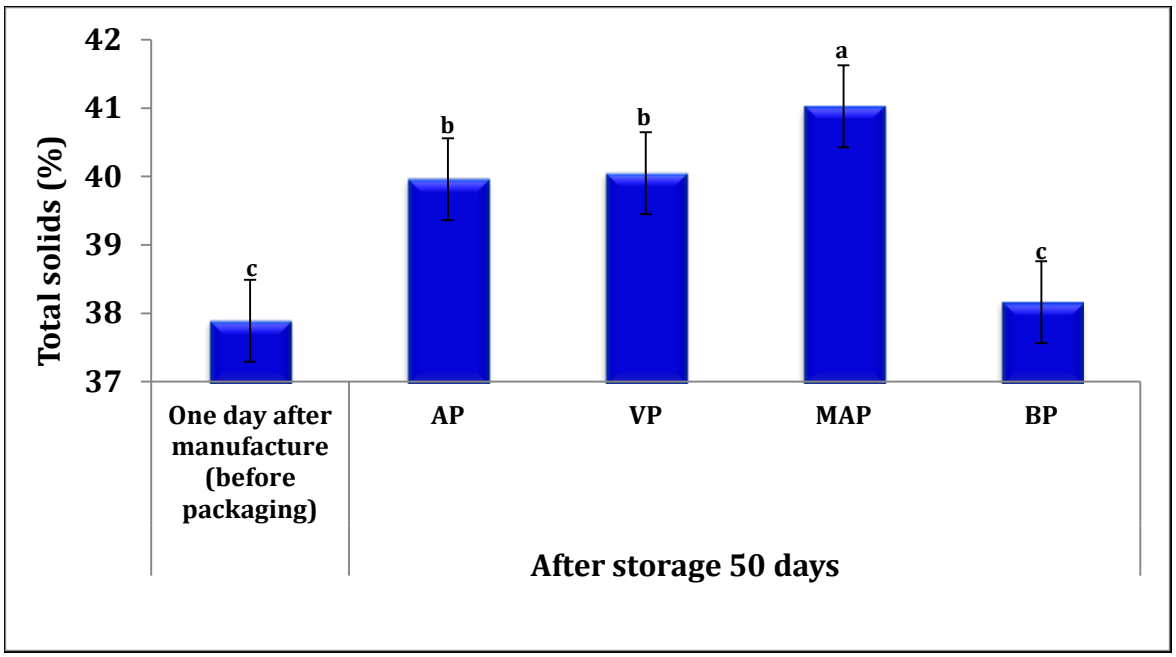

Figure 5.7. Total solids (%) in soft cheese after one day of manufacturing (before packaging), and after 50 days of storage under AP, VP, MAP and BP at 2-5°C (mean ± se) (n=3).

Similar findings have been obtained by Papaioannou *et al.* (2007) and Favati *et al.* (2007) in Greek whey cheese and Provolone cheese respectively. Cheese samples under BP differed with the samples under AP, MAP and VP in their contents of salt (Figure 5.8). This was because of the brine content of salt, whose diffusion to cheese samples and increases the salt the S/M in the cheese under brine packaging. The initial pH value of cheese before packaging was 4.92 ± 0.01, decreased significantly in cheese samples under all packaging methods, without the difference between samples under AP and other systems during storage for 50 days at 2-5°C. The mean value of pH in cheese samples under AP after 50 days of storage was 4.52 ± 0.02 and 4.50 ± 0.017 in cheese samples under MAP (Figure 5.9). The fat contents (%) in cheese samples before packaging and the cheese samples under the brine and air packaging after 50 days were 15.67 ± 0.333%, 16.250 ± 0.333%, 16.280 ± 0.152%, respectively, where the fat content (%) was measured before packaging and after 50 days of storage in samples under BP and AP.

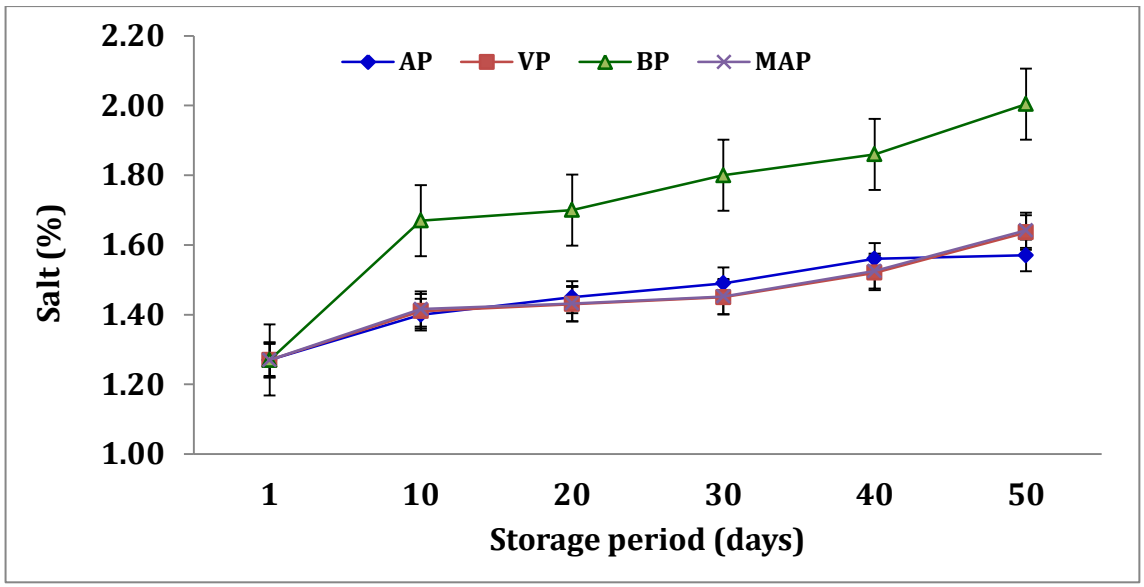

Figure 5.8. Salt content (%) in soft cheese after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

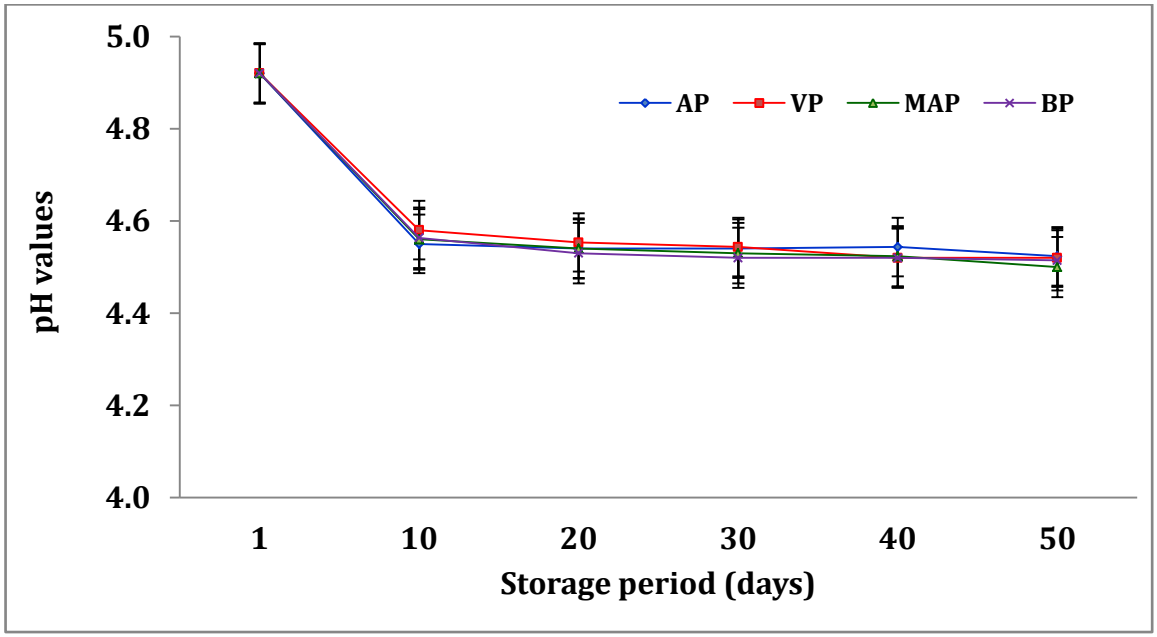

Figure 5.9. pH value of soft cheese after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

The results indicates that the CO_2 does not significantly affect the pH value of cheese samples under MAP, in comparison to the cheese samples under AP, this is in line with the results of Moir *et al.* (1993) in Cottage cheese. Although the environment in VP and MAP are both unsuitable for the growth of starter cultures, but the pH value drop in cheese samples may be through the contribution of LAB because of their

abilities to grow under these methods, due to their facultative characteristics. Similar results have been found by Whitley *et al.* (2000) and Dermiki *et al.* (2008), which found that there was a relationship between the growth of LAB and the pH value of cheese under MAP. On the other hand, the pH value of cheese samples under VP and MAP was nearest to the pH value of samples under AP and BP. The reason was maybe the high levels of M&Y that were present in the cheese samples under AP and BP, and which could be consuming a part of the lactic acid that was produced by the starter cultures and the LAB, in comparison to the low levels of these microorganisms under VP and MAP. Or, it may be because of the absorption of CO_2 on specific spots on the cheese surface, rather than the depth or the cheese matrix totally.

Most foods have a water activity level in the range of 0.2 for very dry foods to 0.99 for moist fresh foods. For foods with relatively high water activity, such as soft cheeses the correct refrigeration is always necessary. Chirife *et al.* (1996) stated that a_w has several limitations and should always be used carefully, and this must include precautions regarding the possible influences on non-equilibrium situation in food. It is considered to be an important measurement of the capacity of various microbes to grow in a particular food, in addition, the pH and temperature of food storage also control the growth of microorganisms.

The mean value of a_w range between 0.954 ± 0.004 and 0.970 ± 0.003. Significant differences ($P<0.05$) between control and the other packaging systems were detected (Figure 5.10). The a_w in cheese samples under all packaging methods was in high level, this was because of the low concentration of salt that was used in cheese, which adversely correlated with a_w, and this is in line with the report of Esteban and Marcos (1989), and similar findings have been reported by Fox *et al.* (2000b), which stated that the salt content in cheese played an important role in the determination of a_w in

cheese, and the control on the growth of microorganisms, in addition to the effect on the enzyme activities.

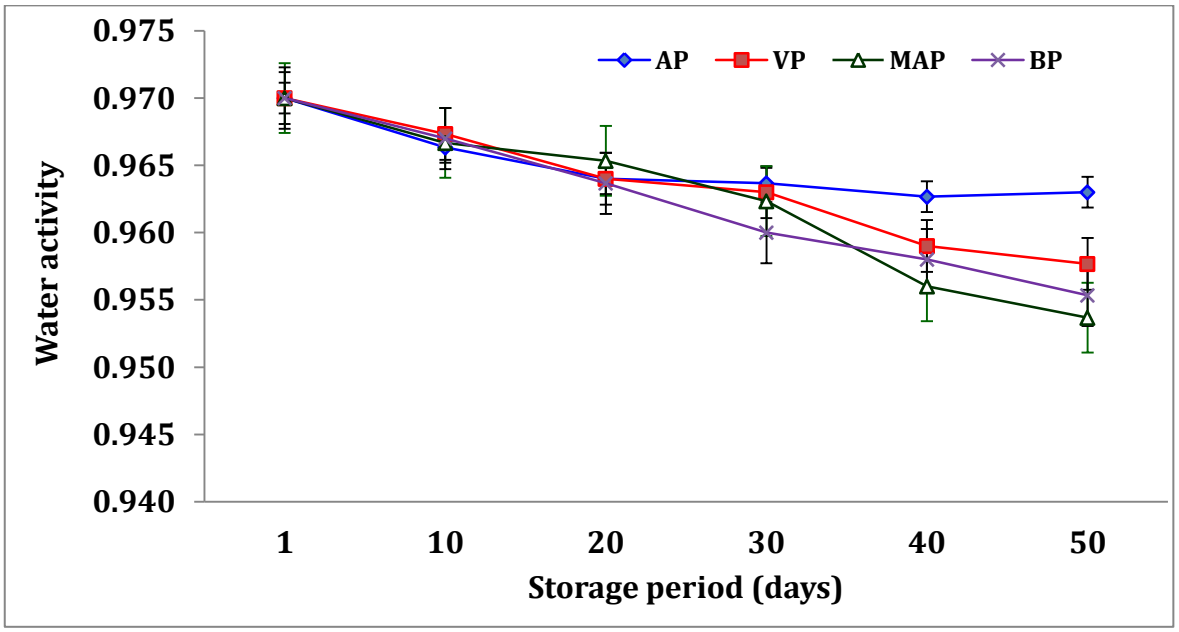

Figure 5.10. Water activity value in soft cheese samples after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se) (n=3).

The mean of a_w in the cheese samples under all packaging was at a minimum critical level of the deterioration of food, through the growth of spoilage microbes. But the level of a_w in cheese samples under VP, BP and MAP was lower than those in samples under AP. Decrease in the storage temperatures, dropping the pH and a_w values of cheese, in addition to the salt used, contributed to retard or prevent the growth of spoilage microbes and some pathogens, as reported by Petruska *et al.* (1990) and Guinee (2004). Organic acids, such as acetic acid and lactic acid are more effective as preservatives in the undissociated state. Lowering the pH of cheese increases the effectiveness of an organic acid as a preservative. The pH can interact with factors such as a_w, salt, storage temperature, redox potential of cheese, and preservatives to inhibit growth of pathogens and other organisms (Mossel. *et al.*, 1995).

5.4.2.1 Thiobarbituric acid value (TBA)

The values of thiobarbituric acid-reactive substances (TBARS) in cheese samples under packaging methods MAP, VP, BP and AP, which were measured, as MA mg Kg^{-1} sample are shown in the Figure 5.11. The storage period affected the cheese content of TBARS significantly, one day after the manufacture of cheese, and after 50 days of the storage at 2-5°C. The mean value of TBARS in cheese samples before packaging was 0.025 ± 0.002 MA mg Kg^{-1} cheese, and after 50 days of the storage, the value was 0.064 ± 0.002, 0.038 ± 0.002, 0.031 ± 0.002 and 0.051 ± 0.031 MA mg Kg^{-1} cheese under AP (control), MAP, VP and BP, respectively. Off-flavour in the cheese, results from deterioration of fat, through the reactive atmospheric oxygen, i.e. oxidative rancidity, or through hydrolytic reaction, catalyzed by lipases from food or microorganisms. The low levels of MA which was produced by Oxy-, and/or lipid free radical generation in cheese samples, indicates to the low degree of deterioration of lipids in the product. Assessment of TBARS is a commonly used method for the detection of lipid peroxidation. This assay is a well-recognized, established method for quantifying these lipid peroxides, although it has been criticized for its reactivity towards other compounds other than MA. MA is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products and reacts with TBA to form a pink pigment that has an absorption maximum at 532nm. Other compounds also react with TBA to form colored species that can interfere with this assay, but little is known about these interfering species (Trevisan *et al*., 2001; Jardine *et al*., 2002). Storage of cheese under all packaging in a dark place, contributed to the formation of low levels of TBARS during their storage for 50 days at 2-5°C, because all types of cheese have sensitivity to light, which causes unwanted changes in their sensory properties of cheese, such as flavour, odour and appearance.

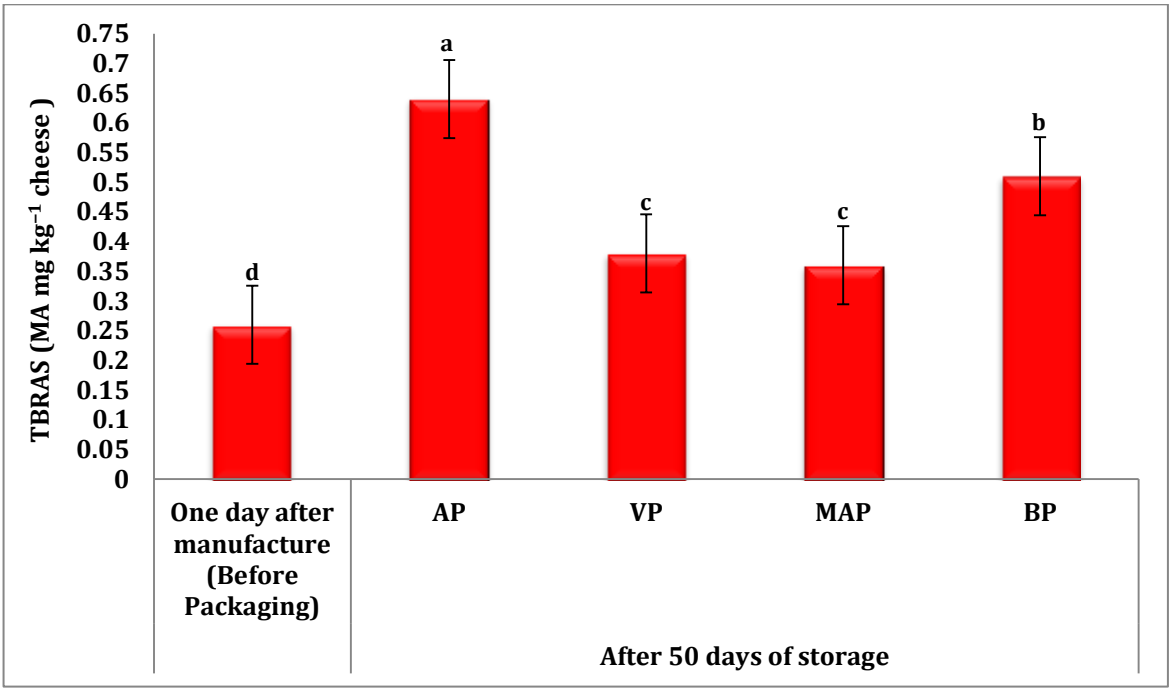

Figure 5.11. TBARS level (MA mg Kg^{-1} cheese) in cheese samples after one of manufacturing (before packaging), and after 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se)(n=3).

Similar results have been obtained by other researchers, including, Kim *et al.* (2003), which observed that the cheese samples of Goat cheese stored under light has significantly more off-flavour, than those samples stored in the dark place,Juric *et al.* (2003), it was stated that the storage of Sliced Samsø cheese, stored up to 3 weeks, and exposed to light (1500 lx), negatively affected the colour of the product. In same trend, Mortensen *et al.* (2004a), it was mentioned that the major quality changes in cheeses under packaging, such as physicochemical changes, are due to oxidative reactions. The low redox potentials in cheese restrain lipid oxidation. Furthermore, the presence of some natural antioxidants in cheese, such as Vitamin E, makes only a small contribution to the development of cheese flavour (Fox and Wallace, 1997; McSweeney and Sousa, 2000; Trobetas *et al.*, 2008). The level of MA in control samples was higher than those found in cheese samples under VP and MAP under cold conditions, where VP and MAP play an important role in reducing or slowing

down the oxidation rate through the removal of air (Dawson and Gartner, 1983; Ladikos and Lougovois, 1990; Nawar, 1998). In this regards, Deeth (2006a) and Deeth and Fitz-Gerald (2006b), stated that lipolysis in milk and milk products causes rancid off-flavours and other problems, and is a constant concern in the dairy industry. The major microbial lipases are produced by psychrotrophic bacteria and have heat resistance characteristics. In general, the oxidation of cheese is a process that must be avoided, however, the process occurs naturally when the product is exposed to air and it is potentiated by heat, light, chemical catalysts or enzymatic actions. In addition, the oxidative changes cause development of off-flavours, loss of nutrients, and even the formation of potentially toxic compounds originating products, which are considered harmful for human health (Santos-Fandila *et al*., 2014).

5.4.2.2 Total volatile basic nitrogen determination (TVB-N)

The levels of TVB-N as nitrogen in the packaged cheeses under AP, VP, MAP and BP conditions ranged between 6.827 ± 0.092 before packaging, and 11.594 ± 0.232 mg 100 g^{-1} cheese under air packaging, after 50 days of storage at 2-5°C. In the cheese samples under VP, MAP and BP, after 50 days of storage were 9.547 ± 0.095, 8.177 ± 0.193 and 5.321 ± 0.015 mg 100 g^{-1} cheese, respectively (Figure 5.12). After 50 days of storage, TVB-N levels in cheese under control (AP) differed significantly with the packaged cheeses under VP, MAP and BP, as well, with cheese before packaging.

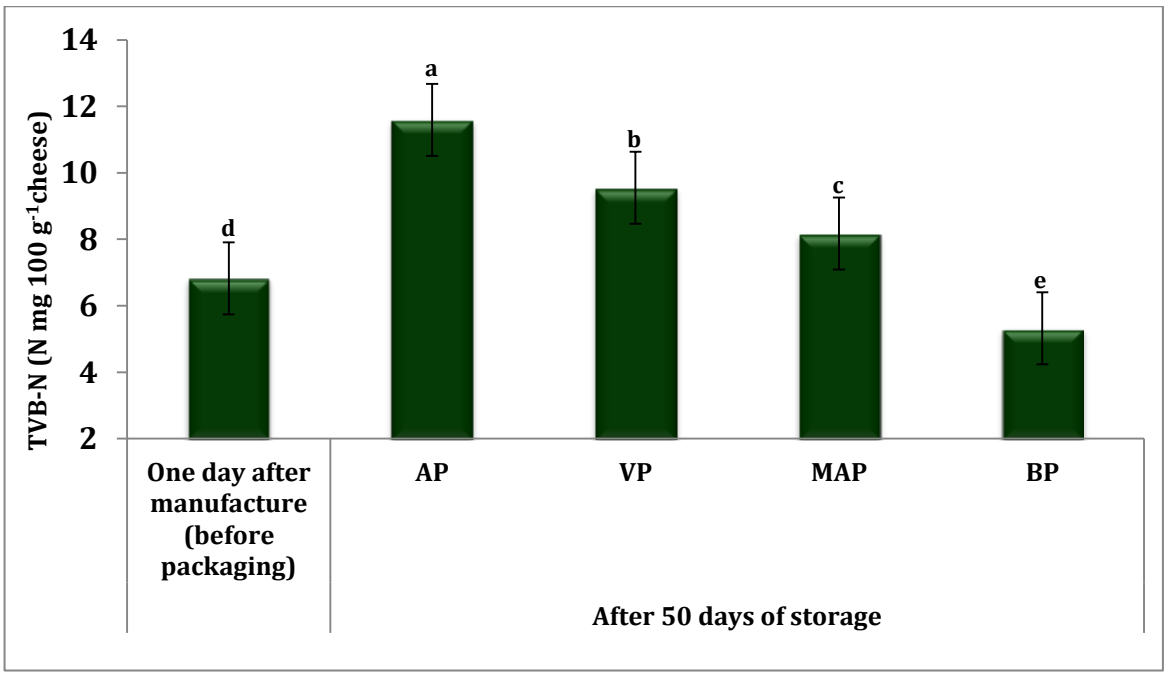

Figure 5.12. Levels of TVB-N (N mg 100 g-1 cheese) in cheese samples after one day of manufacturing (before packaging), and after 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se)(n=3).

The levels of TVB-N in all packaged cheeses were at low levels. The low temperature of storage contributed to slow the microbial activities in cheese and decrease the biogenic amines quantities, which significantly correlated with TVB-N quantities. As well as, these levels did not affect the colour or flavour of the cheese, further reinforcing that the low levels of microorganisms are insufficient in the occurrence of cheese spoilage. These results similar to that reported by Dondero *et al.* (2004), which reported that the low temperatures of storage positively affected the quality and sensory properties of vacuum-packed food products. But, researchers mentioned that the biogenic amines, moulds and yeasts are considered non-objective indicators for assessing sensory quality of foods. On the other hand, the report of Shi *et al.* (2012) stated that the low temperatures contributes to the preservation of the food quality through inhibition of the levels of biogenic amines, which correlated significantly with the levels of total volatile base nitrogen.

5.4.2.3 Trimethylamine determination (TMA)

Biogenic amines have unwanted effect in humans. Although, there is no specific rule about the biogenic amines content in dairy products, it is generally expected that they should not be permitted to accumulate(Linares *et al.*, 2011). Data of TMA value in the cheese samples indicates to the significant difference ($P < 0.05$) between the samples before packaging, and after 50 days of storage at 2-5 °C, and between the cheese samples under VP, MAP and BP, and the samples under air packaging (AP).

As shown in Figure 5.13, the levels of TMA as nitrogen in cheese samples before packaging were 2.745 ± 0.209 mg 100 g^{-1} cheese, and after 50 days of the storage under AP, VP, MAP and BP were 7.165 ± 0.160, 5.208 ± 0.137, 5.096 ± 0.158 and 3.376 ± 0.201 mg 100 g^{-1} cheese, respectively. Formation of trimethylamine (TMA) in cheese is, through the decomposition of proteins by activity of enzymes amino acid decarboxylase that are produced by bacteria, which is used as an indicator to check the spoilage in foods, such as cheese (Santos, 1996; Bodmer *et al.*, 1999). In addition, TMA is a good target for the detection of biogenic amines, because of its volatility (Bota and Harrington, 2006) and cheese is one of fermented food that could be exposed to contamination during the manufacture or storage, therefore, it is likely to contain amines, as stated by (Shalaby, 1996).

The low levels of TMA in cheese samples under all packaging explains that the decomposition of proteins in cheese by the presence of bacteria does not occur in considerable levels during the storage period at 2-5°C. The low degree of storage temperature and the pH value of cheese contributed to a slowdown of TMA levels in cheese. Similar results have been reported by Shi *et al.* (2012).

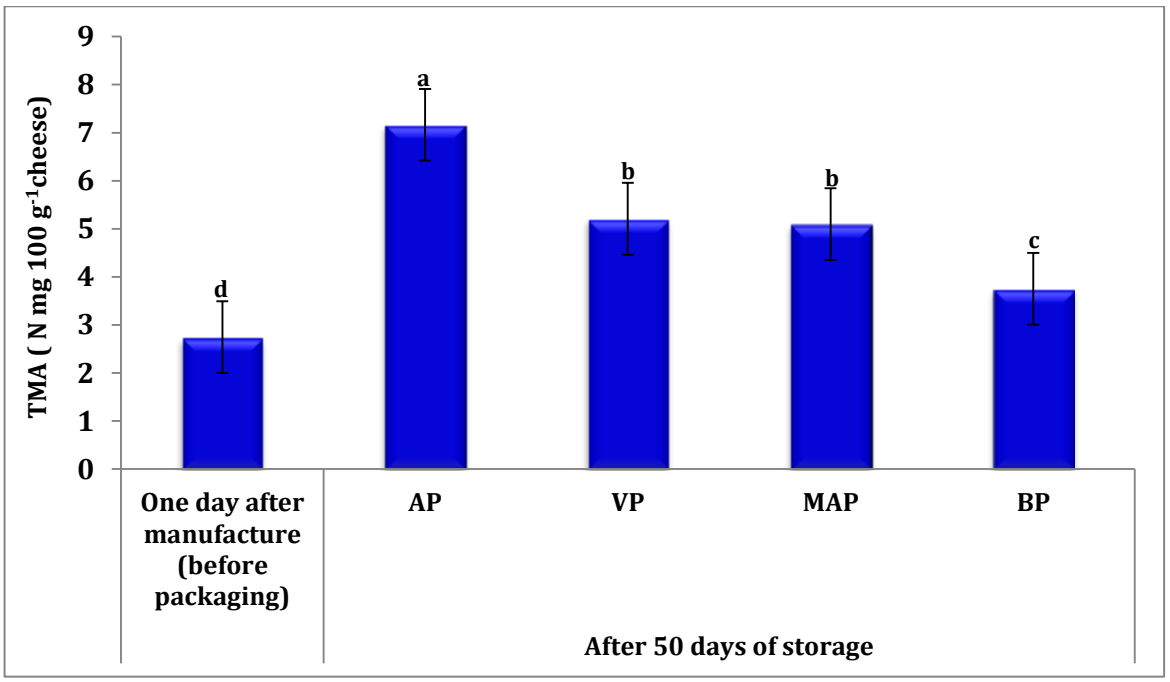

Figure 5.13. TMA values (N mg 100 g^{-1} cheese) in cheese samples after one day of manufacturing (before packaging), and after 50 days of storage at 2-5°C under AP, VP, MAP and BP (mean ± se)(n=3).

Specific indicators, such as fishy odour, which is clear in the case of presence of low levels of TMA, and odour like ammonia, in case of presence of high levels of TMA in a food sample. The low levels of TMA in the packaged cheeses reflect the good correlation between the changes in TMA value with the viable count of bacteria, storage period, storage temperature, and the sensory tests of food (Dondero *et al.*, 2004). On the other hand, the low viable count of *Enterobacteriaceae* in cheese samples under all packaging methods, explains that these microbes contributes to producing some BA, such as histamine, which negatively affect the quality and nutritional value of the product, as mentioned by Martuscelli *et al.* (2005), which observed that the presence of *Enterobacteriaceae* strains in Pecorino Abruzzese cheese have the ability of production of BA. On the other hand, the team found that the presence of specific precursor of amino acids is of relevance to the quantity and quality of BA in cheese. As well, the enzymatic activity of proteases, produced by microorganisms or another source, affects the quality of BA in cheese. In this respect, Pereira *et al*., (2001) suggested that the production of biogenic amine were derived

from decarboxylation of free amino acids arising from catabolism of lactic acid, for example, the decarboxylase enzymes of *Lactobacillus* spp., should be borne in mind during the selection of microorganism for potential probiotic use due to the potential for exciting problems in host health. Immoderate intake of biogenic amines may cause toxicological effects, such as hypotension or hypertension, headache, allergic reactions and nausea (Shalaby, 1996).

5.4.2.4 Colour of soft cheese

Data shows that there are significant differences ($P < 0.05$) in the redness, with slight differences in the yellowness of the sample under AP, VP, MAP and BP (Table 5.1). Colour differences (ΔE_{ab}^*) between cheese samples after one day of manufacture (before packaging) and cheese samples after 50 days of storage at 2-5°C, under AP, VP, MAP and BP were 1.565, 0.574, 0.730 and 1.577, respectively. These differences in the colour attribute of cheese samples under these packaging methods explains that the VP and MAP have a close effect on the colour of cheese samples after the packaging process and storage for 50 days. After 50 days of storage, the differences in colour (ΔE_{ab}^*) between the samples under AP and VP, between samples under AP and MAP, between samples under AP and BP, between samples under VP and MAP and between samples under BP and MAP were 0.518, 0.521, 1.870, 1.097, and 2.15 respectively (Table 5.2). There were increases in L^* value (lightness) in samples under BP, with an increase in a^* values (redness) in cheese samples under VP and MAP, and increasing in b^* value in cheese samples under AP.

Table 5.1. Colour parameters of cheese after 50 days of storage under AP, VP, MAP and BP at 2-5°C (mean ± se) (n=3)

Colour space	Packaging methods			
	AP	VP	MAP	BP
L	16.270 ± 0.06[a]	16.600 ± 0.035[a]	15.767 ± 0.020[a]	17.282 ± 0.020[a]
a	0.740 ± 0.012[c]	0.827 ± 0.051[b]	0.870 ± 0.020[a]	0.829 ± 0.008[b]
b	15.840±0.048[a]	15.100 ± 0.035[a]	15.800 ± 0.012[a]	14.837 ± 0.029[b]

L = Lightness; a = Redness; b=Yellowness; AP = Air packaging; VP = Vacuum packaging; MAP = Modified atmosphere packaging; BP = Brine packaging. Mean values in the same row bearing the different superscripts differ significantly (P <0.05).

Table 5.2. Colour differences (ΔEab*) between cheese samples under AP, VP, MAP and BP after 50 days of storage at 2-5°C

Colour differences	Packaging methods				
ΔE_{ab}^*	AP:VP	AP:MAP	AP: BP	MAP:VP	MAP: BP
	0.518	0.521	1.870	1.097	2.150

CD = Colour difference, AP: VP = CD between AP and VP; AP: MAP = between AP and MAP; AP: BP = between AP and BP; MAP:VP = between VP and MAP; BP:MAP = between BP and MAP. AP = Air packaging; VP = Vacuum packaging; MAP = Modified atmosphere packaging; BP = Brine packaging.

These results are in line with the results obtained by Kristensen *et al.* (2000), Kizilirmak *et al.* (2009), Del Nobile *et al.* (2009) and similar findings have been obtained by Favati *et al.* (2007) in relation to the packaging of cheese under AP, VP and MAP. As storage of cheese samples under all packaging was under dark conditions, in this respect, the results of TBA in cheese samples confirm these results in relation to effect of light on the colour of cheese under dark or light conditions, through their effect on the photo-oxidative reactions in the product (Alves *et al.*, 2007; Trobetas *et al.*, 2008). Similar results have been reported by Ozlem *et al.* (2009), which found no significant differences between the colour of "Crottin de Chavignol" under MAP (CO_2:N_2) (20:80) and AP, and between VP and AP after 15 weeks at 4°C, and Kristensen *et al.* (2000), which studied the effect of light on sensory properties of sliced Havarti cheese, which were packaged using various atmospheres, and the researchers found that the storage of cheese under light contributes to an accelerated deterioration in the odour and taste of cheese, and an increment in the rancid and sour attributes. Thus, protection of soft cheese from light during the storage period contributes to maintaining the quality and sensory properties of the product.

5.4.2.5 Odour of cheese and shelf-life

The results of sensory evaluation of odour attribute of soft cheese stored at 2-5 °C for 50 days under AP, VP, MAP, and BP are present in Figure 5.14. Data shows that the odour attribute of cheese samples under all packaging recorded scores between 3.0 and 5.0, which corresponds to the products at class 1. The storage period significantly affected the odour attribute of cheese under AP and BP after 30 days of storage. Considering a score of 3.5 as corresponding to the end of the shelf life of cheese under air packaging, Figure 5.14 shows that the cheese samples under air packaging and brine packaging change from class 1 (very good) to class 11 (good) (initial off-flavour, but not spoiled) after 30 days of storage, and this was retained until the end of storage, whilst the samples under VP and MAP stayed at class 1 after storage of 50 days. The quality of cheese samples under VP and MAP maintained very good sensory characteristics, and were better preserved than AP and BP, where they remain as class 1 (non-off flavour) until the end of storage. Overall, fishy or ammonia flavour was not recorded in the comments field in the ballot sheet by the panelists, under all packaging methods, including the cheese samples under AP and BP. Cheese samples under AP and BP, although their class changed from class 1 to class 11, remained non-spoiled for human consumption. The situation of cheese contributed to maintaining the quality of the product for a marked amount of time longer, when compared with other soft cheeses, such as “Anthotryros” cheese (Papaioannou *et al.*, 2007), which was stored under MAP with gas mixtures (CO_2%:N_2%)(40:60 and 60:40), Whey cheese "Myzithra Kalathaki" (Dermiki *et al.*, 2008), and Ricotta cheese (Del Nobile *et al.*, 2009). These cheeses are considered more suitable for the growth of spoilage, because the value of pH is higher than 5.0, which contributed to the decrease in the shelf life of these cheeses under MAP and VP, when compared with the cheeses of this study, which have pH value lower than 5.0. The environment of cheese plays an

important role in preventing the spoilage of cheese, and this is compatible with the report of Leroy and De Vuyst (2004) with regards to the function of starter cultures, and their contribution to the microbial safety or offering one or more organoleptic, technological, nutritional, or health benefits.

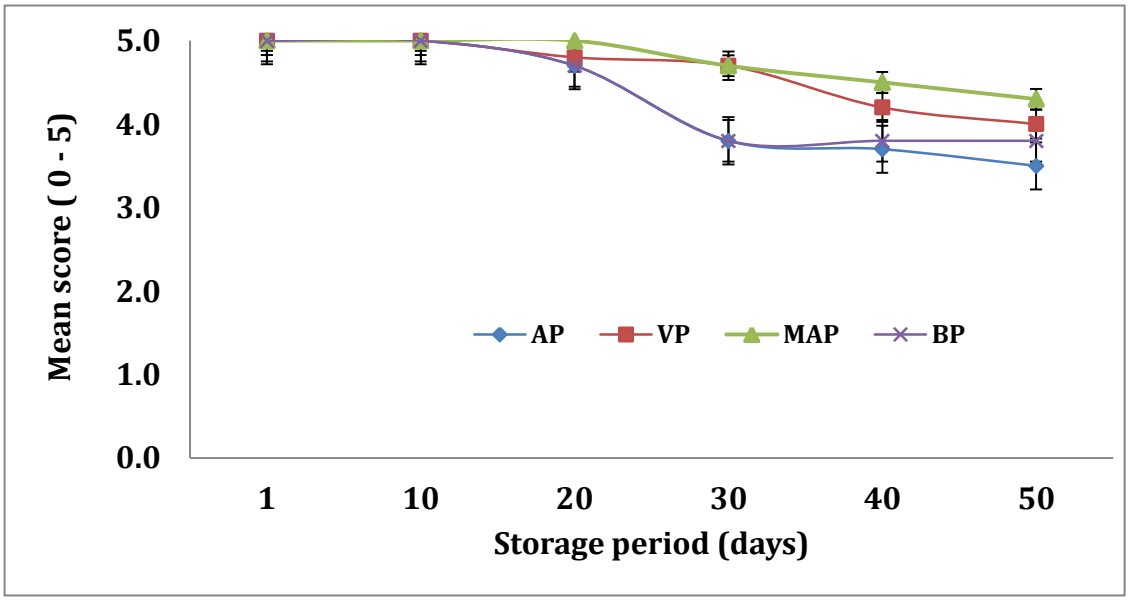

Figure 5.14. Changes in an odour attribute of soft cheese after one day of manufacturing (before packaging), and during 50 days of storage at 2-5°C under AP, VP, MAP and BP.

In this respect, Gonzalez-Fandos *et al.* (2000), stated that the Cameros cheese packaged under CO_2 and N_2 atmospheres, demonstrated the best sensorial quality, but using CO_2 at a level 100%, the product was rejected by panellists after 14 days of storage. The results of Mannheim and Soffer (1996) showed that the presence of CO_2 had no significant effect on the sensory properties of the Cottage cheese. In addition, storage of cheese at dark place contributed to the maintenance of its quality without negatively affecting the sensory properties. Similar findings have been reported by Kristensen *et al.* (2000). On the other hand, exposure of dairy products, such as cheese to fluorescent light contributes to the increase in oxidized derivatives of cholesterol, which may be important in many biological processes, including cholesterol homeostasis content (Addis and Park, 1992).

Overall, the sensory evaluation data for cheeses under all packaging methods were correlated well with the microbiological data. Storage temperature, relative humidity, and light intensity, to which the product is exposed during storage, were considered more important factors in extending the shelf life of cheese (Petersen *et al.*, 1999). As well, it was reported that the quality factors used in determining the shelf-life of fresh cheese, stored at less than 5°C for 1–8 weeks, under MAP, are microbiological characteristics, flavour attribute, and photo oxidation. Storage of soft cheese samples under MAP positively affected the microbial properties, rancidity, and odour attribute, when compared with the cheese samples under AP and BP. Furthermore, it contributed to retarding the growth of spoilage indicator, prevented lipid oxidation without appearance of any development in off-flavour in cheese samples until end of storage. But, with the cheese samples under AP and BP, there was a slight change in the cheese samples that appeared after 30 days of storage. Not only the odour of cheese samples under AP and BP changed after 30 days of storage, but also the microbial load in the samples, and consequently, the change of the score of samples from class 1 to class 11. With regards to lipid oxidation in cheese samples after 50 days of storage, the levels of TBARS were lower in cheese samples under MAP and VP, much more than those in samples under AP and BP. The data from the microbiological analysis and sensory evaluation of the odour attribute of cheese, during the storage of cheese 50 days at 2-5°C under packaging methods used, could conclude that using MAP in packaging of soft cheese with gas mixture 30% CO_2:70% N_2, contributes to maintaining the quality of cheese for a longer period, in comparison with the other packaging methods used. These results similar to that results obtained by Favati *et al.* (2007) for Provolone cheese, Dermiki *et al.* (2008) for whey cheese, Temiz *et al.* (2009) for Turkish whey cheese, where it was found that using the same gas mixture used in this work was the best product to use as a preserver,

and it extended the shelf-life of Provolone cheese by 50%, in comparison to vacuum-packaging and Papaioannou *et al.* (2007) for Greek whey cheese. Moreover, Ozlem *et al.* (2009), stated that the greatest score recorded to the "Crottin de Chavignol" cheese was under gas mixture 20% CO_2 : 80% N_2. On the other hand, with regards to the shelf life for cheese samples under control packaging (AP), the shelf life of the samples was higher than those recorded to the soft cheeses that were manufactured and evaluated by numerous researchers under similar storage condition to that which was applied in this work. The shelf life was 13 days for Mozzarella cheese (Alves *et al.*, 1996), after 7 days for the same cheese was evaluated by Gonzalez-Fandos *et al.* (2000), Fresh goat cheese ‘‘Cameros’’, when the control cheese (air packaged) was unacceptable after 14 days of storage (Olarte *et al.*, 2002), 15 days for Myzithra whey cheese at 4°C (Dermiki *et al.*, 2008), 3 weeks for "Crottin de Chavignol" (Ozlem *et al.*, 2009), 10 days for Turkish whey cheese (Temiz *et al.*, 2009), and 3 days for traditional Stracciatella cheese (Gammariello et al., 2011).

5.5 Conclusions

Based on the obtained results, it can be concluded that the manufacture of non-starter unripened soft cheese with a starter culture strains, and storage of cheese samples under MAP in slices with thickness up to 1.0 cm, could contribute to maintain the cheese quality through the effect of CO_2, and decrease the risk of product with regards to food-borne diseases, and maintaining the health of the consumers. Based primarily on sensory analysis, it can be concluded that the modified atmosphere, containing 30% CO_2:70% N_2, resulted in the extension of the shelf life of soft cheese for 20 days. Packaging the product, using MAP and storage under controlled low temperature, is considered a great hurdle for the growing spoilage by the microorganisms in cheese. Consequently, contributing to retard unwanted changes in the product, and extend its shelf life, is important. Furthermore, lipid oxidation,

trimethylamine, and total volatile base nitrogen were at minimum levels, due to the presence of CO_2 containing atmospheres and storage under controlled low temperatures. In addition, MAP packaging could be the technological approach that contributes to the microbial stability of the Middle Eastern unripened soft cheese. The absence of O_2 under modified atmospheres may affect the safety of foods packaged under MAP, through the growth of anaerobic pathogens. Thus, additional work should be commenced, using various modified atmosphere, on the growth of specific pathogens and patterns. On the other hand, the quality of milk used in the manufacture of cheese plays an important role in the determination of cheese yield. In this experiment, because the pasteurized milk that was used in the manufacture of cheese, was fresh supplied from the farm nearby the place of cheese manufacturing. The yield of cheese according to method that defined by Walstra *et al*., (2005) is the mass of cheese in kg obtained per 100 kg of milk contains starter culture, was 11.2% (11.2 kg).

Chapter 6

Potential effect of prebiotic ingredient (inulin) on the microbial quality and sensory properties of probiotic soft cheese

6.1 Introduction

Probiotic foods are manufactured products the matrix of which must be appropriate, and which must contain a sufficient concentration of live probiotic microorganisms (Saxelin *et al.*, 2003). The application of probiotic bacteria and prebiotics to dairy products confers functional characteristics on these products (Saarela *et al.*, 2000; Meyer *et al.*, 2011). So, functional foods are foods with traditional components and nutrients, but they also contain health-promoting compounds (Niva, 2007). The combination of probiotics and prebiotics in a food product is called a synbiotic (Holzapfel and Schillinger, 2002b). Food products containing functional ingredients, such as probiotic bacteria and prebiotics, have different taste profiles comparing with the non-functional food products (Mattila-Sandholm *et al.*, 1999a). In the food industry, the production and development of healthier foods with desirable sensory properties, could be realised, through the success in the development of cheeses which contain bifidobacteria (Boylston *et al.*, 2004). Prebiotics may have health benefits, and they play an important role in improving the sensory properties of dairy products. However, they may also be added because of their actual technological properties, e.g. as low-calorie sweeteners or fat replacers (Guven *et al.*, 2005; Wang, 2009; Meyer *et al.*, 2011). Prebiotics are short-chain carbohydrates that change the composition, or metabolism, of the gut microbiota, in a beneficial way. Prebiotics offer the potential to modify the gut microbial balance in such a way as to bring direct health benefits cheaply and safely, and is easier to incorporate into the diet than probiotics (Macfarlane *et al.*, 2006). Inulin is a heterogeneous blend of fructose polymers stored in some plants as a carbohydrate, which is widely distributed in

nature. Commercially, it has several qualities. including a neutral, clean flavour, and it contributes to the stability and acceptability of low fat foods (Niness, 1999). Inulin and oligofructose have nutritional advantages and technological characteristics (Franck, 2002b). Therefore, in dairy products, they are used as a fibre or prebiotic ingredients, in order to improve the taste and texture of the final product (Kaur and Gupta, 2002). The effect of inulin on the sensory and textural properties of dairy products depends on the concentration used, and the chain length of the polymer (Van Loo *et al.*, 1995; Meyer *et al.*, 2011). The combination of prebiotic ingredients and probiotic bacterial strains in white soft cheese would result in what is named a synbiotic product, and may contribute to health claims, thus promoting the additional health properties (Effat *et al.*, 2012). Gomes *et al.* (2009), stated that the changes in probiotic cheese manufacturing should be at a minimum level, when compared to conventional products, which makes the production of functional cheeses favourable. The versatility of cheese offers many marketing opportunities for it as a probiotic food carrier. However, the development of probiotic cheeses requires comprehensive knowledge for all processing steps, and their level of influence (positive or negative) on the survival of these microbes, sensory acceptance, chemical stability, and microbiological status during the shelf life (Granato *et al.*, 2010b). The possibility of producing a soft cheese that is capable of presenting a potentially synbiotic effect, due to the incorporation of the probiotic bacterial strains *Lb. acidophilus* LA-5, *Lb. casei* Shirota, *B. animalis* subsp. *lactis* BB12 and the prebiotic ingredient inulin, is really promising. The opinion of the consumers is considered to be an important matter with regards to developing a new food product, and the consumer liking is the key of the success of the product on the market (Bolenz *et al.*, 2003). The objective of study is evaluate the effect of inulin on the survivability of probiotic bacterial strains *Lb. acidophilus* LA-5, *Lb. casei* Shirota, and *B. animalis* subsp. *lactis* BB12, which were

incorporated into soft cheese, and on the cheese attributes, including appearance, texture, aroma, colour, and the overall acceptance of the final product. This study aims to investigate the potential effect of inulin on the microbial quality and the sensory properties of soft cheese manufactured with probiotic bacterial strains BB12, LA-5 and LcS.

6.2 Materials and methods

6.2.1 Determination of the ability of starter culture and probiotic bacterial strains on the growth on M17 media with inulin *in vitro*

The effect of inulin on the microbiological quality and sensory properties of a probiotic soft cheese, the ability of the starter culture and probiotic bacterial strains to grow on M17 media with inulin, as a sole source for carbohydrates, was assessed.

6.2.1.1 Preparation of M17 agar with inulin and lactose separately

M17 broth without sugar (CM0817 Oxoid Ltd., Basingstoke, Hampshire, UK) was used in the preparation of M17 agar with lactose, following the manufacturer's instructions by dissolving 37.25g of M17 broth in 950 ml distilled water. 12g agar No.1 (LP0011, Oxoid Ltd., Basingstoke, Hampshire, UK) was added to the broth. Media was autoclaved at 121°C for 15 min. Lactose solution 10% (w/v) was prepared by dissolving 10g lactose (29002, BDH, UK) in 90 ml distilled water. Fifty ml lactose solution was added to 950ml of melted media at 50°C, using sterilized filter and mixed gently before pouring. For the preparation M17 agar with inulin, the same procedure was applied with the replacement of lactose with chicory inulin powder.

6.2.1.2 Bacterial strains inoculation

To investigate the ability of starter culture and probiotic bacteria strains to grow on M17 agar in the presence of a prebiotic ingredient (inulin) before its application in soft cheese, two media of M17 agar, including, M17 agar with lactose, and with inulin were prepared. Overnight cultures of starter culture and probiotic bacterial strains

were inoculated into M17 broth with lactose and inulin separately. Tubes were incubated aerobically at 30°C for starter cultures, at 37°C at 5% CO_2 for probiotic strains LA-5 and LcS, and anaerobically for strain BB12. A streak was made from overnight cultures on M17 agar with lactose and inulin separately for starter cultures, on MRS agar for probiotic strains LA-5 and LcS, and on MRS agar plus 0.05% L-cysteine for probiotic strain BB12. A streak was made by using a metal loop which was sterilised using a Bunsen burner and cooled. After overnight incubation of tubes containing MRS broth with probiotic strains BB12, LA-5, LcS and M17 broth with starter strains, separately, a streak for each strain was made on the surfaces of M17 agar and MRS agar by using a metal loop following submersion in each tube. After that, a loop was submerged into test tubes containing overnight cultures of strains BB12, LA-5, LcS and SC, separately. A streak of each strain was made on the surface of M17 and MRS agar. A loop was immersed into the test tubes which contained broth with overnight cultures of probiotic and starter strains separately, then a streak of each strain was made on the surface of M17 and MRS agar. Plates of starter strains were incubated aerobically at 30°C and at 37°C at 5%CO_2 for probiotic strains LA-5 and LcS, and anaerobically for strain BB12. After 24 hours, the growth of strains was observed on M17 and on MRS agar.

6.2.1.3 Growth of starter culture and probiotic strains on M17 broth with lactose and inulin separately and on MRS media

To study the growth density (OD^{590nm}) of overnight cultures of starter cultures and probiotic bacterial strains, a plate reader (TECAN, Infinite 200, Reading, UK) and spectrophotometer (Unicom Hexois, spectrophotometer, UK) were used. In addition to an enumeration of the bacterial strains, serial dilutions between 10^{-1} to 10^{-9} were prepared with maximum recovery diluents (MRD) and poured on the M17 agar for the enumeration of starter culture strains, and the plates were incubated aerobically

at 30°C for 48 hours. MRS agar was used for the enumeration of probiotic bacterial strains LA-5 and LcS. Plates were incubated 72 hours at 5% CO_2 at 37°C. Probiotic strain BB12 was grown on MRS agar with 0.05% L-cysteine, where the plates were incubated anaerobically at 37°C. As well, probiotic bacteria strains LA-5 and LcS were inoculated on the MRS broth overnight, and the tubes were incubated at 37°C at 5% CO_2, and on MRS broth with 0.05% L-cysteine for strain BB12 at 37°C anaerobically. An optical density of the overnight cultures was estimated at A^{590nm}. After that, probiotic bacterial strains were enumerated on M17 agar with lactose and inulin separately, through the preparation dilutions of overnight cultures of strains between 10^{-1} and 10^{-9} with MRD. Plates were incubated for 48-72 hours at 37°C at 5% CO_2 for the strains LA-5 and LcS, and anaerobically for strain BB12.

6.2.1.4 Measurement the acidification activity of starter culture strains in the presence of inulin

Acidification activity of starter culture strains was measured by applying the method of Ayad *et al.* (2004), by the change in pH value (ΔpH) during a period of incubation. Overnight culture of starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* were inoculated in pasteurized skimmed milk 9.5 (w/v) at level (2%) in the presence of inulin at levels 0, 3, 4, 5, 6, and 7 % (w/v). Milk samples were incubated aerobically at 30°C. After 24 hours, the pH value was measured, using a pH meter. The acidification rate was calculated as a ΔpH (ΔpH= pH zero time - pH at time). The experiment was carried out twice.

6.2.2 Manufacture of probiotic soft cheese with and without inulin

Two probiotic soft cheeses were manufactured, where the first was a soft cheese with combination of starter culture and probiotic bacterial strains (PSC), the second was with a starter culture and probiotic bacteria strains in the presence of inulin (IPSC). Sixty litres of sterilised commercial semi-skimmed milk was purchased from local

shops (Tesco supermarket), and was used in the manufacture of cheeses. The method of Rodrigues *et al.* (2011) was applied in the curd manufacturing with minor modifications. Milk was collected and divided into two parts, each part was of 30 liters (part for each cheese), and heated to 36 ± 1.0°C in the sterilized stainless steel vats. Food-grade calcium chloride $CaCl_2$ (C/1280/53, Fisher Scientific, UK), was added at a level of 5.0g $10L^{-1}$ cheese milk for both cheeses. For IPSC, chicory inulin powder was added to the cheese milk at a level 4.0% (w/v). Starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* were added to both cheeses at level 1% with probiotic bacteria strains *Lb. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB12 (Chr. Hansen's Laboratory, Copenhagen, Denmark) and *Lb. casei* Shirota (LcS). Liquid Chymosin rennet (Chr. Hansen's Laboratories, Copenhagen, Denmark), was added at level 5ml $10L^{-1}$ cheese milk. Milk was left for 5-6 hours at 36 ± 1°C, until the setting of the curd, and after that, the curd with whey for the both cheeses, were transferred to the cool room (2 – 5°C) and left overnight. On the next day, the curd for both cheeses were cut gently, using sterilized knives under aseptic conditions, and then the curd left 15 minutes to rest before draining the whey. After draining the whey, the curd was transferred to the cheesecloths, which were sterilized with a standard solution of sodium hypochlorite, warm water, and boiling water. The curd for both cheeses was left hanging overnight on a stainless steel holder, to drain the whey by gravity. After most of the whey had been drained, salt was added to the curd at a level 1.5% (w/w). After that, the curd for both cheeses was left hanging for 3 hours, to drain the whey fully. The cheeses were stored for 14 days under vacuum packaging at 2-5°C, using sterilized polystyrene containers. A schematic diagram of the manufacture of a probiotic soft cheese, with and without inulin is illustrated in Figure 6.1.

6.2.3 Sampling and cheese tests

Cheese samples were taken weekly from the cool room and transported to the microbiological laboratory on ice, inside insulated Styrofoam boxes for conducting the following analysis.

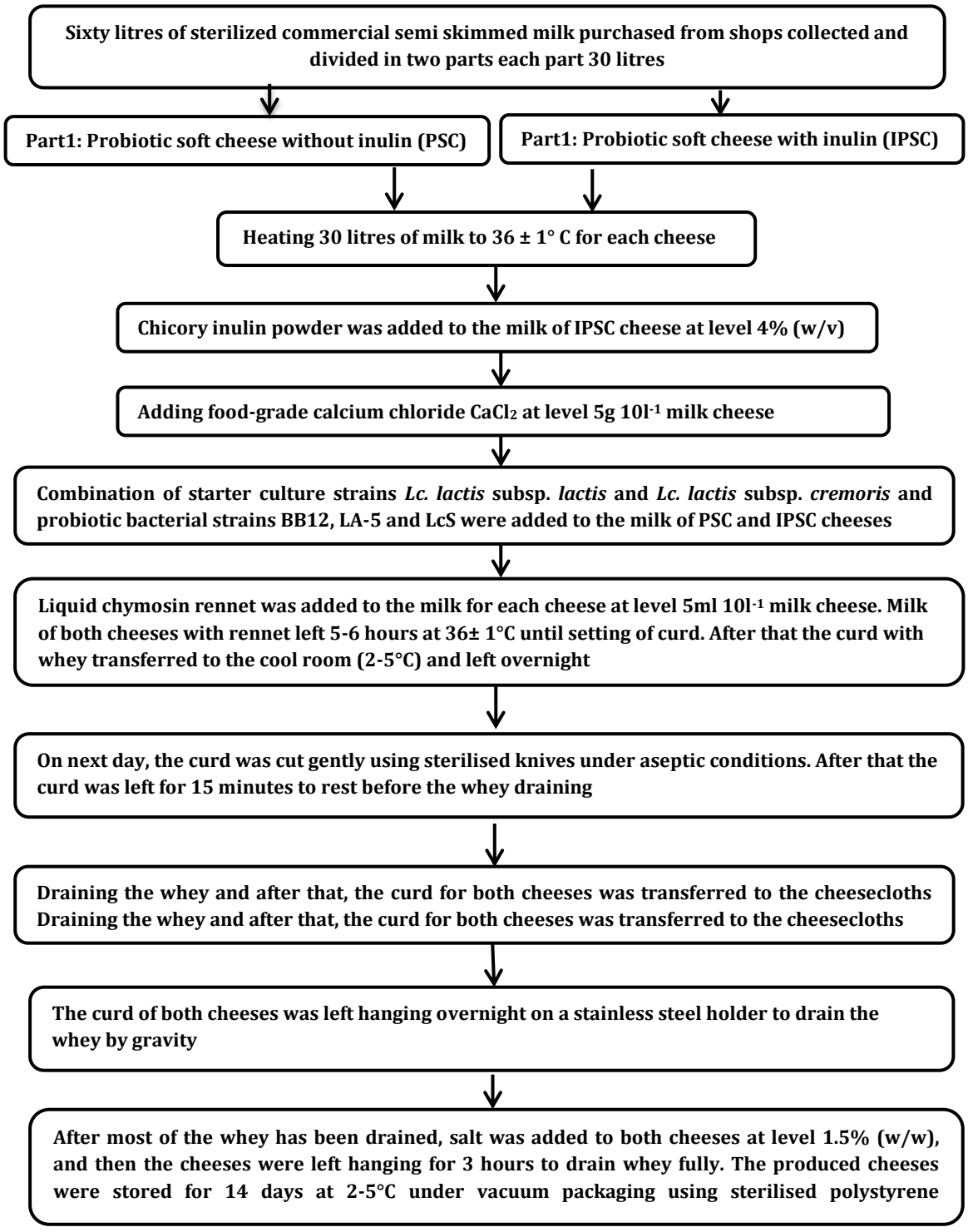

Figure 6.1. A schematic diagram of the manufacture of probiotic soft cheese with and without inulin

6.2.3.1 Microbiological analysis of probiotic soft cheese with and without inulin

The methods of microbiological analysis, including, the viable count of MAB, LAB, PB, M&Y and *Enterobacteriaceae*, were carried out by applying the same methods of microbiological analysis of soft cheese that applied in chapter 4 (section 4.2.6) and chapter 5 (section 5.2.3).

6.2.3.2 Physicochemical analysis of probiotic cheese with and without inulin

The pH value, fat content, total solids, salt content, and water activity were determined after one day of the manufacturing, and after 7 and 14 days of storage at 2-5°C, by applying the same procedures that were applied in chapter 4 (section 4.2.7) and chapter 5 (section 5.2.4.1.4).

6.2.3.3 Determination of protein concentration in cheese whey

Measurement of total protein in whey of cheese with and without inulin was carried out according to the method of Ronald *et al.* (2005). The Biuret reagent was prepared by adding 3 g of $CuSO_4.5H_2O$ (Sigma, Ltd, UK) and 9 g of sodium potassium citrate to 500 ml of 0.2N NaOH solution, followed by the addition of 5 g of KI. The resulting solution was completed to 1 litre with 0.2N NaOH. Protein standard and whey of cheese with and without inulin were added at level 200 µl to 7 separate tubes. 1800 µl of biuret reagent was added to each tube and mixed well and incubated at room temperature for 30 min to develop the colour. Standards 2 to 7 were measured at 540 nm and standard 1 used as blank. A linear fit was applied to the results of standard solutions to obtain the standard curve (Absorbance at 540nm vs. protein concentration). The resulting calibration curve mostly exhibits a linear relationship. The samples of whey of cheese with and without inulin were measured in

quantification mode. Using the calibration curve the concentration of protein in the whey samples was calculated.

6.2.3.4 Hydrolysis of inulin in probiotic cheese and whey

Hydrolysis of inulin in cheese and whey was conducted, following the method of Böhm *et al.* (2005). Cheese and whey were prepared for determination of their content of inulin. Ten g of cheese samples, with and without inulin, are mixed with 90 ml of distilled water, after that, the sample is blended using a food blender. Five ml of the solution and 5ml of whey from the soft cheese with inulin was placed in test tubes, and 5ml of 0.6N hydrochloric acid (HCl) (H1758, Sigma-Aldrich Ltd, UK) was added to each tube. Controls were prepared by adding 5ml of 0.6N of HCl to the tubes containing 5ml of solution of whey and cheese without inulin. Two tubes, each tube containing 5ml of 2% and 5% standard fructose solutions (F3510, Sigma-Aldrich Ltd, UK), were prepared. After addition of 5ml of 0.6N hydrochloric acid to all tubes, the tubes were placed in a water bath (Grant, UK) 85°C, for 45 minutes. Then, the tubes were directly cooled to the room temperature using cold water.

6.2.3.4.1 Preparation of DNS reagent

Ten g 3,5-dinitrosalicylic acid (Sigma Aldrich, Ltd, UK), 182g Rochelle salt (Sodium potassium tartrate) (Sigma Aldrich, Ltd, UK), 2 g Phenol (Fisher Scientific, UK), 0.5 g Sodium sulphate (Na_2SO_4) (Sigma Aldrich, Ltd, UK) and 10 g of NaOH (Oxoid, Ltd, UK) were dissolved in 800 ml distilled water and was adjust to a total of 1 litre. The reagent was stored in refrigerator at 4°C in a brown coloured bottle.

6.2.3.5 Preparation of standard curve of fructose

The standard curve of fructose was prepared by preparation of 10 mM solution fructose using standard powder of fructose (F3510, Sigma-Aldrich Ltd, UK). Twenty microliter of the DNS reagent was transferred by pipette into each of seven 3ml tubes.

Twenty microliter of a cheese whey of IPSC, the standard solutions of inulin [2% and 5% (w/v)] and 20µl of PSC and cheese whey of the PSC, were placed into a tube containing DNS. The tubes were labelled appropriately. The seventh tube was prepared as control by the pipette 20µl of distilled water and mixed with the content of the tubes. The tubes were incubated at 60°C for 10 min, then, the tubes were removed from the water bath, and 80µl of distilled water was added to each tube. The absorption (A^{540nm}) of the contents of each tube was recorded for results, and the concentration of fructose was calculated, using the standard curve of fructose.

6.2.4 Texture profile analysis (TPA) of probiotic cheese with and without inulin

Texture characteristic hardness, adhesiveness, and cohesiveness were determined by texture profile analysis (TPA), using a stable microsystems texture analyser. The height and diameter of the cheese samples which were prepared for testing were 20mm. The cross head and the chart speeds of the instrument were pre-speed 3.0mm sec^{-1}, speed 1.0mm sec^{-1} and post–speed 10mm sec^{-1}. The test was carried out at 21 ± 1.0°C.

6.2.5 Sensory properties evaluation of probiotic soft cheese

The sensory properties evaluation of the cheese samples were achieved with a scoring test by applying the method of British Standard Institution (1986), through the participation of 25 panellists from the food and nutrition department's staff, postgraduate and undergraduate students. Panellists were asked to evaluate their preference of cheese attributes, including appearance, flavour, colour, texture, and the overall acceptance. Cheese samples were presented to the panellists in disposable plastic cups, with a plastic knife and a cup of water, for cleaning their palates between the samples. A coded sample was written on the cup in the standard panel room, using white fluorescent light and a temperature of (21 ± 2°C). Before the participation

in the session, the panellists were briefed on a consent form, before their signature was received. The process was carried out in two sessions of 90 minutes each. All ballot sheets were collected, and the data received was statistically analysed.

6.3 Statistical analysis

The effect of cheese type and the storage period on the microbiological and chemical properties of cheese was measured using the analysis of variance (ANOVA) balanced ANOVA of Minitab 16 Software. Difference between means was carried out by using the Least Significant Difference (L.S.D), where level significance was ($P<0.05$). Comparison between the two cheeses was carried out using t test (paired t test) in relation to the scores of the preference test that was used for evaluating the cheese attributes.

6.4 Results and discussion

6.4.1 Microbiological analysis

6.4.1.1 Growth of starter culture and probiotic bacterial strains on M17 media with lactose and inulin separately and on MRS media

The data presented in Table 6.1, Figure 6.2, Figure 6.3, shows the ability of the starter culture and probiotic bacterial strains to grow in M17 media (broth and agar) containing inulin as the sole source of carbohydrate for the metabolism, instead of lactose, which is used as a carbon source in this media. Probiotic strain BB12, which is strictly anaerobic, was inoculated on media with 0.05% L-cysteine, then incubated anaerobically using a gas jar at 37°C, because the plate reader machine records the turbidity of the growth of aerobic and facultative microorganisms only. The results of this study indicate that substituting the inulin for lactose support the growth of probiotic bacterial strains *in vitro*. Significant differences ($P<0.05$) between the starter strains and probiotic bacterial strains, with regards to their growth on the M17 agar with lactose and inulin separately, and in broth were found. On the other

hand, probiotic bacteria strains LA-5 and LcS were inoculated on MRS broth and on MRS with 0.05% L-cysteine for strain BB12, and incubated anaerobically overnight at 37°C, and after that, the strains were enumerated on the M17 with inulin and lactose separately. No significant differences were found between the probiotic bacterial strains (P>0.05).

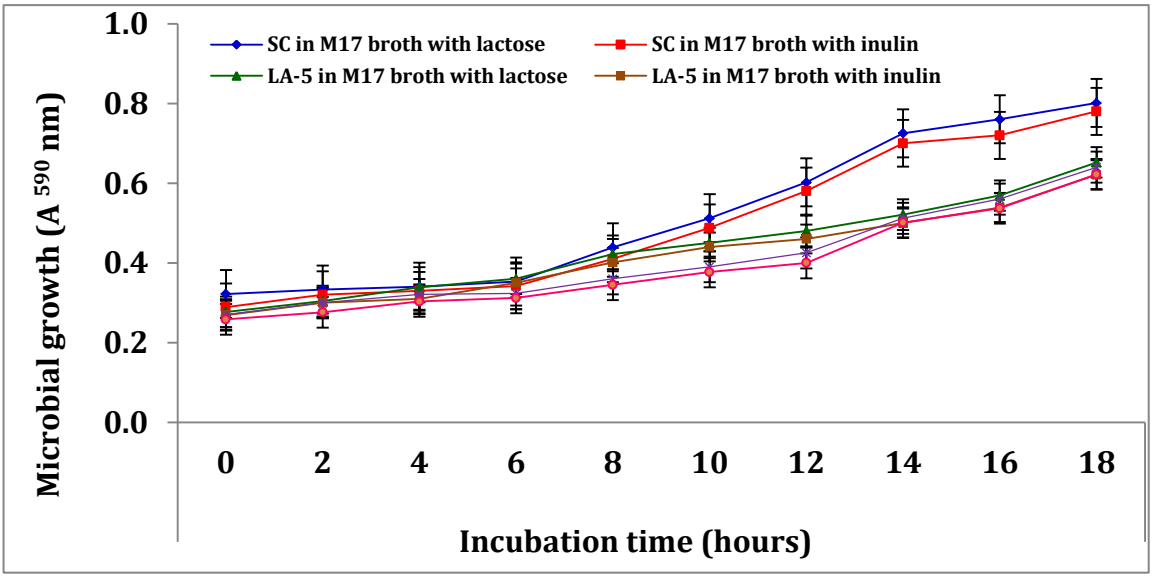

Figure 6.2. Growth density of starter culture and probiotic bacterial strains on M17 broth with lactose and inulin separately, after incubation 18 hours under aerobic conditions at 37°C using a plate reader at (A_{590nm}). (mean ± se) (n=3).

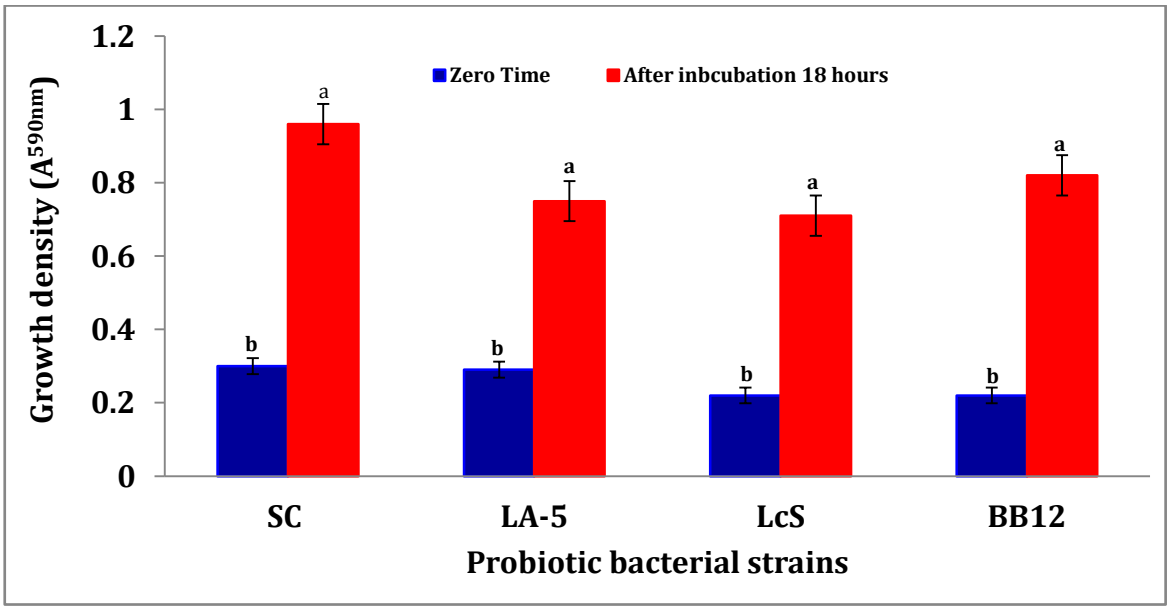

Figure 6.3. Growth density of probiotic bacteria strains on the M17 broth with inulin at zero time, and after 18 hours of aerobic incubation at 30°C for starter culture (SC), and at 37°C at 5% CO_2 for strains LA-5 and LcS and anaerobically for strain BB12 by detection optical density (A_{590nm}). (mean ± se)(n=3).

The growth of all strains on both media was satisfactory. These results are similar to those obtained by Su *et al.* (2007), reporting that the probiotic strains of *Lb. acidophilus* L10, *B. animalis* subsp*. lactis* B94 and *Lb. casei* L26 had the ability to grow in basal media containing inulin. Figure 6.3, indicate growth of probiotic strains in inulin media. Also, as shown in Figure 6.4, starter culture strains have the ability to ferment some carbohydrates regardless of the presence of several levels of inulin between 3% and 7% (w/v). The pH value of milk, under all levels of inulin used, decreased with slight differences after 24 hours of aerobic incubation at 30°C. In this regard, many researchers used this property in the application of inulin with probiotic strains *Lb. acidophilus* and *Bifidobacterium* spp*.,* in some dairy products, such as ice cream (Akın *et al.*, 2007). It was stated that the addition of inulin at level 10% (w/w) to ice cream enhanced the growth of *Lb. acidophilus* and *B. lactis,* and increased the viable count in comparison with the control samples.

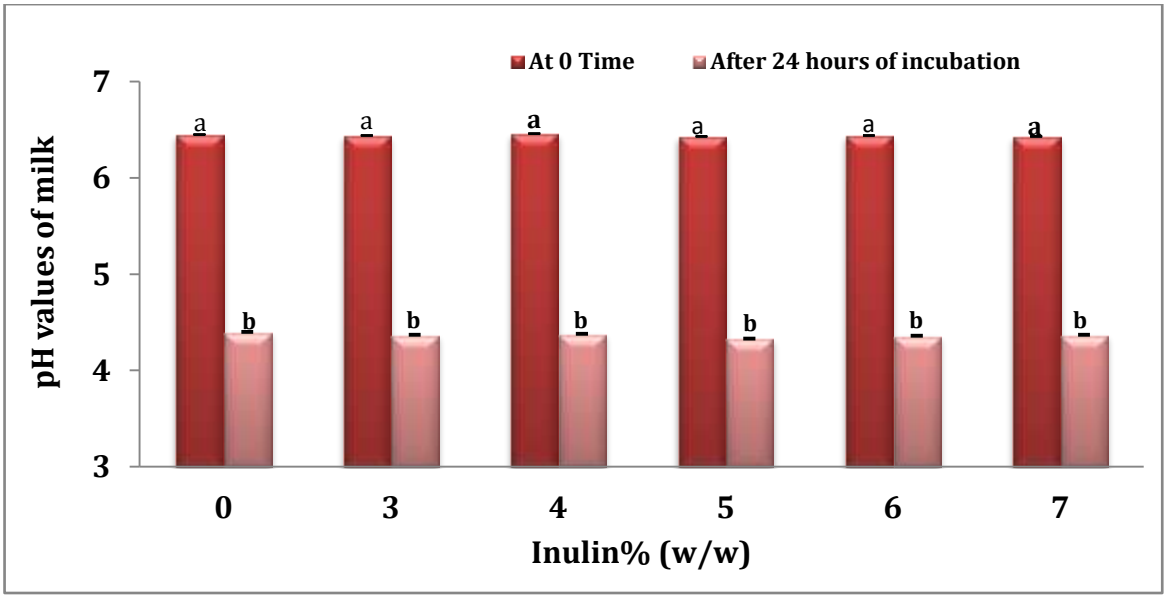

Figure 6.4. Fermentation of milk, by starter culture in the presence of inulin at levels 0, 3, 4, 5, 6 and 7% (w/w), after 24 hours of aerobic incubation at 30°C (mean ± se) (n=3).

As well, Cardarelli *et al.* (2007), mentioned that the manufacture of petit-suisse cheese with starter culture and probiotic bacteria strains *B. lactis* and *Lb. acidophilus in vitro*, with prebiotic ingredients inulin and oligofructose contributed to increased growth rates of probiotic strains in cheese, in addition to the production higher levels of lactic acid. Furthermore, similar findings have been reported by Nazzaro *et al.* (2012a), which stated that the presence of pectin or inulin with glucose did not affect the viability of probiotic strain *Lb. acidophilus* DSM 20079. The presence of pectin or inulin contributed to the increased stress resistance of cells against gastrointestinal juices. On the other hand, the results obtained by Nazzaro *et al.* (2012b), with probiotic strain *Lb. plantarum* subsp. *plantarum*, where the viable count of strain was 10.07, 10.28, and 10.42 log cfu ml^{-1} for pectin, inulin, and glucose respectively. The aforementioned data encourages the application of inulin into a probiotic soft cheese spread, which could act as an improver of the sensory properties for the product, including texture, colour, aroma, appearance, and overall acceptance.

6.4.1.2 Microbiological analysis of probiotic cheese with and without inulin

The data illustrated in Table 6.2 and Table 6.3, shows that the initial viable count (log cfu g^{-1}) of MAB, PB, and LAB in the both cheeses, after one day of the manufacture of cheeses, were high, and reached to 8.596 ± 0.151, 8.556 ± 0.146 and 8.966 ± 0.155 log cfu g^{-1}, respectively, without much difference between the cheeses. The storage period significantly influenced the viable count of the microbes ($P<0.05$). The stages of the manufacture of cheeses, which included the setting of the curd, storage of the curd overnight in a cool room, cutting the curd, draining the whey by gravity using cheesecloth, and salting. These stages took around 30 hours, which contributed to the increase levels of the initial viable count of these microorganisms in the both the cheeses. These treatments mainly

contributed to increase the initial load of these microorganisms before packaging and storage of the products.

The results reported by Temelli *et al.* (2006) were similar to that obtained in this work, which found that the microbiological contamination sources in PB during the manufacture of Turkish white cheese, are cheese making place and the packaging materials. Cheese vat and knives used in cutting the curd and cheesecloth, and are considered sources of contamination in MAB. The air of the cold room and the cheese manufacturing area are the contamination sources in M&Y.

On the other hand, the matrix of cheeses is considered to be suitable environments for the growth of psychrotrophic microorganisms, which are associated with cheese, and are able to grow within the cheese matrix. But the storage of cheeses, by using vacuum packaging, contributed to slow down the growth of these microbes without significant increase during the storage of cheeses. Similar findings have been reported by Breseford (2007), with regards to the growth of these microorganisms in most cheese varieties, during the manufacture and ripening of cheese. On the other hand, Leitner *et al.* (2008), indicated that there are mechanisms, such as enzymatic activity produced by some microorganisms may affect quality of milk, consequently on the quality of cheese.

The viable count of M&Y in PSC was higher than those in IPSC, and the initial viable count was 5.286 ± 0.114 log cfu g^{-1} in PSC and 5.230 ± 0.10 log cfu g^{-1} in IPSC. The viable count decreased significantly during the storage period of the cheeses (Table 6.2 and Table 6.3). Cappa and Cocconcelli (2001), stated that fungi play an important role in the spoilage of dairy products, such as cheese and yoghurt. Breseford (2007) stated that cheese contamination with moulds and yeasts causes

the formation of coloured spots on the cheese surface which negatively affect the flavour and aroma of the product. In addition, liquefaction of the curd was observed, beside production of gas. In this respect, during the manufacture and storage of both cheeses, and during the sensory evaluation of the final products after 14 days storage at 2-5°C under VP, these negative phenomena were not noted on the cheeses.

Ledenbach and Marshall (2010) mentioned that the existence of PB and M&Y in cheese, before the storage at low temperatures affects the quality of product. In addition, the high moisture in soft cheese, fat, protein, and the low levels of salt and the pH value. These characteristics of soft cheese are considered suitable environment for the growth of yeasts, as mentioned by Fleet (1990), when stated that yeasts are frequently found within the microflora of many types of cheese. This because of their resistance to low pH and moisture, high level of salt and low storage temperatures. And the report of Kurtzman (2006), it was stated that the best growth of yeast is in low pH values, which is generally 5.5 or lower, and the presence of sugars, organic acids, and other easily metabolized carbon sources. Furthermore, it was noted that the yeast spoilage often occurs through their growth on the surface of products, such as cheeses. On the other hand, Eliskases-Lechner and Ginzinger (1995) reported that the composition of the yeast flora, which are present in a fresh cheese seems to be rather varied and strongly dependent on the place of the manufacture of cheese. No significant differences between the cheeses were found, with regards to the viable count of M&Y ($P > 0.05$), but significant differences were observed during the storage period. The viable count of *Enterobacteriaceae*, during the cheese manufacturing process, and during the storage for 14 days at 2-5°C was < 1.0. Although the length of cheese manufacturing duration is considerable, the results confirms that the process was

undertaken in a hygienic conditions, with due precautions taken to prevent the contamination of cheese in these microorganisms. Storage of cheeses using VP and low temperatures drop the pH value of the cheeses, through lactose fermentation in milk to lactic acid, by the starter culture strains, and to lactic acid and acetic acid, by LAB and bifidobacteria (Tamime, 2002), which contributes to retarding the growth of microbes that contaminate the product, and reducing the growth rate of spoilage indicator.

Table 6.1. Growth of starter culture strains and probiotic bacterial strains on M17 broth and agar with lactose and inulin separately. Incubation at 37°C at 5% CO_2 for strains LA-5 and LcS, anaerobically for strain BB12 and aerobically at 30°C for starter culture strains. (mean ± se) (n=3)

Bacterial group	Growth of starter culture on M17 broth and agar in presence of lactose and inulin			
	M17 media with lactose		M17 media with inulin	
	Optical Density^ (A^{590}) on M17 broth with lactose	Viable count # (log cfu ml^{-1}) on M17 agar with lactose	Optical Density^ (A^{590}) on M17 broth with inulin	Viable count # (log cfu ml^{-1}) on M17 agar with inulin
Lc.lactis* subsp*. lactis and cremoris	0.629 ± 0.208^{aA}	10.168 ± 0.241^{aA}	0.606 ± 0.207^{aA}	9.702 ± 0.304^{aA}
***Lb. acidophilus* LA-5**	0.492 ± 0.161^{aB}	8.793 ± 0.149^{aB}	0.480 ± 0.158^{aB}	8.318 ± 0.279^{aB}
***Lb.casei* Shirota LcS**	0.461 ± 0.155^{aB}	8.327 ± 0.151^{aB}	0.447 ± 0.154^{aB}	8.618 ± 0.166^{aB}
***B. animalis* subsp*. lactis* BB12**	0.519 ± 0.188^{aB}	8.742 ± 0.177^{aB}	0.485 ± 0.069^{aB}	8.370 ± 0.183^{aB}

^Mean of OD at zero time and after 18 hours of incubation in M17 broth with lactose and inulin separately, # =Mean of viable count (log cfu ml^{-1}) after incubation 48 hours on M17 agar with lactose and inulin separately for starter culture strains and for 48-72 hours for probiotic bacterial strains, ^{a} =Difference between media, A&B=Different between strains.

Table 6.2. Viable counts (log cfu g^{-1}) of microbial groups in PSC and IPSC, after one day of manufacturing and after 7 and 14 days under vacuum packaging at 2-5°C. (mean ± se)(n=3)

Microbial groups	Viable count (log cfu g^{-1}) during the storage period (days)		
	1	7	14
Mesophilic aerobic bacteria	8.596 ± 0.151^{a}	8.218 ± 0.117^{a}	8.072 ± 0.163^{b}
Psychrotrophic bacteria	8.556 ± 0.146^{a}	8.226 ± 0.158^{a}	7.828 ± 0.240^{b}
Lactic acid bacteria	8.966 ± 0.155^{c}	9.272 ± 0.259^{b}	9.637 ± 0.232^{a}
Enterobacteriaceae	<1.0	<1.0	<1.0
Moulds and yeasts	5.258 ± 0.124^{a}	3.421 ± 0.177^{b}	2.461 ± 0.142^{c}

Mean values in the same row bearing the same superscripts not different significantly (P >0.05), values in the same row bearing the different superscripts differ significantly (P <0.05).

Table 6.3. Viable counts (log cfu g^{-1}) of microbial groups in PSC and IPSC, after one day of manufacturing and after 7 and 14 days under vacuum packaging at 2-5°C. (mean ± se) (n=3)

Cheeses	Microbial groups	**Viable count^ (log cfu g^{-1}) during the storage period (days)**		
		1	7	14
Probiotic soft cheese without inulin (PSC)	Mesophilic aerobic bacteria	8.680±0.124^{aA}	8.240±0.129^{bB}	8.120±0.173^{bB}
	Psychrotrophic bacteria	8.492±0.243^{aA}	8.206±0.191^{bB}	8.000±0.164^{bB}
	Lactic acid bacteria	8.907±0.219^{bD}	8.925±0.340^{bD}	9.361±0.207^{aC}
	Moulds and yeasts	5.286±0.114^{aA}	3.607±0.122^{bB}	2.574±0.100^{cC}
	Enterobacteriaceae	<1.0	<1.0	<1.0
Probiotic soft cheese with inulin (IPSC)	Mesophilic aerobic bacteria	8.512±0.180^{aA}	8.200±0.243^{bB}	8.020±0.056^{bB}
	Psychrotrophic bacteria	8.619±0.133^{aA}	8.245±0.128^{aB}	7.830 ±0.078^{bC}
	Lactic acid bacteria	9.025±0.078^{cD}	9.619±0.130^{bB}	9.913±0.144^{aA}
	Moulds and yeasts	5.230±0.100^{aA}	3.236±0.116^{bB}	2.349±0.070^{cC}
	Enterobacteriaceae	<1.0	<1.0	<1.0

^ Viable counts (log cfu g^{-1}) of each microbial group in both cheeses during storage period. Mean values in the same row bearing different superscripts differ significantly (P <0.05). PSC= Probiotic soft cheese without inulin; IPSC= Probiotic soft cheese with inulin

Ledenbach and Marshall (2010) observed that introducing desirable microorganisms, such as LAB in the manufacture of dairy foods, such as cheese, and the use of a suitable packaging system with limited oxygen quantities, controls the growth of undesirable microorganisms and retards spoilage through lactose fermentation and lactic acid production. On the other hand, Mortensen *et al.* (2004b), found that the use of appropriate methods for cheese packaging and storage contributes to reducing the undesirable changes that are caused by the presence of harmful microorganisms. In this regards, inhibition activities of starter culture and probiotic bacterial strains LA-5, BB12 and LcS, which have a good survival in both cheeses, may contribute to the inhibition these microbes. On the other hand, Martins *et al*., (2009) found that probiotic strain BB12 exhibited an antagonistic activity against pathogens, such as *E.coli* ATCC 4328 and *Listeria monocytogenes.* Therefore, it can be concluded that the incorporation of probiotic strains in soft cheese, as an adjunct cultures with starter culture strains, may contribute to make the product safer, through their inhibition activities toward unwanted microorganisms. This is clear in the viable count of MAB and PB in the both cheeses, as illustrated in Table 6.2 and Table 6.3, when the growth of these microbes declined during the storage period of 14 days. O'Riordan and Fitzgerald (1998), found that the production of inhibitory compounds, by probiotic bacterial strains of *Bifidobacterium* contributed to reduce the levels of *Pseudomonas* in cottage cheese at refrigeration temperature. As well as, the report of Buriti *et al.* (2007c) mentioned that the application of probiotic strain *Lb. paracasei* subsp. *paracasei* into fresh cream cheese, appeared as inhibitory activities against microbial contaminants, with or without the addition of inulin.

With regards to the viable count of LAB in both cheeses, these results are similar to the results obtained by Papaioannou *et al.* (2007), Dermiki *et al.* (2008), Gammariello *et al.* (2009b) and Temiz *et al.* (2009), which stated that these microorganisms have

facultative characteristics, and they can grow under vacuum packaging in Stracciatella cheese, Greek whey cheese, and Turkish white cheese. In addition, these microbes have ability to grow with a low pH value. Furthermore, the presence of probiotic bacteria in cheeses, which will contribute to the rising, numbers of these microbes. On the other hand, an antimicrobial compound, which may be produced by LAB, supports these microbes with a competitive benefit over other organisms. The use of antibiosis of LAB is the best choice for not only improving the microbial safety of the food products, but also in a probiotic preparation, because of their natural adaptation to the gut environment. Moreover, numerous LAB bacteriocins are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in several foods, such as nisin which inhibits the growth of some psychrotrophic bacteria in cottage cheese (Soomro *et al.*, 2002; De Vuyst and Leroy, 2007). In addition to the use of LAB and their metabolic products, which are usually considered to be the safe materials (Zacharof and Lovitt, 2012). The viable count of LAB was high, during the manufacture and storage of product, without the differences between the both cheeses. De Vuyst and Leroy (2007), stated that the application of lactic acid starter culture strains, which have able to produce of inhibition substances during food manufacturing, contributes to increase the competitiveness with pathogens. The results confirm the negative correlation between the viable count of LAB and the viable count of MAB and PB, which was found during the enumeration of these microorganisms in both cheeses during the storage period at 2-5°C.

The viable count of M&Y decreased significantly during the storage time, due to the unavailability of oxygen under VP, which is considered the essential factor to the growth of these microbes. In addition, the application of a good hygiene conditions and sanitation measures during the manufacture and storage of the product, were contributed to preventing cheese contamination in yeasts, as stated by Guerzoni *et al.*

(1998). On the other hand, the presence of yeasts will result in specific correlation which positively or negatively affects the final product (Viljoen, 2001).The viable count of M&Y in both the cheeses after 14 days of storage was lower than those found in Feta cheese, which was produced by three different Danish dairies, as tested by Westall and Filtenborg (1998), who found that the viable count was between 10^4 and 10^6 cfu g^{-1}, which was caused by the production areas. In this regards, Jakobsen and Narvhus (1996) and Fröhlich-Wyder (2001) mentioned that the yeasts play an important role as a spoilage organisms in dairy products, such as cheese, because of their resistance to stresses, in addition, the conditions of the cheese manufacturing contribute to the cheese contamination with yeasts, in particular with the cheese surface.

The application of a good hygiene conditions during the manufacture of the product, the storage, during sampling and the carrying out of tests, contributed to reducing the viable count of *Enterobacteriaceae* in both products, to less than that which were found in numerous cheeses, produced by some researchers. According to the Guidance on the interpretation of results for hygiene indicator organisms in ready-to-eat foods, which was reported by a Health Protection Agency (2010c) and a Health Protection Agency (2009), both the cheeses were considered to be satisfactory. In this regards, the manufacture of whey cheese, with probiotic bacterial strains *B. animalis* and *Lb. casei* by Madureira *et al.* (2011), contributed to fighting foodborne pathogens, and food spoilage microbes, by inhibiting activity, through the production of antimicrobial substances, and consequently, the delay or prevention of the growth of contaminates, and to make the product safer.

With regards to the survival of starter culture and probiotic bacterial strains in both cheeses, in general, the mean of viable counts of the starter culture strains were between 8.364 ± 0.236 log cfu g^{-1} in IPSC, one day after manufacturing, which

significantly decreased to 5.441 ± 0.072 log cfu g^{-1} in IPSC, and 5.582 ± 0.174 log cfu g^{-1} in PSC, after 14 days of manufacturing, without any significant differences between the cheeses (Figure 6.5). The mean of viable count of probiotic strain BB12, was between 7.059 ± 0.127 log cfu g^{-1}, after one day of manufacturing, and 7.286 ± 0.115 log cfu g^{-1}, after 14 days of manufacturing in PSC, and between 7.260 ± 0.139 and 7.615 ± 0.205 log cfu g^{-1} in IPSC, with significant differences between the (strain BB12 was added to cheese at level ~10^{12} cfu ml^{-1}, because of this strain anaerobic, and conditions and period of the manufacture of cheeses, may affect the survival of strain in comparison with other strains). The viable count of probiotic strain LA-5, was between 7.985± 0.059 and 8.492± 0.115 log cfu g^{-1} PSC and ranged from 8.157± 0.141 to 8.492± 0.115 log cfu g^{-1} IPSC (Figure 6.5). These finding similar to those reported by Cardarelli *et al.* (2008), which found that the viable count of probiotic strain *B. animalis* subsp. *lactis*, varied from 7.20 to 7.69 log cfu g^{-1}, and from 6.08 to 6.99 log cfu g^{-1} for strain *Lb. acidophilus* in petit-Suisse cheeses, which were manufactured with oligofructose, and/or inulin, at levels above 8.90 g 100 g^{-1}. The increase in the viable count of probiotic strain BB12 in the probiotic soft cheese spread with inulin may contribute to enhance the formation of synbiotic, which was characterized as the products with interesting nutritional properties. Therefore, it could be considered to be a probiotic soft cheese, which contains probiotic bacterial strains and inulin, as a health-enhancing food, as reported by Gibson and Roberfroid (1995), which reported that the prebiotics ingredients could contribute to enhance the growth and survival of the probiotic strains in GIT, and also, influence the metabolic levels in these strains. On the other hand, Buriti *et al.* (2007c) found that the use of inulin in a combination with probiotic strain *Lb. paracasei* subsp. *paracasei* in the manufacture of fresh cream cheese, have not affected the growth and viability of probiotic strains in a synbiotic fresh cream cheese. In regards to the viable count of starter culture strains, the

cheese packaging method (Vacuum Packaging) affected the survival of starter culture strains, since these microorganisms need anaerobic conditions for their growth.

The mean viable count of probiotic bacterial strains LA-5 and BB12 differed significantly between the cheeses after 14 days of storage. No insignificant differences in the viable count of strain LcS were detected after 14 days of storage. The mean of viable count of probiotic strains LcS in PSC was between 7.408 ± 0.166 log cfu g^{-1}, after one day of manufacturing, and 7.651 ± 0.118 log cfu g^{-1} after14 days of manufacturing, and that ranged from 7.301 ± 0.174 log cfu g^{-1} after one day of manufacturing, to 7.534 ± 0.120 log cfu g^{-1} after 14 days of manufacturing in IPSC, without significant differences between the cheeses.

Although higher viable count values were generally observed for strains LA-5 and BB12 in IPSC when compared with PSC, probiotic strain LA-5 did not significantly grow in either of the cheeses during the storage of 14 days. Following success for other types of dairy products, there is drive for incorporating probiotic strains of *Bifidobacterium* into different varieties of cheese, which is still considered to be a challenge in the dairy industry (Gomes *et al.,* 2009). With regards to the viability of probiotic strain BB12 in cheese, several factors could affect the survival of bifidobacteria in these products, including interactions with other starter and probiotic strains, salt, and temperature. However, the pH value and oxygen significantly effect on *Bifidobacterium* during the storage period (Crittenden, 2004). The potential combination between probiotic bacterial strains used, particularly, *Lb. acidophilus* LA-5 and/or *B. animalis* subsp. *lactis* BB12 and inulin in IPSC, may result in obtaining a symbiotic because inulin is readily available for fermentation, and result in benefits to the host that the live microorganism and prebiotic offer. In this respect, Cardarelli *et al.* (2007) found that the combination of probiotic strains *Lb. acidophilus* and *B. lactis,* and the prebiotics ingredients inulin and/or oligofructose,

resulted in relatively capable functional Petit-Suisse cheese. In terms of the viability of probiotic strains, it has to be stated that the viability of probiotic bacterial strains in cheese is a complex phenomenon. Apart from the viability of probiotic strains in the product until the time of consumption, the survival of the strains after exposure to GIT conditions, is also crucial (Karimi *et al.*, 2011). Furthermore, the methodology of adding probiotic bacterial strains as direct vat set contributes to improving the performance of the probiotic strains, as mentioned by Bergamini *et al.* (2005), who found that the direct addition of probiotic strains *Lb. acidophilus* and *Lb. paracasei* subsp*. paracasei* to milk, during the manufacture of semi-hard Argentinean cheese, improved the performance of strains, in comparison to the strains within a substrate composed of milk and milk fat. During the storage period of cheeses, the growth and survival of LAB, and the probiotic bacterial strains in both the cheeses which were assessed showed significant correlations with the acidifying profiles that was obtained throughout the storage as expected, where negative and positive correlations were found between the viable count of LAB and the pH value of cheeses, which were r= - 0.658 and P= 0.003. A higher acidification rate was observed with the strains LcS and BB12, than those obtained with LA-5. The correlation between the pH value of cheeses and the viable count of probiotic bacterial strains LA-5, LcS and BB12 were r= -0.388 and P= 0.112, r= - 0.549 and P= 0.018 and r=-0.506 and P= 0.032, respectively. Similar results have been found by Rodrigues *et al.* (2011), who stated that in spite of the differences observed between probiotic bacterial strains and synbiotic of curd matrix with 20g L^{-1} milk of 50:50 fructooligosaccharides:inulin and probiotic strains *B. lactis* B94, *Lb. casei* 01 and *Lb. acidophilus* LA-5, it had not significantly affected the acidification process, , and it was in confluence with the report of Saarela *et al.* (2000), with regards to the survival of probiotic strains under the conditions of the cheese manufacture.

With regards to the yield of the cheeses, the most common way to determine cheese yield is the determination of the mass of cheese using a parameter Kg, obtained from 100 Kg of milk containing starter culture strains during the manufacture of cheese (Walstra *et al.*, 2005). Presence of inulin in probiotic soft cheese, contributed to increase the yield of cheese compared with cheese without inulin, where the yield of PSC was 9.8% and for IPSC was 14.11%.

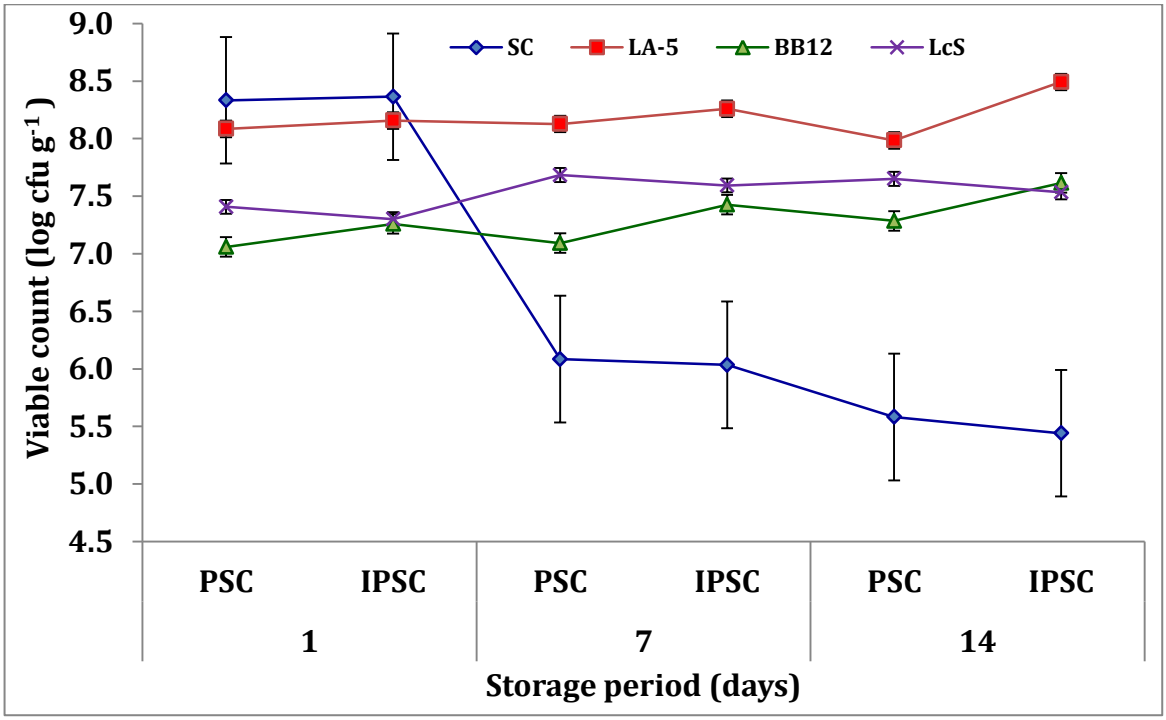

Figure 6.5. Viable counts (log cfu g^{-1}) of starter culture and probiotic bacterial strains in PSC and IPSC, after one day of manufacturing, and after 7 and 14 days of storage under vacuum packaging at 2 -5°C (mean ± se)(n=3).

On the other hand, Ross *et al.* (2002b) reported that cheese contains a complex combination of microbes, which changes with time, initially containing large numbers of starter LAB, and then, during maturation, an increasing number of non-starter lactic acid bacteria. Therefore, the viability of probiotic bacteria in cheese is a complex phenomenon. In addition, the cheese matrix has significant influences on success of the delivery of probiotics into the intestine. In this respect, despite a decrease in the viable count of starter culture during the storage period, there was a decrease in the pH value of cheeses with the progress of storage time. This decrease

could be produced through the contribution of probiotic bacterial strains. In addition, LAB and NSLAB, which have the ability to grow and ferment lactose, contributed to a drop in the pH value of cheese, and increased the acidity of cheese with and without inulin during the storage period.

6.4.2 Physicochemical analysis of probiotic soft cheese with and without inulin

As shown in Figure 6.6, the mean of total solids in PSC, after one day of manufacturing, and after 7 and 14 days of storage under vacuum at 2-5°C, were 31.342 ± 0.145, 31.626 ± 0.152 and 31.744 ± 0.024%, respectively, whilst in IPSC were 33.198 ± 0.179, 33.382 ± 0.144 and 33.449 ± 0.175%, respectively. The mean of total solids in PSC during 14 days of storage was 31.573 ± 0.199%, and was 33.343 ± 0.075% in IPSC, with significant differences between the cheeses ($P < 0.05$). The average fat percentage in IPSC after one day of cheese manufacturing was 8.333± 0.167% and after 14 days of storage was 8.833± 0.167%, whilst the average after one day of manufacture of PSC, was 11.200 ± 0.167%, and was 11.333 ± 0.333 % after 14 days of storage. No significant differences between the cheeses were found ($P > 0.05$), with regards to the pH value, where the value ranged between 4.426± 0.004 in PSC after 14 days of storage, and was 4.449± 0.007 after one day of manufacture, and between 4.448 ± 0.005 after one day of manufacture, and 4.428 ± 0.010 after 14 days of storage in IPSC.

The mean value of pH during storage 14 days in PSC was 4.437± 0.007, and 4.439± 0.006 in IPSC. The mean salt content in cheeses after one day of manufacture was 1.404 ± 0.011%, and after 14 days of storage it was 1.427 ± 0.011% in the PSC, and ranged in the IPSC from 1.399 ± 0.010% after one day of manufacture, to 1.426 ± 0.010% after 14 days of storage, without significant differences between the cheeses. The average water activity for both the cheeses ranged between 0.968 ± 0.003 in IPSC, and 0.972 ± 0.002 in the PSC (Figure 6.6). The differences between the cheeses are

clear in total solids of cheeses. These results are similar to the results obtained by Moghari *et al.*, (2014) who found that the application the *Lb. acidophilus* LA-5 and *B. lactis* BB-12 into Iranian ultrafiltered-Feta cheese as a function of inulin concentration. There were no significant differences in the physicochemical analysis of cheese with and without inulin (except for total solids), which was higher in cheese manufactured with inulin at levels 1.5 and 3% (w/w) than that found in cheese without inulin. Storage period had no effect on the percentage of TS in the cheeses ($P > 0.05$). The pH value of cheeses also did not differ between both the cheeses.

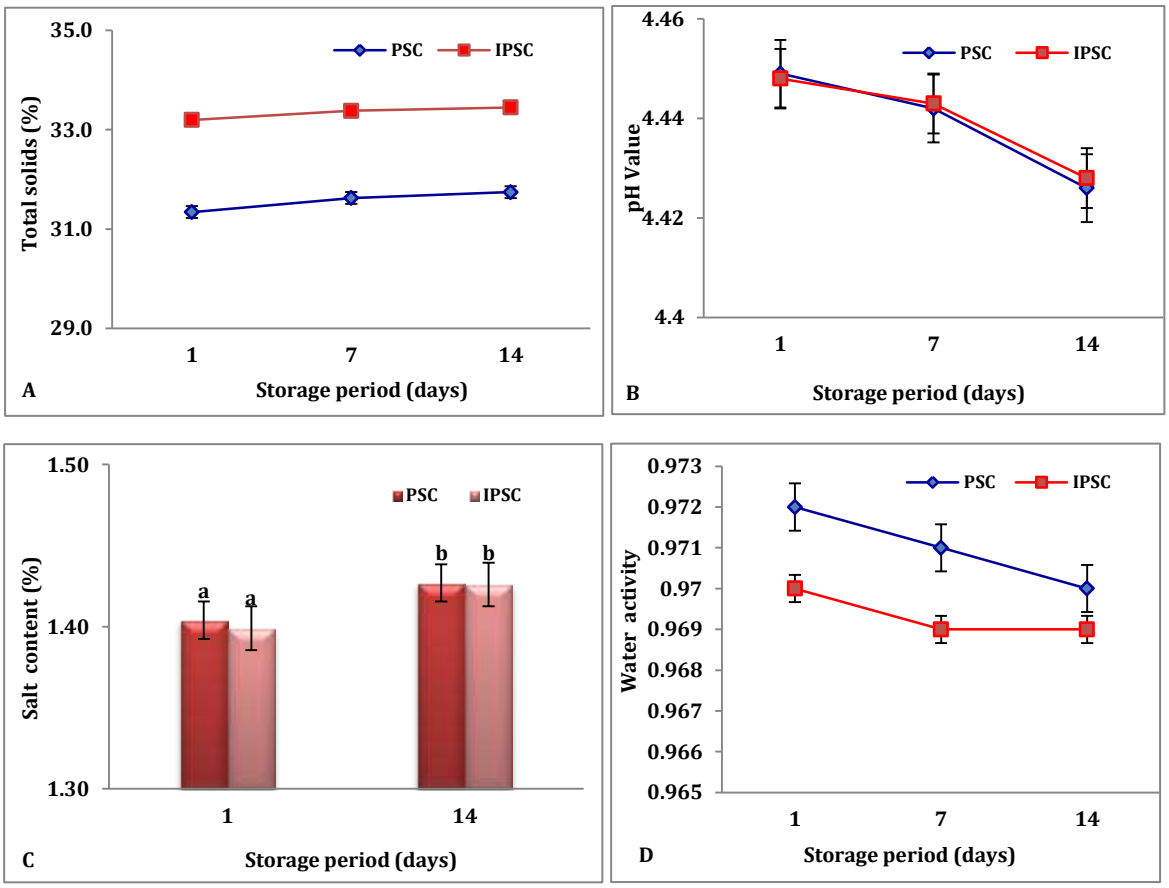

Figure 6.6. Physicochemical analysis of probiotic soft cheese with and without inulin, after one day of manufacturing, and after 7 and 14 days of storage at 2-5°C. (A) total solids (%), (B) pH value, (C) salt content (%) and (D) water activity. (mean ± se) (n=3).

The decrease in the pH value during the storage of cheese was through the fermentation of lactose, where these results were compatible with the results of the milk fermentation, through the starter culture strains *in vitro,* in the presence of

inulin with lactose, together in milk. Despite decreasing the viable count of starter culture strains during storage, the presence of LAB and probiotics bacterial strains contributed to a decrease in the pH value in both cheeses, without an effect of the inulin on the activity of LAB, including probiotic strains with regards to the fermentation of lactose to lactic acid, by homofermentative strains, and to lactic acid and acetic acid, by heterofermentative strains like probiotic strain BB12, in the presence of inulin. In this regards, Olson and Aryana (2012) reported similar findings, by demonstrating that the prebiotics did not have a major effect on the rate of decrease of pH, by *Lb. acidophilus* in skim milk. The results of Rodrigues *et al.* (2011) demonstrated no significant differences were found in the pH value of the curd matrices that was manufactured from sterilized milk with probiotic bacterial strains *B. lactis* B94, *Lb. casei* 01 and *Lb. acidophilus* LA-5. The synbiotic curd matrix contained a combination of prebiotic ingredients inulin and fructooligosaccharides at a ratio of 50:50, during the storage period 60 days at 12°C. In this respect, the correlation between the viable count of LAB and the pH value ($r = -0.632$, $P=0.005$) suggests that the existence of LAB beside the probiotic strains contributed to decreasing the pH value of cheese during storage. As well, lactic acid produced by *Lactobacilli* strains could be used as food preservatives, flavouring agent, and emulsifier (Mack *et al.*, 1999). With regards to the a_w of the cheeses, and according to Mathlouthi (2001), determination of water content, whatever the accuracy of the analytical method, is not adequately informative in regards the stability of the food product tested. Water activity (a_w) gives a supplement of information as it accounts for the availability of water for degradation reactions. The understanding of why some products are more stable than others at the same a_w needs interpretation of water structure. In particular the interactions such as hydrophilic and hydrophobic

interactions between water and the components of the foodstuff and the effect of the soluble molecules of the food on the hydrogen bonding in solvent water.

With regards to the availability of water for the degradation reactions in the food products, the high levels of a_w of both the cheeses was because of the use of low concentrations of salt in both the cheeses, due to the a_w which adversely correlated with levels of salt in the product (Guinee, 2004).

According to the report of Food and Drug Administration (2013), in regards to the evaluation and definition of potentially hazardous food factors that influenced microbial growth, the pH value of PSC and IPSC were lower than the minimum value for the growth and to prevent the production of toxin for numerous pathogens, including, *Staphylococcus aureus* (toxin) *Clostridium botulinum* (growth and toxin), *Vibrio parahaemolyticus*, *Shigella* spp., *Campylobacter* spp., *Bacillus cereus*, *Vibrio vulnificus* and *Clostridium perfringens.*

6.4.3 Protein concentration in cheese whey

The detection of protein in the whey of cheese, with and without inulin by using estimation of the absorbance at A^{540nm}, was made. The protein levels in the whey of cheese, with and without inulin, were expressed as mg per ml of whey of both cheeses, and are shown in Figure 6.7. No significant differences ($P > 0.05$) between the two wheys of the cheeses, in relation to the whey content of protein for both cheeses, t test was noted. The level of protein in whey solutions from cheese, with and without inulin was 2.171 ± 0.080 and 2.172 ± 0.040 mg ml^{-1}, respectively. The biuret reaction can be used for both qualitative and quantitative analysis of protein and it is influenced by the presence of peptide bonds in proteins. When a solution of proteins is treated with cupric ions Cu^{++} in a moderately alkaline medium, a purple colour or a blue-violet complex from Cu^{++} and peptide complex is formed, which can be assessed quantitatively by a spectrophotometer in the observation area.

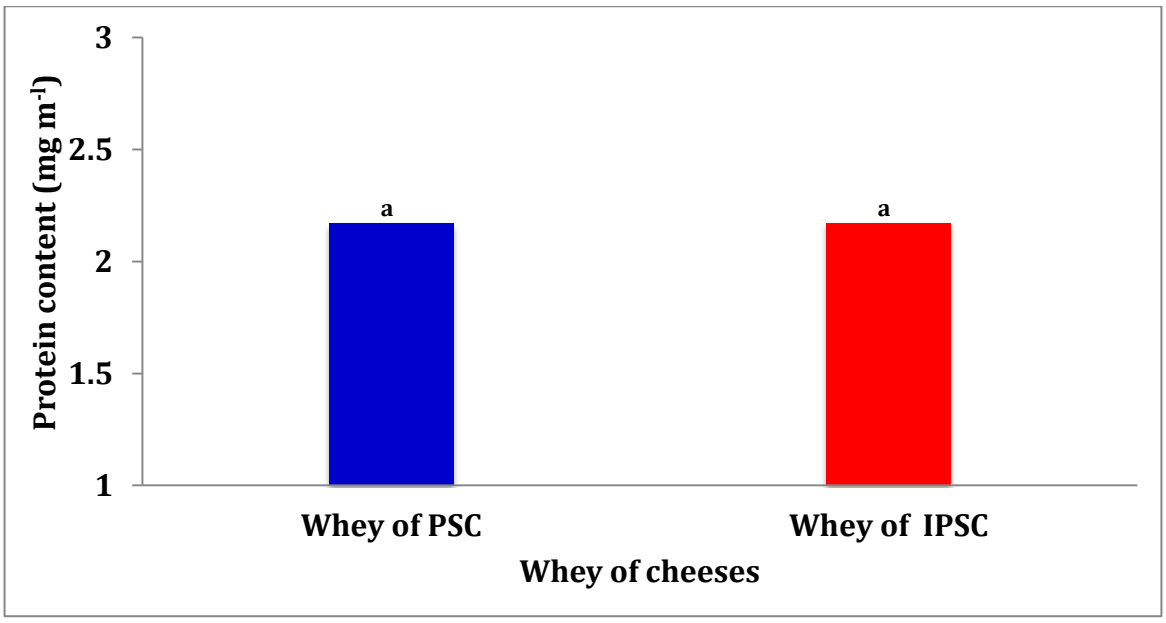

Figure 6.7. Protein concentration (mg ml^{-1}) in whey of probiotic soft cheese with and without inulin.

So, the biuret reagent is an alkaline copper sulphate solution. Each copper ion forms a complex with 5 or 6 peptide bonds (Burtis *et al.*, 2012). The determination of total protein in food highlights an analytical challenge (Mariotti *et al*., 2008). In relation to determination of total proteins in whey of PSC and IPSC and as mentioned by Mariotti *et al*., (2008) proteins are difficult to define biochemically because they can contain a wide diversity of amino acids that comprise the primary structures of proteins. Secondary and tertiary structures could differ, which are dependent on proteins status or the methods of purification used. Regardless of the structure, the amino acid composition is relevant for nutritional purposes, and dairy proteins are regarded as being of high quality. Cheese whey is the material obtained as a by-product of cheese production during curding and draining. It can be concentrated or dried to produce a material high in proteins, with variables quantities of lactose or acid, depending on the type of cheese and process. Whey protein is commonly marketed and used as a dietary supplement and various health claims have been attributed to it in the alternative medicine community (Marshall, 2004). Its components are beta-lactoglubolin (~65%), alpha-lactoalbumin (~25%) and bovine serum albumin (~8%).

These are soluble in their native forms, independent of pH value (Haug *et al*., 2007). In the dairy industry, whey proteins are considered to be a material with functional properties that could contribute to dairy product characteristics and/or the human health promotion, and its applications in food and dairy products are gaining importance (Shenana *et al*., 2007). Technologically, several methods have been developed for using cheese whey; one of these methods is the manufacture of Ricotta cheese. The production of this product has been considered to be one of the most economical ways for the use of whey. The benefits of whey lies in its high levels of essential amino acids, branched chain amino acids, particularly leucine which contributes to increased muscle protein synthesis, in addition, weight loss, loss of body fat, and decreasing plasma insulin and triglyceride (Etzel, 2004). As well, whey protein has reported anticancer effects, particularly for urogenital cancers (Temime *et al*., 2003); it is also used in stimulate the growth of LAB, which is considered one of important applications for preparation of probiotic cultures, that grows slowly in milk. It is nutritive highly valued by-product in the cheese industry, which is still not used fully exploited in the food industry, although, it contains proteins of the highest biological value, good content of minerals and group B of vitamins (Drgalić *et al*., 2003). Furthermore, the amino acid cysteine in whey protein plays an important role in the synthesis of glutathione in the human body which is considered one of the most important cellular antioxidants.

In relation to the use of colorimetric methods in the measurement of total proteins in food, the Biuret method is fast and simple. The presence of proteins or peptides with 3 or more amino acids, with any other compounds with 2 connecting –CO–NH– groups, such as succinic diamide and malonic diamide, are required for completing the interaction in this method (Kołakowski and Sikorski, 2001; Hortin and Meilinger. 2005;Kohn *et al*., 2005). Hortina and Meilinger, (2005) reported that this specificity

may not be unlimited because there are a variety of compounds which are interactive in this method, such as some amino acids including histidine, asparagine, threonine, and serine, in addition to dipeptides, and other compounds. They also stated that the absorbance at 540 nm can be influenced by interactions of copper with both the peptide spine and side-chain amino acids. Some techniques, including isolation of protein or extraction of interfering materials such as lipids by using an appropriate solvent, are required for more complex matrices. The use of more specific sample preparation contributes to minimize the interference of the matrix. (Owusu-Apenten, 2002). The sensitivity of the biuret method is low in comparison to other colorimetric methods, and may be unsuitable for some applications, but it may not matter during analysis of protein-based ingredients which commonly have high levels of protein (Chang 1998; Krohn 2005).

6.4.4 Inulin concentration in cheese and whey of cheese with inulin

Calculating the amount of fructose present in the cheese and whey of cheese samples was achieved using a standard curve. The quantity of fructose was 13.45 ± 0.054 g $100g^{-1}$ probiotic soft cheeses spread with inulin (IPSC) and 6.35g ± 0.037 100 ml^{-1} whey of cheese. The data refer to the finding that there was a loss in the quantity of inulin in whey during the drainage process (Figure 6.8).The application of inulin in probiotic soft cheese may contribute to the protection the probiotic strains during consumption of cheese, through increased stability of strains at low pH values and high levels of bile salts, as demonstrated by the study of Araújo *et al*., (2010) who found that the application of probiotic bacterial strain *Lb. delbrueckii* H2b20, into cottage cheese containing inulin, showed good survival and desirable resistance to low pH values and high levels of bile salts when the cheese was exposed to conditions simulating those found in the GIT.

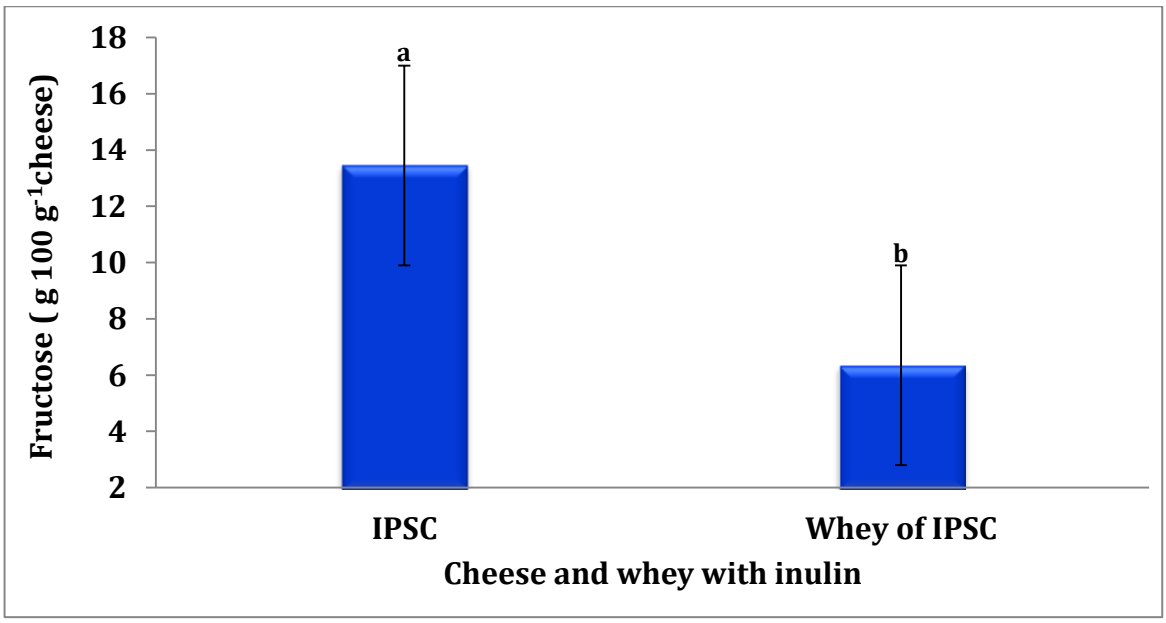

Figure 6.8. Fructose concentration (g $100g^{-1}$ cheese) in the whey and probiotic soft cheese with inulin (mean ± se) (n=3).

With regards to the beneficial effects of the probiotic soft cheese containing inulin, in addition to the health benefits of the probiotic strains in the product, it has been proposed that the beneficial effect of inulin could be due to the ability of bifidobacteria to change the colonic environment by antagonising harmful microorganisms via the production of bacteriocins, the successful competition for substrates or adhesion sites on the gut epithelium, and stimulation of the immune system. Polysaccharides and whey proteins found in food products, such as cheese both contribute to food stability, texture and shelf life. Potential interactions between proteins polymers and polysaccharides, such as inulin, could significantly affect the stability of the whole system (Dickinson and Stainsby, 1982). These interactions between macromolecules are specific or non-specific; either repulsive (i.e. electrostatic, hydration, steric discords) or attractive (i.e. hydrophobic, hydrogen bonds, Van der Waals, disulfide bonds); either weak (i.e. hydrogen bonds, Van der Waals, disulfide bonds) or strong (i.e. hydrophobic, hydration, steric repulsions). On the other hand, numerous researchers indicated that there are specific interactions which occur between whey proteins and polysaccharides, which may contribute to

formation of complex with improved characteristics (Dickinson and Galazka, 1991; Nagasawa *et al*., 1996; Mishra *et al*., 2001; Tesch *et al*., 2002; Akhtar and Dickinson, 2003; Neirynck *et al*., 2004; Einhorn-Stoll *et al*., 2005). In this regards, the loss in inulin quantities during the manufacture of the probiotic soft cheese with inulin, could be through the occurrence the interaction between inulin and whey protein of cheese, consequently, losing the inulin during the whey draining process.

6.4.5 Evaluation of the sensory properties of probiotic soft cheese without and with inulin

Statistically, no significant differences in the means preference scores for the evaluation the cheese attributes were found. The range of score1 (like extremely) for PSC was between 32%, and 44% and between 40% and 60% for IPSC. Score 7 (dislike extremely) was 0% for PSC, and between 0 and 7% for IPSC for all attributes. The highest score for both cheeses were in appearance and colour attributes, where in IPSC it reached to 52% and 60% respectively. For all the cheese attributes, the levels of score 4 and score 7 ranged between 0% and 8%. As shown in Table 6.6 and Table 6.7, the percentage of score 1, score 2 (like more) and score 3 from the total score for PSC, was 37%, 31% and 23%, respectively, whilst the percentage between score 4 (neither like or dislike) and score 7 ranged between 0% and 4%. As well as, for IPSC, when the highest percentage was for score 1, it reached 48%, 34% for score 2, 10% for score 3, and between 0% and 3% for score 4 and score 7. The mean scores of cheese attributes are shown in Table 6.4.

The mean scores of cheese attribute for both cheeses which were obtained from sensory evaluation ranged between 1.480 ± 0.127 for colour attribute of cheese with inulin, and 2.440 ± 0.271 for the appearance attribute for cheese without inulin. No significant differences between the cheeses were observed in the cheese attributes which were assessed, where the P values ranged between 0.153 for colour attribute

and 0.637 for texture attribute. The data indicated that the presence of the probiotic bacterial strains in soft cheese, with and without the prebiotic ingredient (inulin), contributed to the production of soft cheeses with good sensory acceptance, in regards the cheese attributes which were evaluated.The aforementioned results, which were obtained from evaluation of the attributes for the both cheeses by the assessors, indicate that there was high acceptability. Statistical analysis of the values of frequencies of cheeses attributes, by using T test (t paired), to compare between the attributes of cheeses, was made. The results indicate no significant differences between the cheese attributes which were evaluated. The mean scores that were recorded by the assessors for the attributes of cheeses evaluated were between score 1 and score 3 (Table 6.4). In addition, a high correlation was obtained between cheese attributes, which was evaluated using the preference test. High correlation was obtained between cheese attributes, which was evaluated using the preference test (Table 6.5).

Similar findings have been obtained by Buriti *et al*., (2005a), who stated that the incorporation of probiotic bacterial strains *Lb. acidophilus* in Minas fresh cheese made it perform better in the sensory evaluation of the product, which is considered to be a suitable carrier for the delivery of *Lb. acidophilus*.

Table 6.4. Mean scores of cheese attributes obtained from sensory evaluation of PSC and IPSC using 7-points score after storage 14 days at 2-5°C (mean ± se)(n=25)

Cheeses	**Cheese attributes**				
	Appearance	**Aroma**	**Texture**	**Colour**	**Overall Acceptance**
PSC	2.080±0.230	2.000±0.200	2.440±0.271	1.840±0.197	2.200±0.252
IPSC	1.800±0.238	1.800±0.153	2.240±0.323	1.480±0.127	1.960±0.261

PSC = Probiotic soft cheese without inulin, IPSC = Probiotic soft cheese with inulin.

Table 6.5. Correlation between cheese attributes obtained from sensory evaluation of PSC and IPSC, after storage 14 days at 2-5°C using 7-points score

Cheese attributes					
	Appearance	**Aroma**	**Texture**	**Colour**	**Overall acceptance**
Appearance	–	0.862	0.910	0.865	0.949
Aroma	–	–	0.838	0.850	0.874
Texture	–	–	–	0.859	0.949
Colour	–	–	–	–	0.856

Table 6.6. Scores obtained for PSC in the sensory evaluation according to preference of cheese attributes, performed after storage 14 days at 2-5°C (n=25)

	Scores of cheese attributes (%)				
Score	**Appearance**	**Aroma**	**Texture**	**Colour**	**Overall Acceptance**
1	36	36	32	44	32
2	36	36	20	36	36
3	20	20	32	16	24
4	0	0	8	0	0
5	8	8	4	4	4
6	0	0	4	0	4
7	0	0	0	0	0

score1=like extremely, 2 = like more, 3= like moderately, 4 = neither like or dislike, 5= dislike moderately, 6=dislike more, 7 =dislike extremely. PSC = Probiotic soft cheese without inulin.

In this regards, the results of Kasımoğlu *et al.* (2004) stated that the vacuum packaging for probiotic white cheese, manufactured with starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* and probiotic strain *Lb. acidophilus* 593N, contributed to obtaining the highest sensory scores for the product, when stored for 90 days at 5°C. Therefore, *Lb. acidophilus* could be used in the manufacture of probiotic white cheese to shorten ripening time and vacuum packaging is the best storage method. On the other hand, the high potential for a synergistic between probiotic strains and prebiotic ingredients could be obtained through the selection of a suitable combination between each other, and it contributes to promoting their growth, as reported by Ranadheera *et al.* (2010).

Table 6.7. Scores obtained for IPSC in the sensory evaluation according to preference of cheese attributes, performed after storage 14 days at 2-5°C (n=25)

	Scores of cheese attributes (%)				
Scores	**Appearance**	**Aroma**	**Texture**	**Colour**	**Overall Acceptance**
1	52	40	44	60	44
2	32	40	28	32	40
3	8	20	12	8	4
4	4	0	0	0	4
5	0	0	8	0	4
6	4	0	8	0	4
7	0	0	0	0	0

score1=like extremely, 2 = like more, 3= like moderately, 4 = neither like or dislike, 5= dislike moderately, 6=dislike more, 7 =dislike extremely. IPSC = Probiotic soft cheese with inulin

6.4.6 Texture profile analysis of probiotic cheese spread with and without inulin

The data in Figure 6.9, Figure 6.10 and Figure 6.11 show the effect of inulin on the texture of probiotic soft cheese spread through assessment of the hardness, cohesiveness, and adhesiveness of the both cheeses, using a texture analyzer. Texture attributes were influenced by the nature of the cheese matrix that resulted due to the use of inulin. The mean of hardness was 110.07 ± 6.51 for PSC, and 286.48 ± 56.65. The mean of cohesiveness for PSC and IPSC was 1.064 ± 0.271, and 1.171 ± 0.024, respectively. The mean of adhesiveness of cheeses was 434.2 ± 38.9, and 1649.74 ± 712.4 for PSC and IPSC, respectively. As shows in Figure 6.9, the hardness of a probiotic soft cheese spread, which was manufactured with inulin, was more than those of cheese which was manufactured without inulin. Statistically, there were significant differences between the hardness of the cheeses ($P< 0.05$).

These results similar to that results obtained by Buriti *et al.* (2007b), which stated that the use of inulin at level 10 % of fresh cream cheese curd contributed to increasing the score of all cheese attributes. As well, similar to that results obtained by Lucey *et al.* (2003) and Pinho *et al.* (2004), with regards to the differences in the hardness and cohesiveness of cheese, it may result from the differences in the composition of cheeses, and the procedures of the cheese manufacture. The data of

the sensory properties evaluation, which are illustrated in Table 6.6 and Table 6.7, show that the scores which were obtained by using a preference test for the texture attribute of IPSC was 44% and 32% in PSC. This was compatible with the instrumental testing of the both cheeses with the same scores for the overall acceptance attribute.

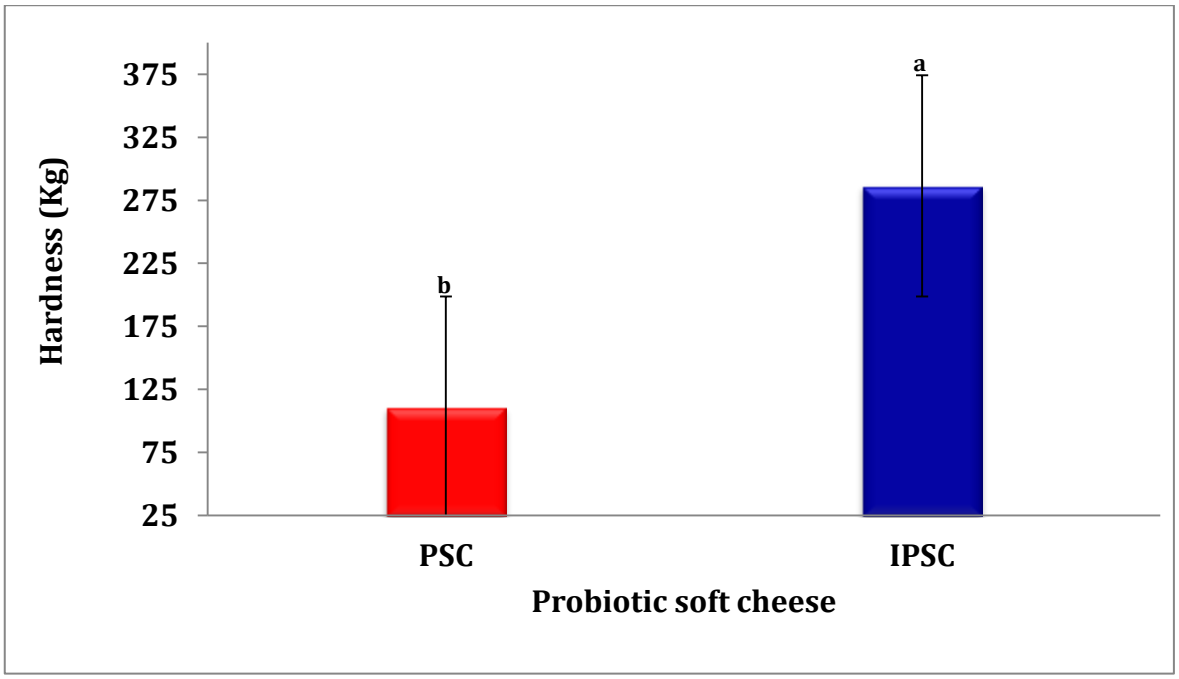

Figure 6.9. Hardness (Kg) of probiotic soft cheese with and without inulin, after 14 days of storage at 2-5°C under vacuum packaging. Measurement carried out at 21 ± 1°C using stable micro-systems texture analyser (mean ± se).

The results are in line with the results obtained by Cardarelli *et al.* (2008) with regards to the application of probiotic bacteria strains to Petit Suisse cheese with inulin, which contributed to the increase in the acceptability of the product after 21 days of the storage. Buriti *et al.* (2007a), reported that the application of strains *Lb. acidophilus* LA-5 and *B. animalis* BB12 into Minas fresh cheese, affected the instrumental texture and the sensory properties. In this regards, Gomes and Malcata (1999) stated that the use of a combination of bifidobacteria and *Lb. acidophilus* cultures, or other LAB, is considered to be helpful, with regards to the non-appearance of defects in certain sensory and texture attributes. Furthermore,

improvement of the nutritional values of "bifidus" products, besides increasing the growth rates and reduction of fermentation period, could be seen. The behaviour of the IPSC texture profile, after 14 days of storage, was different from what was observed for PSC. In this regards, the results of the researchers differ, in regards to the effect of probiotic addition on the textural properties of cheese.

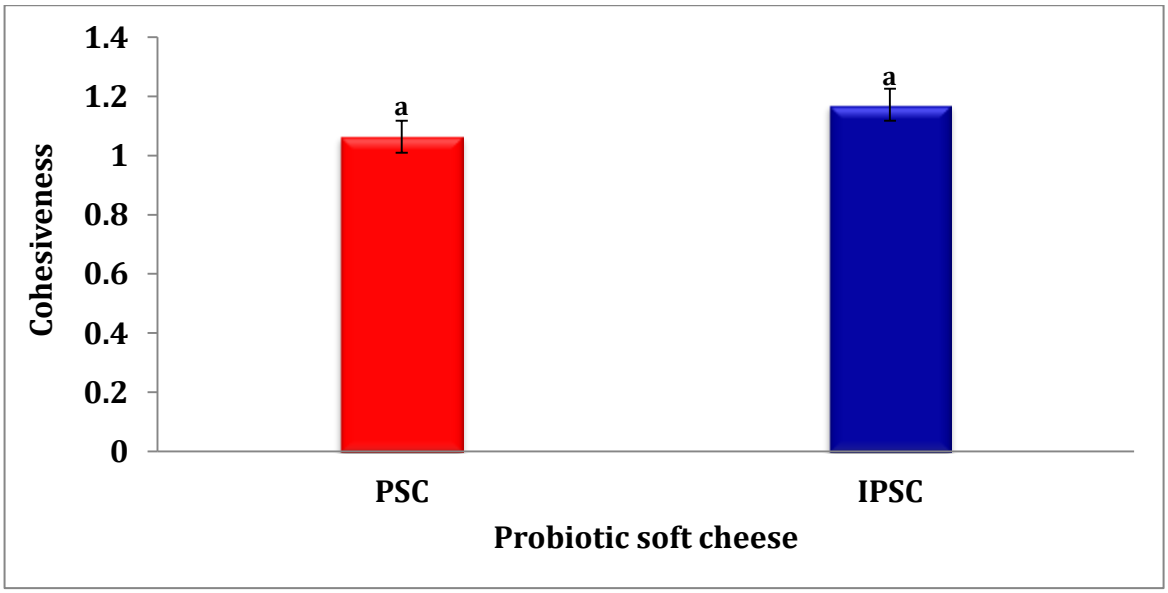

Figure 6.10. Cohesiveness of probiotic soft cheese with and without inulin, after 14 days of storage at 2-5°C under vacuum packaging. Measurement carried out at 21 ± 1°C using stable micro-systems texture analyser (mean ± se).

Some researchers indicated no significant change in the textural characteristics (Dinakar and Mistry, 1994), whilst others report positive textural changes, or improvement in the textural characteristics, when compared to the control (Ryhänen *et al.*, 2001; Katsiari *et al.*, 2002a). It seems that the species and strain of probiotic bacteria used, as well as the compositional and process factors in the production and storage of cheese are the primary causes of the differences aforementioned.

With regards to the cohesiveness and adhesiveness of both cheeses, significant differences between the cheeses in the adhesiveness attribute were observed, where the P value was 0.022, but no differences were found in the cohesiveness attribute, where the P value was 0.526. Data in Figure 6.9, Figure 6.10 and Figure 6.11 illustrate that the correlation between the hardness and cohesiveness was not significant, but it

was significant with adhesiveness. No significant correlation between the adhesiveness and cohesiveness was found, in spite of the significant correlation between some of the sensory textural parameters and rheological assessments.

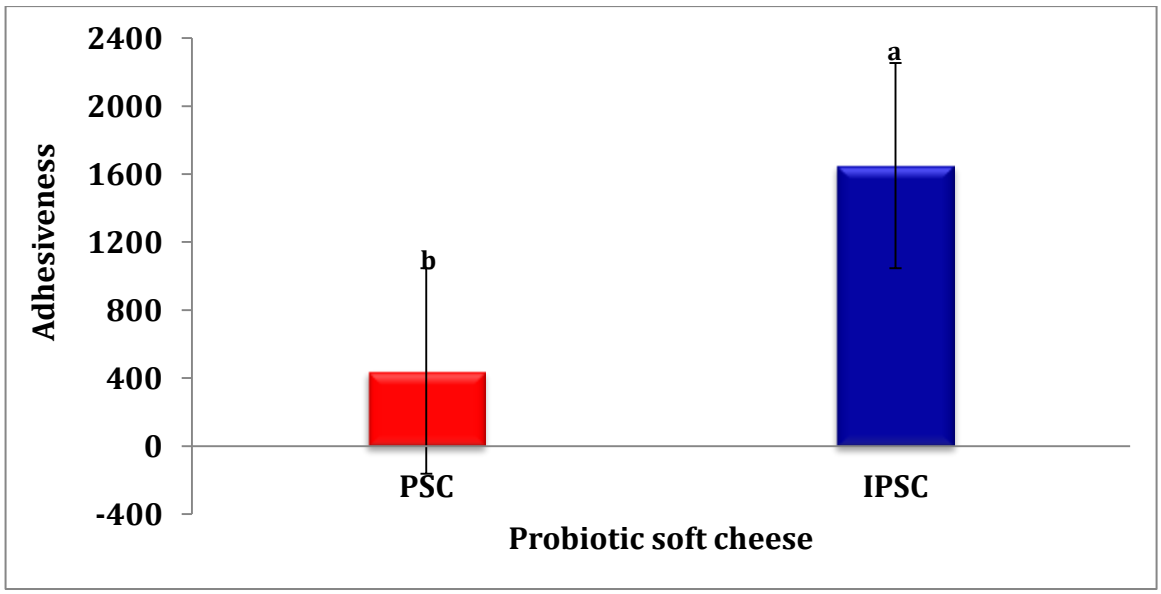

Figure 6.11. Adhesiveness of probiotic soft cheese with and without inulin, after 14 days of storage at 2-5°C under vacuum packaging. Measurement carried out at 21 ± 1°C using stable micro-systems texture analyser (mean ± se).

Delahunty and Drake (2004) stated that the use of the texture analyser for texture analysis was considered to be a complete substitute to the sensory evaluation. But the relation between the sensory awareness and instrumental measurements is important, because in some cases an instrumental test would be more cost-effective and/or suitable than sensory testing.

6.5 Conclusions

Starter culture and probiotic bacterial strains have the ability to growth in both M17 media broth and agar, with lactose and inulin together, and separately. The results demonstrated in this study demonstrate the capability of starter culture strains *Lc. lactis* subsp. and *Lc. lactis* subsp. *cremoris* to ferment lactose in the presence of inulin. The viable cell numbers of the three probiotic bacterial strains under assessment, in both cheeses, with and without inulin, attained, in general, values between 10^7 and

10^9 cfu g^{-1}, throughout the 14 days storage period, with an increase in the viable count of LAB, and decrease in the viable count of mesophilic and psychrotrophic bacteria in both cheeses.

The presence of inulin did not affect the dropping of the pH values of the cheese used. Inulin could be considered to be a good candidate for improving the sensory properties of cheese and increasing its acceptability. The success of incorporation of bifidobacteria in soft cheese spread, with and without inulin, will encourage and contribute to the development of healthier foods, with desirable sensory characteristics. The survival of probiotic bacterial strains in cheese, containing inulin, could represent a valid support and stimulate the strains, to produce higher amount of some bio-components with beneficial effects for human health.

Prolonging the period of the cheese manufacturing contributes to increasing the initial microbial loads, which may affect the behaviour of the beneficial microbes used, and increase the probability of cross-contamination of the cheese by unwanted microbes. Regarding the method for the packaging of probiotic soft cheese spread, the use of the vacuum, low pH value, and low storage temperatures, are considered to be important factors contributing to the delay of the growth of unwanted microbes in both cheeses during the shelf life. Practically, this type of cheese would be ideally manufactured from sterilized milk and packaged in aseptic conditions, in systems such as such as that for the Tetra Brik, to control the cross contamination that would be otherwise expected to occur during the manufacture process.

Chapter 7

General Discussion

Worldwide concerns over food safety, the quality of perishable food, in particular soft cheese, have elevated the importance of food microbiology to acceptable levels that has affected the consumers positively. Consequently, there is a growing need for comprehensive information about the occurrence and survival of the food-borne pathogens associated with dairy products, which allows better shelf life prediction. This study aimed to evaluate whether cheese starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* and probiotic bacterial strains *Lb. acidophilus* LA-5, *B. animalis* subsp*. lactis* BB12 and *Lb. casei* Shirota (LcS), and a prebiotic ingredient (inulin), could play a positive role in improving the safety and the quality of soft cheese, and also whether they modulate the sensory properties and extending the shelf-life of the product. Chapter 3 explains the results obtained from the study of some technological and functional characteristics of probiotic bacterial strains *Lb. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB12, and *Lb. casei* Shirota *in vitro*. Salt is considered to be one of the important ingredients used in the manufacture of most cheeses. It affects the viability and activity of both beneficial and harmful microorganisms in the product. Therefore, the salt resistance of the probiotic bacterial strains under study was assessed by studying their ability to grow in MRS broth and agar in the presence of salt at levels 0% as control and 3, 5, 8, and 10% (w/w). By assessing the relative growth into the criteria of Vinderola *et al.* (2002a), the growth of probiotic bacterial strains were negative at levels 8 and 10%, weak at 5%, and positive at 3%. These results agree with the results of Centeno *et al.* (1996a). Inactivation of probiotic strains at the high levels of salt is through the effect of salt on the cell membrane and its enzymes and components. In addition to cytoplasmic membranes and the enzymes inside the cell, which are responsible for metabolic

process, comparison with the low levels of salt, as reported by previous studies (Lanyi, 1974; Ventosa *et al.*, 1998; Wood, 1999), in addition the effect on nucleoids of bacteria. Alternatively, changing the cell membrane fluidity and lipid composition can affect the activity of bacteria, as stated by Denich *et al.* (2003). Furthermore, the high salt concentrations contribute to the inhibition of the bacterial enzymes, such as lipases, esterases, and proteinases (Gobbettia *et al.*, 1999a). On the other hand, the combined action of salt, which affect the Gram-negative and Gram-positive bacteria, as well, contribute to obtain better conditions for the food storage. Factors that affect the production of bacterial enzymes are the level of salt used, pH value and storage temperatures, in addition to the growth stage of cultures (Marquis, 1968; Membré and Burlot, 1994). In order to survive in the human GIT, probiotic bacterial strains must tolerate many environmental conditions (Chowdhury *et al.*, 1996), including, tolerance to the bile salts and low pH, which are considered to be an important requirement for the application of probiotic bacterial strains and for providing their health benefits to the consumers (Anal and Singh, 2007). One of the important criteria to be fulfilled and can be used as a probiotic is its ability to resist the effect of bile salts in the gastrointestinal tract (Lee and Salminen, 1995). However, there are no reports on the exact concentration to which a selected strain should be tolerant. On the other hand, a probiotic strain should survive transit through the stomach where the pH is low around 1.5 to 3. Therefore, tolerance to extremely acidic conditions is another important characteristic of probiotic strain (Dunne *et al*., 2001; Guo *et al*., 2009). It was observed that at pH 3.0, *Lactobacillus* showed better survival, even after 4 h of incubation. However, it was found that the percentage of survival decreased with decrease in the pH value. Therefore, the tolerance of probiotic bacterial strains to bile salts and acid also investigated in chapter 3. Probiotic bacterial strains differed in their survival under the bile salt levels tested. Resistance to bile is related to bile

salt hydrolase (BSH), an enzyme which helps in hydrolysis of conjugated bile, consequently reducing its toxic effect (Du Toit *et al.,* 1998). The greatest effect of the bile salt on the survival of strains was at 1% after incubation for 24 hours at 37°C, when compared to the control (0.0%), 0.3%, and 0.5%. Gómez Zavaglia *et al.* (2002), stated that the effect of bile salt is on the surface properties, and consequently, the changes occur in the metabolism of microbes. In addition, bile can affect the macromolecule stability as mentioned by Begley *et al.* (2005). Probiotic strain LA-5 was more resistant, compared to probiotic strains LcS and BB12, and depending on their sensitivity to the simulated gastric juice, they ranked as follows: LcS, BB12 and LA-5. In this regards, it was concluded that probiotic strain BB-12 shows high gastric acid and bile tolerance compared to other bifidobacteria, these properties improves its potential to provide a health benefit to the host. The effect of probiotic strains on the human body depends on their viability and physiological activity in the intestine. With regards to resistance of strains to an acidic conditions, the results are in line with the results of previous studies that carried out by Chou and Weimer (1999) who observed that the growth density of strain *Lb. acidophilus* ATCC 11975 at low pH was lower than those observed by strains *Lb. acidophilus* ATCC 43121, *Lb. acidophilus* ATCC 33200, and *Lb. acidophilus* ATCC 4962 and Dunne *et al.* (1999) who showed that the probiotic strain *Lb. salivarius* UCC118 has the potential to successfully transit the human stomach and may possess the ability to reach the intestinal environment in which they may function effectively. But, when exposed to human gastric juice the bifidobacteria strains proved significantly less acid resistant than those of lactobacilli. Most microbes have a low rate of survival and viability in acidic environments, such as that occurring in the human stomach, where the pH value ranges between 1.5 and 3.5 (Huage *et al*., 2014). Probiotics are a heterogeneous group of microorganisms where variability with respect to growing, survival and functionality features can be

expected (Mishra and Prasad, 2005). In this work, probiotic bacterial strains differed in their resistance to low pH value (pH 2) after exposure for 3 hours. Probiotic strain LA-5, was more resistant, when compared to the strains LcS and BB12, where the levels of all strains were decreased to varying degrees. Similar findings have been reported by Charteris *et al.* (1998a), who found that the probiotic strains of *Lb. casei* and *Bifidobacterium,* which when test for their resistance to bile salts *in vivo* showed significant reduction in their viability, in comparison with strains of other LAB. In general*, Bifidobacterium* cultures are more sensitive to acidic environment than *Lactobacillus* cultures and this is reflected through their poor tolerance to human gastric juice (Ross *et al*., 2005). The viability of bifidobacteria under acidic conditions depends on the pH value, the period of the exposure to acid and the species and strains used (Charteris *et al*., 1998). In fact, strains of LAB already are applied in many dairy products, such as cheese (Garcia-Ruise *et al*., 2014). Probiotic strains must survive within the GIT and able to tolerate the acidic environmental of the stomach, as well resistance to the digestive enzymes and bile acid at the start of small intestine. The viability of probiotic strains influenced strongly under acidic conditions, therefore, dairy products, such as cheese are preferred for use as carriers of probiotic bacterial strains to enhancement their survival in gastric juice, because of a buffering or protective effect for these products (Erkkilä and Petäjä, 2000; Shah, 2000; Cotter and Hill, 2003; Jensen *et al*., 2012). In order to choose the best combination of starter culture and probiotic bacterial strains for the application into cheese, the possible inhibitory activity by starter strains against the probiotic bacterial strains and versa, and / or between probiotic bacterial strains with each other should be considered. Tamime, (2008) stated that the interaction between starter cultures and probiotic bacterial strains are another characteristics which must be considered to obtain a great viable count at time of the consumption of the dairy products, such as cheese.

On the other hand, probiotic strains are influenced by other microorganisms during the fermentation process that continue for long time, but during a short fermentation time, the growth of most probiotic strain has hardly started and they do not appear to be affected. In this regards, a positive interaction between some probiotic bacterial strains is also known e.g. between probiotic bacterial strains of *Bifidobacterium* spp. and *Lb. acidophilus*. Because the presence like this activity may affect the ability of the strains to survive in the cheese, thus, failing in achieving the target of their application. As well as, numerous LAB change the environment and makes it unsuitable for the growth of not only pathogenic and spoilage microbes, but also potentially probiotic strains (Vinderola *et al*. 2002a,b). This inhibitory activity occurs through several ways, some of them, is the production of acids including lactic and other organic acids, hydrogen peroxide, bacteriocins and nutrient competition (Shah 2000a). In the present research, two methods were used for the detection of the presence of the possible inhibitory interactions between the strains. These assays revealed that there was no inhibition activity between probiotic bacterial strains *Lb. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB12 and *Lb. casei* Shirota (LcS) with each other and with the starter strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. It has been stated by Tharmaraj and Shah (2003), that the enumeration of probiotic strains in the presence of several types of similar organisms in the product is difficult. In present study, the enumeration and differentiation of probiotic bacteria and starter culture strains was carried out on HHD agar. Before studying the growth of strains on HHD agar, the strains were inoculated, separately and together into HHD broth, and tubes were incubated overnight at 37°C using gas pack containing CO_2 and H_2 atmosphere, in order to observe the ability of the strains to change the original colour of HHD broth, and the degree of the change, through the fermentation of fructose. Images of the probiotic bacteria and starter culture strains on HHD agar, and on

reference agars that were used for strains, were taken after their incubation for 72 hours at 37°C. The viability of starter cultures and probiotic bacterial strains could be enumerated on the reference agar, including, M17 and MRS agar, in addition to HHD agar.

Probiotic bacterial strains were enumerated separately using MRS agar as a reference agar for strains LA-5 and LcS and MRS agar with 0.05% L-cysteine for strain BB12. Starter culture strains were enumerated on reference agar M17. The enumeration of starter and probiotic strains together were applied on HHD agar. The results obtained indicated to that there were no significant differences in the viable count of strains BB12, LA-5 and starter strains (SC) on reference agars and HHD agar, but the viable count of LcS on MRS and HHD agar differed significantly. Enumeration of multiple strains of LAB and bifidobacteria together on one plate of HHD agar is easier and more economical than using separate plates for each strain.

Chapter 4 involves the manufacture of three types of soft cheese, including the cheese without starter culture (NSC), cheese with starter culture strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* solely (SCS), and the cheese with starter culture strains and probiotic bacterial strains *Lb. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB12 and *Lb. casei* Shirota LcS (PSC). Mehaia (1993) and Centeno *et al.* (2002), stated that the use of starter cultures during the manufacture of traditional soft cheese contributes to the flavour, body, and texture of the cheese, and helps to repress the growth of pathogenic and spoilage bacteria in the product. In this regard, the application of starter culture and probiotic bacterial strains in unripened soft cheese contributed to maintaining the microbiological characteristics and sensory properties during storage of 21 days at 2-5°C, when compared to NSC. Therefore, early acidification during cheese manufacture contributed to the reduction of the growth of unwanted microorganisms, which affect the quality and safety of the

product. Similar findings have been reported by Abd El-Salam and Benjamin (2003), with regards to the role of starter culture strains during the cheese manufacturing procedure. There is an increasing demand for new products in the food market. Hedonic scales and preference tests are common instruments used when decisions on market introductions are made. Sensory analysis is also used for quality control, for the comparison of product prototypes to understand consumer responses in relation to product's sensory attributes, and for product matching. It may also be used to track product changes over time with regard to understanding shelf life and packaging effects and to investigate the effects of ingredients or processing changes on the final sensory quality for the product and to research consumer perceptions of products (Murray *et al.,* 2001). As mentioned by numerous researchers, cheese is a promising food matrix for probiotics. But, strain selection and possible process regulations should be carefully applied to maintain probiotic cell viability during cheese manufacture and storage to maximum levels, with limit possible changes in organoleptic properties of the final product (Yerlikaya and Ozer, 2014). Vaclavik and Christian (2008) stated that the evaluation of food quality by using both objective and sensory properties tests is equally important for the measurement of food, and should correlate and complement each other. By questioning people and understanding their opinions about food products, a reliable method is applied to understand what they think about a particular type of food. This could be applied by asking the panellists to taste a food products and give their opinion on it, and it was carried out in this chapter using the preference of cheese attributes appearance, texture, flavour, colour and overall acceptance, and the intensity of cheese attributes of flavour, hardness, acidity, colour, and crumbliness. Numerous analytical methods have been established to evaluate the sensory properties of foods. Descriptive

analyses are considered one of the successful methods which have been used in the evaluation of cheese (Drake and Civille, 2003).

There were no significant differences in the intensity of most cheese attributes, where the differences were in colour and crumbliness attributes, and the colour of the PSC and SCS was closer. The scores of the test showed the intensity of cheese attributes, which were between score 2 and 5, with a high percentage of moderate score 4. By using the preference test for cheese attributes, NSC differed with the both PSC and SCS in colour attribute, without any difference between PSC and SCS. The scores ranged between 2 and 4, which referred to the high acceptance for all cheeses by the panellists. It could be said that only a few probiotic cheeses have been successfully developed for the market in comparison with yoghurts or fermented milks because of product quality that can be affected by the addition of some probiotic bacteria (Grattepanche *et al*., 2008). Chapter 5 involved the study of extending the shelf life for Middle Eastern style soft cheese, which is mostly manufactured by using rennet solely. The cheese was manufactured with starter culture strains that applied in chapter 4, and packaged under four packaging systems, including air packaging (AP) as a control, vacuum packaging (VP), modified atmosphere packaging (MAP), and brine packaging (BP), and then stored at 2-5°C for 50 days. The MAP method has been used by numerous researchers for several types of cheeses for this target by using mixtures composed of gases N_2 and CO_2 at varying levels (Mastromatteo *et al.*, 2010). Because of the white colour of soft cheese, which is sensitive to environmental changes and because the gases CO_2 and N_2 are colourless and odourless, they a suitable. On the other hand, CO_2 has a high solubility in both water and fat. In general, the inhibitory effects of CO_2 increase with decreasing temperature due to the increased solubility of CO_2 at lower temperatures (Jay et al., 2005), which contributes to the drop in the pH value of the product which affect undesirable microorganisms.

This method have advantages and disadvantages (McMillin, 2008). The permeability of the packaging materials used the exposure of the packed products to light and the storage temperatures used affects the quality of the packed product. During storage period, the microbial characteristics of the packaged products, dependent on the quality of milk used. The survival of heat resistant microbes during cheese manufacturing, and the microbial contamination after manufacturing process, plays an important role in determining the shelf life of the product, as reported by Cutter (2002), Del Nobile *et al.* (2009), and Mastromatteo *et al.* (2010). Data on the microbiological and physicochemical analysis of cheese samples under MAP suggests that this system was effective for delaying the growth of unwanted microbes, including PB, MAB and M&Y, and delaying or preventing the occurrence of unwanted changes, which result from the growth of these microorganisms in cheese samples, in comparison to the changes that occurred in cheese samples under control and brine packaging. Delaying or preventing these unwanted changes were through the work of gas mixes (30% CO_2:70% N_2) used, the low temperature during storage (2 - 5°C), low pH value (< 5.0) and low water activity. On the other hand, MAP can affect the quality of the product, mainly are to CO_2, which dissolves into the product and increases the level of carbonic acid in the product (Sivertsvik and Jensen, 2005). On the other hand, CO_2 may be interacting with lipids in the cell wall of unwanted microorganisms and consequently reduces the activity of microbes, or affect the bacterial cell directly. Nitrogen, being an inert gas, has no direct antimicrobial properties. It is typically used to displace oxygen in the food package either alone or in combination with CO_2, thus having an indirect inhibitory effect on aerobic microorganisms (Christopher and Hotchkiss, 2002). There were some differences between the physicochemical properties of cheese samples. Thiobarbituric acid – reactive substances (TBARS), the secondary lipid oxidation products, in soft cheese were also assessed. However, only

certain lipid peroxidation products generate MA, and it is neither the sole end product of fatty peroxide formation and decomposition, nor a substance generated exclusively through lipid peroxidation. TBA reactivity and oxidative lipid degradation support the suggestion that the MA assessment and the TBA test will offer some experimental information on the complex process of lipid peroxidation. On the other hand, and in particular in the biological systems, the use of MA analysis and/or the TBA test and interpretation of MA content in the sample, and TBA test response in study of lipid peroxidation, requires caution and discretion (Janero, 1990).Total Volatile Base Nitrogen (TVB-N) and Trimethylamine (TMA) were determined, using TCA- extract by steam distillation. The levels of TBARS, TVB-N and TMA in the samples under MAP were lower than those detected in cheese samples under AP, VP and BP, which increased during the storage period. Some factors contribute to the decrease the quantities of TMA, TVB-N and TBARS in cheese, including, the control on the storage conditions, the low pH value of cheese, storage under dark conditions, and low levels of unsaturated fatty acids in cheese, which contributes to decreased lipid oxidation process, which is basically a free - radical chain, which occur in three stages. These stages are beginning, spreading and termination, through oxidation of unsaturated fatty acids, and the formation of odourless, tasteless hydroperoxides. In addition, the stability of the final product depends on the levels of polyunsaturated lipids, total surface area of lipids, and the nature of surface materials, as stated by O'Connor and O'Brien (1995), German (1999), and Park (2001), or through the interaction between CO_2 and high-intensity light, which negatively affects the colour of the product through oxidation of the pigment particles (Colchin *et al.*, 2001). In addition, the decrease in a redox potential, and lowering the initial viable count of microbes in cheese contributes to low levels of TMA, TVB-N and TBARS, which reflect the effect of high viable count of microbes, that were observed in cheese samples

under air packaging, which contributes to spoilage of the product, in comparison to those samples that were packaged under MAP system. Descalzo *et al*., (2012) and Orczewska-Dudek *et al*., (2012) stated that the application of suitable conditions in the manufacture of cheese, such as using low or medium temperatures in the manufacture and packaging processes, and a proper storage temperature during the storage of cheese. As well, the use of appropriate container, contributes to reduce oxidation levels in the product. During the sensory evaluation of the odour attribute of cheese, the samples stored under MAP remained in the class 1 (score > 4.0) until the end of storage, in comparison with the samples under the BP and AP, where their class was changed to class 11 after 30 days of storage (score < 4.0). The colour of cheese samples under control differed with the samples under VP and MAP, which were close, although the lightness parameter of the cheese samples under BP was high, when compared to those that were recorded in other packaging systems. The results of the microbiological analysis and the sensory evaluation for an odour attribute during the storage period, in addition to the levels of TBARS in cheese under all packaging systems, concluded that the MAP method contributes to delaying the unwanted changes, and keeping the quality of the cheese for a long time, when compared to the samples used under other systems. In this respect, cheese characteristics, such as pH and water activity, contributed to increasing its shelf life more than those observed in some types of cheese, stored using this system with high levels of CO_2, in order to extend their shelf-life, such as Ricotta Cheese under 95% CO_2 (Del Nobile *et al.*, 2009) the team found that the quality loss of the product was after 8 days of storage 95 CO_2:5 N_2 at 4°C and Grated Graviera cheese under 100% CO_2, 100% N_2 or 50% N_2:50% CO_2 (Trobetas *et al.*, 2008) they found that the shelf life of cheese samples packaged using 100% N_2 or 50% N_2:50% CO_2 was extended by more than 2.5 weeks under dark conditions. The application of a prebiotic ingredient

(inulin) to probiotic soft cheese was the subject of Chapter 6. Inulin is a functional food ingredient which offers a unique combination of nutritional properties and significantly improves organoleptic characteristics. The importance of inulin for modern food industry is basically due to its characteristics in improve the texture attribute, particularly in the low fat dairy products, such as cheese, besides that, it used as a fat and sugar replacer (Meyer *et al*., 2007). It also contributes to enhancing the health characteristics of food products and thus its properties as a dietary fibre and prebiotic are important (Tungland and Meyer, 2002). The texture attribute of cheese plays important role in the determine identity of product and consumer acceptance (Drake *et al.,* 2003). In this work, the application of inulin in probiotic soft cheese contributed to the improvement of product texture, in comparison to probiotic soft cheese without inulin and stored under the same conditions. Because inulin's possess very specific technological characteristics that enable its use in food industry in the development of novel functional food products with improved nutritional and organoleptic characteristics. Therefore, it has wide applications in various types of foods, such as fresh cheese (Frank 2002; Kuar and Gupta 2002; Gibson *et al*., 2004; Čepo 2012; Lugovska *et al*., 2013).

In this respect, Shah (2004) stated that the combination of prebiotics and probiotic bacteria contributed to modulate the gastrointestinal microbiota, and they have the potential to supply functional food for health benefits. As well the development of the functional foods lies in the use of probiotics, prebiotics, and synbiotic in the modification of the activity of the GIT (Rafter, 2002). In this regards, the application of inulin into the probiotic soft cheese under study, in order to improve the sensory properties of the product, depend on its characteristics, and the degree of polymerization. The synbiotic was prepared by the application of inulin into a probiotic soft cheese, which was manufactured with a combination of starter culture

strains *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris,* and probiotic bacterial strains LA-5, BB12 and LcS, and storage was at 2 - 5°C for 14 days under vacuum packaging. Before the application of inulin into the probiotic soft cheese, a study of the ability of starter and probiotic strains to grow on M17 broth and agar media, which contained inulin and lactose separately, was carried out. The ability of starter culture strains to ferment milk in the presence of inulin reflects their ability to ferment milk during the manufacture of soft cheese with inulin. Similar findings was obtained by Su *et al.*, (2007). The application of inulin into soft cheese did not affect the microbiological characteristics of the product, in comparison with cheese without inulin, but there was an effect during the storage period. Some physicochemical characteristics of PSC and IPSC differed, including, a_w and TS. Considering the survival of strains SC, LA-5, BB12 and LcS, the cheeses differed in their load of strains LA-5 and BB12, where a viable count of strains LA-5 and BB12 in cheese with inulin, after 14 days of storage, were higher than those found in cheese without inulin. This is important in regards to the quality of cheese and the consumers health, it was concluded that the production of acetate and lactate by probiotic strain BB12 directly were offering antagonistic action, rather than those resulting from pH lowering. In addition, survival of strain BB12 through the GIT has been shown support a healthy gastrointestinal microbiota (Jungersen *et al*., 2014). On the other hand, the results obtained by El-Kholy *et al*., (2010) on Domiati cheese, indicated that the probiotic strain LA-5 significantly affect survival of *S. aureus* and *E. coli* O157:H7 during cheese storage.

PSC and IPSC did not differ in their protein content. There was loss in the quantity of inulin with whey of cheese with inulin, which may be lost through its interaction with whey protein that were lost during draining during manufacturing process; however, the level of inulin which was retained in curd had a significant impact on the texture

of final product. Buriti *et al*., (2007a) and Gomes *et al*., (2009) stated that the use of combined cultures of such as *Lb. acidophilus* or other lactic acid bacteria is advantageous, due to absence of certain sensory and texture defects and improvement of nutritional value. The inclusion of probiotic bacteria may contribute to the distinct flavour and texture characteristics thus changing cheese quality. However, if the sensory properties do not match the costumer expectations, the product will fail in the market (Yerlikaya and Ozer, 2014). Sensory quality of the food products is considered the most important quality parameter and important in the sense of represent consumer like (Albenzio *et al*, 2013). The sensory evaluation of cheese attributes of appearance, texture, flavour, aroma, and overall acceptance, showed that the highest average sensory evaluation points were recorded in both probiotic soft cheeses made with and without inulin. All cheese attributes that have been assessed by the panellists were obtained on scores ranging between 1 and 3, which refer to a high acceptance for probiotic soft cheese, with and without inulin. The application of inulin in probiotic soft cheese contributed to increasing the hardness of IPSC, and similar results have been obtained by Kaur and Gupta (2002). Soft cheese is generally not aged for long periods and does not have a long shelf life.

These characteristics reflect the performance of these strains in the product, regarding to their effect on the quality and sensory properties of the final product, and as well in the human body after consumption of probiotic soft cheese, in relation to offering their health benefits for consumers. In this respect, the probiotic bacterial strains used in this study, showed differences in regards to responses for some tests which related to their functional and technological characteristics such as salt and bile salts. All cheeses obtained high acceptance by panellist, significant differences in colour and crumbliness attributes of NSC with both SCS and PSC, without differences between SCS and PSC were detected by the panellists during evaluation the sensory

properties of cheeses after 21 days of storage at 2-5°C. The manufacture of soft cheese with starter culture and packaged using MAP, contributed to extending the shelf life of the product about 20 days, in comparison to other packaging methods used, including AP, VP and BP, through delaying or preventing unwanted changes that caused by unwanted microorganisms. The storage of soft cheese under low temperature with low pH value of cheese in dark place, contributed to control the formation of TMA, TVB-N and TBARS that causes spoilage of the product during the storage of product. Inulin is a functional food ingredient which offers a unique combination of nutritional properties and important technological advantages. Thus, its application in combination with probiotic strains BB12, LA-5 and LcS into soft cheese contributed to improve the quality and sensory properties of the product, such as hardness attribute. In addition, there is also potential for formation of a synbiotic with strains BB12 and LA-5.

Chapter 8

Future Work

1- Study the potential to improve the technological and functional characteristics of probiotic bacterial strains, using the microencapsulation or biofilm techniques, in order to develop their survival under unsuitable environments, such as heat treatments, bile salts, and acidic conditions, and maintaining their capacity during the passage through the GIT.

The hypothesis: The application of microencapsulation or biofilm techniques could contribute to improving probiotic stability and offer protection of strains toward the unsuitable environments, which may encounter during the manufacture stages of cheese and a high acid stomach during consumption of the product.

Rationale: Scientific research regarding the use of the probiotic bacteria for technological and therapeutic purposes has been fast growing over the years and has generated significant interest to scientists and health specialists. Due to considerable beneficial health effects, these microorganisms are increasingly incorporated into the dairy products, such as cheese, however, many reports confirmed their poor survival and stability in the product. As probiotics are highly sensitive to many environmental factors, and most commercial strains are selected on the basis of their technological properties. Their survival in the GIT tract is also questionable. To overcome these problems, microencapsulation techniques are using an alginate microparticulate system and present the potentiality of various coating polymers such as chitosan and polylysine for improving the stability of this microencapsulation. In addition, offer adaptation and protection to the probiotic cells before encountering a high acid stomach environment and therefore can be utilized as an effective microencapsulation technique. Of special significance is the ability of microorganisms

to attach and grow on food and food-contact surfaces under favourable conditions. Biofilm formation is a dynamic process and different mechanisms are involved in their attachment and growth. These microorganisms initially are placed on the surfaces and later get attached, grow and actively multiply to form a colony of cells. In this respect, forming the organic polymers considered more importance through their contribution to a suitable colonization of microorganisms.

2- Incorporation of selected probiotic bacterial strains into soft cheese, and studying their survival after cheese consumption, in order to evaluate their resistance during the passage through the GIT, and an appraisal of the capacity of soft cheese to act as a good vehicle for the delivery of these strains to the consumers.

The hypothesis: The application of probiotic bacterial strains into soft cheese may contribute to protect them during consumption of product.

Rationale: Incorporation of probiotic bacteria in milk-based food systems, including soft cheese provides challenges in terms of maintaining viability and probiotic functionality during manufacture and shelf-life. In contrast to yogurt and fermented milk products. Indeed, numerous studies demonstrated that cheese can be an excellent carrier of some health-promoting bacteria, and that it also has convinced an advantages over yogurt and fermented milk as a delivery system for some probiotic strains. During this time, the added probiotic bacteria would need to grow and/or survive in sufficient numbers to deliver the probiotic effect in the GIT of consumers. Therefore, maintaining the survival of probiotic bacterial strains after consumption of probiotic cheese with selected organisms, with known viable counts at time of consumption, and the remaining portion of strains after consumption of the product, reflect the ability of soft cheese on carrying and protect the probiotic bacterial strains. Some probiotic mechanisms presuppose viability and physiological activity of the

probiotic at the target site. Since the target site may not be well-defined, and due to difficulties measuring the viability *in situ*, fecal recovery is often used to confirm viability of probiotics in the GIT.

3- Identification and enumeration of selected probiotic bacterial strains and pathogenic microorganism, such as *Listeria monocytogenes,* during the manufacture and storage of probiotic soft cheese, using the Polymerase Chain Reaction technique (PCR).

The hypothesis: The polymerase chain reaction (PCR) technique could be applied for the direct detection of the selected probiotic strains and *Listeria monocytogenes* in probiotic soft cheese.

Rationale: *Listeria monocytogenes* is a Gram-positive, non-spore forming, facultative bacterium that is now an established food-borne pathogen known for causing the disease listeriosis in humans. Apart from displaying typical symptoms associated with gastrointestinal infections, listeriosis is also characterized by flu-like symptoms. The primary way of infection is through the ingestion of contaminated food. Therefore, food serves as an important vehicle in the transmission of infection, which still plays a decisive role in the prevalence and continuation the cases of listeriosis around the world. The presence of *L. monocytogenes* in the environment poses a challenge in reducing cases of listeriosis. The food industry is incapable in producing food free of this pathogen. Its wide distribution increases the chances of cross–contamination between appliances or several products during processing. This organism also has the ability to colonize surfaces, forming biofilms that remain attached to equipment used in food production. The rapid identification of *L. monocytogenes* is important so that the appropriate antibiotic therapy can be initiated. Currently, molecular methods that assist the identification of selected probiotic

bacterial strains and *Listeria* to the species level in cheese include, Polymerase Chain Reaction (PCR) for detection of genomic divisions for *L. monocytogenes* and their correlation with strains, and restriction endonuclease analysis, has been employed to directly characterize the microorganism without the need for isolation.

4- Use of selected prebiotics ingredients in soft cheese manufacture, with selected probiotic strains. Study of the effect of ingredients on survival of strains, quality, and the sensory properties of cheese. In addition, detecting the levels of ingredients in the product, using High-Performance Liquid Chromatography (HPLC).

The hypothesis:

1-Incorporation of prebiotic ingredients oligofructose and polydextrose in probiotic soft cheese may improve organoleptic characteristics of the product and survival of the selected probiotic strains.

2- The use of high-performance liquid chromatography technique would assist the separation, identify and quantify each ingredient in cheese.

Rationale: The prebiotic ingredients, such as oligofructose and polydextrose, are considered functional food ingredients that offer a unique combination of nutritional properties and important technological benefits. They are found in many vegetables and fruits and can be industrially obtained from chicory roots. They can be used to fortify foods with fibre without negative effects and improve the flavour and sweetness of low calorie foods. In addition, they can influence the textural and organoleptic characteristics of the food products, such as cheese. As well as, combining probiotics with these ingredients in soft cheese could improve the survival of the bacteria crossing the upper part of the GIT, thus enhancing their effects in the large bowel. The use of High-performance liquid chromatography is for separation these ingredients in cheese and identifies the quantity of each ingredient.. This

technique depends on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each ingredient in the cheese sample interacts slightly differently with the absorbent material, causing different flow rates for the different ingredients and leading to the separation of the components as they flow out of the column.

5- Study the potential to use of Tetra Brik method in the packaging of probiotic soft cheese. This method could contribute to keeping the quality of the product, through protect the product from spoilage, offer the consumer the convenience, and extend the shelf life of the product. Moreover, protect the product from oxygen, flavours and light.

The hypothesis: The use of the Tetra Brik packaging system in the packaging of the probiotic soft cheese may assist in extending the shelf life of the product without adverse effects.

Rationale: Food packaging supports the preservation of the food products through the prevention of product spoilage and wastage, and by protecting products until consumption. The principal roles of packaging are to contain, protect/preserve food and inform the user. In this regards, packaging of soft cheese is considered an important matter in relation to preservation of products which have a short shelf life, through extending its shelf life. Therefore, it is essential to choose an effective method, such as the method of cheese production with tetra brik structure and preparing it for distribution. Carrying out the suggested method is through concentrating milk containing whey by separating the whey from the milk to obtain dry matter content in the concentrated milk corresponding to dry matter contents of the final product of between 30-60%. Concentration of milk should preferably be carried out through using an ultra-filtering device with a filter having a pore size such

that all proteins, fats and other constituents necessary for cheese production Packaging the concentrated milk using a packaging machine that continuously forms the packaging material into a tube. The tube is filled with contents, sealed transversely at selected locations, and severed to form individual, distribution-ready packages.

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Appendix A

A.1 Data relevant to chapter 2

Characteristics of desirable probiotic bacterial strains
Possess the "Generally Regarded As Safe"(GRAS)status
Production of antibacterial substances
Their metabolic activity must be beneficial
They must be non-pathogenic
They should stimulate the immune system with suitable cytokine stimulation
They must deliver recombinant proteins and peptides to the gastrointestinal tract.
They must not carry transmissible antibiotic resistance genes
They must be able to interact with the host microbiota and be competitive with the microbial pathogens
Costs should be low and they must maintain their viability and stability during manufacturing and storage
They must be easy to incorporate into the products and be resistant to the physicochemical processing

Appendix B

Sensory evaluation forms and photos

B.1 Sensory assessment of probiotic soft cheese (chapter 4)

Sensory evaluation of soft cheese with probiotic bacteria – Probiotic cheese

Panellist initials/ number……

Preference test of soft unripened cheese with probiotic bacteria – Probiotic cheese

Please taste each of the samples and indicate how much you're interesting in each attribute on the left by ticking one box per column. Write the product code at the top of each table

Product Code **Date :**

Attributes	**Hedonic (preference) scale**						
	Like extremely	**Like a lot**	**Like moderately**	**Neither like or dislike**	**Dislike moderately**	**Dislike a lot**	**Dislike extremely**
Appearance							
Aroma							
Texture							
Colour							
Overall acceptance							

Comments

269

Sensory evaluation of probiotic soft cheese (intensity of attribute)

Panellist initials/ number _____

Intensity of sensory attributes of unripened soft cheese with probiotic bacteria – Probiotic cheese

Please assess the intensity of the following attributes for each sample and mark the box that you feel that describes how intense each attribute is.

Product code **Date:**

Attributes	**(Intensity) scale**						
Flavour	Very weak	Weak	Slightly weak	Moderate	Slightly strong	Strong	Very strong
Hardness	Soft	Semi soft	Slightly soft	Moderately soft	Slightly hard	Semi hard	Hard
Colour	Unclean extremely	Very unclean	Little unclean	Moderate	Little clean	Very clean	Clean extremely
Acidity	Very weak	Weak	Slightly weak	Moderate	Slightly strong	Strong	Very strong
Crumbliness	Very crumbly	Semi crumbly	Slightly crumbly	Moderate	Slightly homogeneous	Semi homogeneous	Homogeneous

Comments

B.2 Sensory assessment of soft cheese (chapter 5)

Assessment of odour attribute of starter soft cheese

Please test of each sample and indicate how much you're interesting in odour attribute by ticking one box per product

Date:

Very bad = 1, Bad = 2, Moderate = 3, Good =4, Very good = 5

Cheese code	1	2	3	4	5

271

B.3 Sensory assessment of probiotic soft cheese with and without prebiotic ingredient inulin (chapter 6)

Sensory Evaluation of Soft Cheese with and without Prebiotics Ingredient (inulin)

Panellist number .....

Preference test of soft unripened cheese with probiotic bacteria and prebiotics (inulin)

Please assess each of the samples and indicate how much you are pleasure in each attribute on the left by ticking one box per column. Write the product code at the top of each table

Product Code: **Date:**

	Hedonic (preference) scale						
Attribute	**like extremely**	**like more**	**like moderately**	**neither like or dislike**	**dislike moderately**	**dislike more**	**dislike extremely**
Appearance							
Aroma							
Texture							
Colour							
Overall acceptance							

B.4 Probiotic soft cheese with and without inulin (chapter 6)

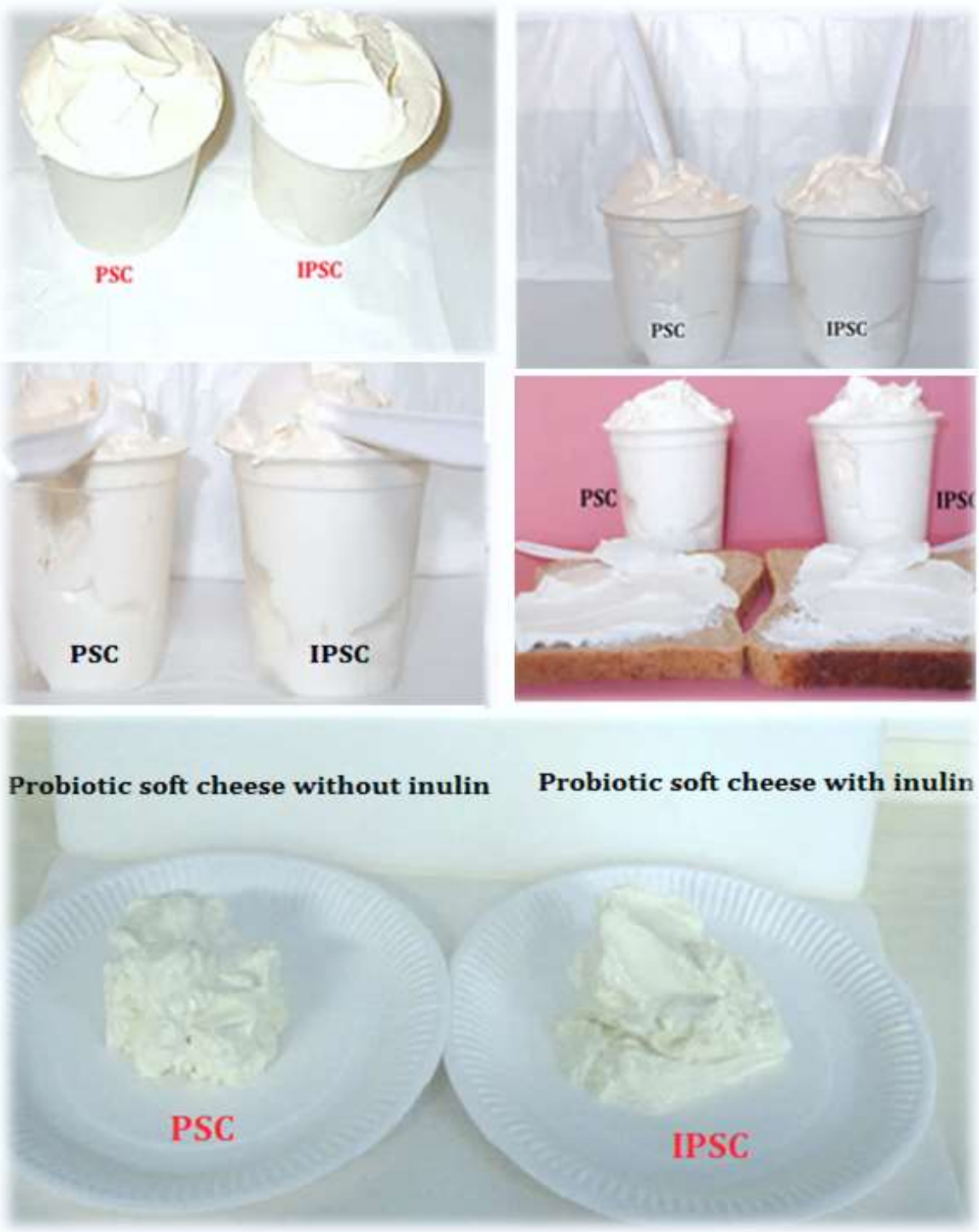

Photo 1: Probiotic soft cheese with and without inulin

B.5 The participants in sensory evaluation of probiotic soft cheese (chapter 6)

Photo 2. Panellists during a session of sensory properties evaluation of probiotic soft cheese with and without inulin.