Supplementation of Beverage, Yogurt and Probiotic Fermented Milk with Lentil and Pea Flour and Study of their Microbial, Physical and Sensory Properties after Production and During Storage

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This thesis is dedicated to my beloved husband, Omid

Abstract

Yogurt and probiotic supplementation with pulse ingredients is a very novel idea with a great potential to improve physico-chemical and nutritional properties of fermented dairy foods. Pulses are the dry seeds of legumes; including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and lupins (*Lupinus perennis)*. They are an excellent food source with numerous health-promoting benefits due to their nutritional composition such as complex carbohydrates, proteins, vitamins and minerals. Therefore, pulses could serve as prebiotic components to improve growth and stability of probiotic bacteria in fermented foods.

1-4% of pulse fractions including pea protein and fiber, chickpea and lentil flour as well as soy flour and protein concentrate were selected, for a comparison study. Supplements were characterized for their functional properties including water holding capacity, fat absorption capacity, protein solubility, emulsifying and foaming properties. Some novel foods were designed based on pulse ingredients and selected food matrices including orange juice, apple juice, yogurt (fermented with two commercial starter cultures) and two probiotic fermented milk (*Lactobacillus rhamnosus* or *Lactobacillus acidophilus)*. Preliminary results indicated that functional properties of pulse ingredients are varied upon their protein content and pH of the food carrier. The physical and sensory properties of supplemented beverages with 1% and 2% pulse fractions gave comparable results in terms of turbidity, cloud and visual stability, color and sensory attributes for both orange and apple juices in comparison with control samples, after production and during 28 days storage. All supplements improved the acidification rate of yogurt and probiotic cultures, but the highest effects were obtained with probiotic supplementation with lentil and soy flour. Preliminary results lead us to focus and develop the main studies on yogurt and probiotic supplementation with some selected pulse ingredients, namely pea and lentil flours.

For the main study, supplemented yogurt and probiotic fermented milk were designed. For this purpose, skim milk (9.5 % w/v solid content) was supplemented with 1-3% (w/v) lentil flour, pea flour or skim milk powder (for comparison) and they were inoculated with yogurt starter

cultures (*S. thermophilus* and *L. delbrueckii ssp bulgaricus*) or probiotic (*L. rhamnosus*). Acid production was monitored during the fermentation. All samples were stored at 4˚C and microbial growth, physical properties (pH, syneresis, and color), rheological properties (dynamic oscillation temperature sweep test at 4-50 ˚C), and sensory properties (only for yogurt; concerning flavour, mouth feel, overall acceptance and color) were studied after production or during 28 days of refrigerated storage.

Yogurt supplementation with 1-3% lentil flour enhanced acid production during fermentation, but the microbial population (CFU) of both *S. thermophilus* and *L. delbrueckii ssp bulgaricus* were in the same range in all lentil flour and skim milk powder supplemented yogurts. The average pH of samples decreased from 4.5 to 4.1 after 28 days of storage. Syneresis in 1-2% lentil flour supplemented yogurts was significantly higher than all other samples. With respect to color, ``a`` and ``L`` values did not significantly differ in all samples and remained constant after 28 days whereas, ``b`` value increased as a result of lentil supplementation. Yogurt with 3% lentil flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the non-supplemented control yogurt. 1-2% lentil flour supplemented yogurt showed comparable sensory properties in comparison with 1-2% skim milk powder supplemented yogurt and the control sample.

Probiotic fermented milk supplementation with 1-3% lentil flour enhanced acid production during fermentation, and the microbial population (CFU) of *L. rhamnosus* were comparable with non-supplemented control sample after production, while the CFU of 2% and 3% lentil supplemented probiotic were as high as the 1% skim milk supplemented sample, after 28 days storage. The average pH of samples decreased from 4.5 to 3.9 over 28 days storage. Syneresis in 1-3% lentil flour supplemented probiotic was significantly lower than all other samples. All lentil flour supplemented samples had significantly lower “L” values and higher “b” and “a” values in comparison with skim milk supplemented samples. Probiotic fermented milk with 1-3% lentil flour showed higher storage (G′) and loss (G″) moduli in comparison with all other samples.

Yogurt supplementation with 1-3% pea flour enhanced acid production during fermentation and the microbial population (CFU) of *S. thermophilus* and *L. delbrueckii ssp bulgaricus* were in the same range in all pea flour and skim milk powder supplemented yogurts, after production and during 28 days of storage. Pea flour supplementation enhanced survival of *L. delbrueckii ssp bulgaricus* after 28 days storage. The average pH of yogurt samples decreased from 4.5 to 3.75 after 28 days of storage. Syneresis in 1-2% pea flour supplemented yogurts was significantly higher than all other samples. With respect to color values ("a", "b" and "L"), after production and 28 days storage, pea flour supplementation did not alter redness or greenness of yogurts, but the yellowness in pea flour supplemented yogurt was significantly higher than other samples. Pea flour supplemented yogurt had the same lightness as other samples after production and after 28 days storage. Yogurt containing 1-3% pea flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the control sample. 1-2% pea flour supplemented yogurt showed comparable sensory properties, except for flavour; in comparison with 1-3% skim milk powder supplemented yogurt and control sample.

Probiotic fermented milk supplemented with 1-3% pea flour enhanced acid production during fermentation, and the microbial populations (CFU) of *L. rhamnosus* were comparable with skim milk supplemented and non-supplemented control sample after production and after 28 days storage. At day 28, the CFU of 3% pea flour supplemented probiotic fermented milk was the highest followed by 3-2% SM and 1-2% PF supplemented samples. The average pH in all samples decreased from 4.5 to 4.04 over 28 days of storage. Syneresis in 1-3% pea flour supplemented probiotic was significantly lower than all other samples. With respect to color, pea flour supplementation slightly changed the color which was not as light as skim milk supplemented samples and they showed more yellowness in final product after production and storage. Probiotic fermented milk with 1-3% pea flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the non-supplemented control samples.

Résumé

La fortification de yogourt ou de ferments laitiers probiotiques avec des légumineuses est une idée très originale ayant un fort potentiel pour améliorer les propriétés physico-chimiques et propriétés nutritionnelles des produits laitiers fermentés. Les légumineuses d'intérêt comprennent le haricot (*Phaseolus vulgaris*), le pois (*Pisum sativum*), la lentille (*Lens culinaris*), le pois chiche (*Cicer arietinum*) et les lupins (*Lupinus perennis*). Ils sont une excellente source alimentaire avec de nombreux avantages pour la santé en raison de leur composition nutritionnelle notamment leurs contenus en glucides complexes, protéines, vitamines et minéraux. Par conséquent, les légumineuses pourraient servir de composants prébiotiques pour améliorer la croissance et la stabilité de bactéries probiotiques dans des aliments fermentés.

Des concentrations de 1-4% de protéines de pois et de fibres, de pois chiche, de farine de lentilles ainsi que de la farine de soja et de concentré de protéines de soja ont été sélectionnés pour une étude comparative préliminaire. Ces ingrédients ont été caractérisés pour leurs propriétés fonctionnelles, y compris la capacité de rétention d'eau, la capacité d'absorption des graisses, la solubilité des protéines, propriétés émulsifiantes et moussantes. Des breuvages ont été conçus incorporant certaines légumineuses à des matrices de jus d'orange, de jus de pomme, de yogourt (fermenté avec deux levains commerciaux) et de lait fermenté (avec deux probiotiques *Lactobacillus rhamnosus* ou *Lactobacillus acidophilus*). Les résultats préliminaires indiquent que les propriétés fonctionnelles des ingrédients des légumineuses choisies sont variées sur la base de leur teneur en protéines et du pH du transporteur alimentaire. Les propriétés physiques et sensorielles de boissons supplémentées avec 1% et 2% des fractions des légumineuses ont donné des résultats comparables en termes de turbidité, d'homogénéité de la suspension et de stabilité visuelle, de couleur et d'attributs sensoriels pour les jus d'orange et de pommes en comparaison avec des échantillons témoins, et ce après la production et pendant les 28 jours de stockage. Tous les suppléments ont amélioré la vitesse d'acidification des cultures de yogourt et de probiotiques, mais les effets les plus importants ont été obtenus avec une la fermentation probiotique ajoutée de lentilles et de farine de soja. Les résultats préliminaires nous ont amené à se concentrer et à développer les principales

études sur le yogourt et les fermentations laitières probiotiques incluant certains ingrédients légumineux choisis dont la farine de lentille et la farine de pois.

Pour l'étude principale, le yogourt supplémenté et le lait fermenté probiotique ont été choisis. Pour ce faire, du lait écrémé (9,5% w contenu v / solide) a été supplémenté avec 1-3% (p / v) de farine de lentilles, farine de pois ou de lait écrémé en poudre (pour comparaison) et ils ont été inoculés avec des cultures de yogourt (levain à yogourt) (*S. thermophilus* et *L. delbrueckii* ssp *bulgaricus*) ou culture probiotiques (*L. rhamnosus*). La production d'acide a été suivie au cours de la fermentation. Tous les échantillons ont été conservés à 4 ˚ C et la croissance microbienne, les propriétés physiques (pH, la synérèse et la couleur), les propriétés rhéologiques (essai dynamique de balayage d'oscillation aux températures de 40-50 ˚ C), et les propriétés sensorielles (uniquement pour les yaourts; concernant la saveur, la sensation en bouche, l'acceptation générale et la couleur) ont été étudiés après la production ou au cours des 28 jours d'entreposage frigorifique.

La fortification des yogourts avec 1-3% de farine de lentilles a favorisé la production d'acide pendant la fermentation, alors que la population microbienne (UFC) de *S. thermophilus* et *L. delbrueckii ssp bulgaricus* étaient dans la même gamme dans tous les yaourts avec farine de lentilles et de lait en poudre écrémé. Le pH moyen des échantillons a diminué de 4,5 à 4,1 après 28 jours de stockage. La synérèse dans 1-2% des yaourts avec farine de lentilles était significativement plus élevée que pour tous les autres échantillons. En ce qui concerne la couleur, les valeurs 'a' et 'L' n'étaient pas différentes significativement dans tous les échantillons et sont demeurées constantes après 28 jours alors que, la valeur 'b' a augmenté par l'ajout des lentilles. Les yogourts à base de farine de lentilles (3%) ont exprimés des résultats rhéologiques plus élevés pour les modules conservation (G) et perte (G˝) en comparaison avec des échantillons préparés avec 1-3% de poudre de lait écrémé et le yogourt témoin non-supplémenté. Le yogourt préparé avec 1-2% de farine de lentilles a montré des propriétés sensorielles comparables au yogourt supplémenté avec 1-2% de poudre de lait écrémé et l'échantillon témoin.

Le lait fermenté probiotique préparé avec 1-3% de farine de lentilles a encouragé la production d'acide pendant la fermentation, et la population microbienne (UFC) de *L. rhamnosus* était comparable avec l'échantillon témoin de contrôle non-supplémenté, tandis que l'UFC des probiotiques préparés avec 2% et 3% de lentilles ont été aussi élevés que les échantillons préparés avec 1% de lait écrémé, après 28 jours de stockage. Le pH moyen des échantillons a diminué de 4,5 à 3,9 après 28 jours de stockage. La synérèse dans les probiotiques préparés avec 1-3% de farine de lentilles était significativement la moins élevé de tous les autres échantillons. Tous les échantillons préparés avec la farine de lentilles avaient des valeurs "L" significativement plus faibles alors que les valeurs "b" et 'a' étaient les plus élevés en comparaison avec les valeurs des échantillons supplémenté avec du lait écrémé. Les laits fermentés probiotiques à base de 1-3% farine de lentilles ont exprimé des résultats rhéologiques de stockage (G) et de perte (G ˝) plus élevés en comparaison avec tous les autres échantillons.

Les yogourts préparés avec la farine de pois 1-3% ont encouragés la production d'acide pendant la fermentation et la population microbienne (UFC) de *S. thermophilus* et *L. bulgaricus delbrueckii ssp* étaient dans la même gamme et ce pour tous les yaourts préparés avec la farine de pois et de lait en poudre écrémé, après la production et pendant les 28 jours de stockage. L'ajouta de farine de pois a renforcé la survie de L*. bulgaricus delbrueckii ssp* après 28 jours de stockage. Le pH moyen des échantillons de yaourt a diminué de 4,5 à 3,75 après 28 jours de stockage. La synérèse des yaourts préparés avec 1-2% de farine de pois était significativement plus élevée que tous les autres échantillons. En ce qui concerne les valeurs de couleur ("a", "b" et "L"), après la production et 28 jours de stockage, la farine de pois n'a pas modifié sur le plan de la rougeur ou de la verdeur, mais la couleur jaune des yaourts préparés avec la farine de pois était significativement plus élevée que les autres échantillons. Le yaourt supplémenté avec la farine de pois avait la même pâleur que d'autres échantillons après la production et après 28 jours de stockage. Les yogourts à base de farine de pois 1-3% ont exprimé des résultats rhéologiques de stockage (G) et de perte (G ˝) plus élevés en comparaison avec des échantillons préparés avec 1-3% de poudre de lait écrémé et l'échantillon témoin de contrôle. Le yaourt supplémenté avec 1-2% de la farine de pois a eu des propriétés sensorielles comparables, à

l'exception de la saveur; en comparaison avec le yaourt préparé avec de 1-3% de poudre de lait écrémé et l'échantillon témoin de contrôle.

Le lait fermenté probiotique et préparé avec de la farine de pois 1-3% a augmenté la production d'acide pendant la fermentation, et les populations microbiennes (UFC) de *L. rhamnosus* étaient comparables avec des échantillons de témoin de lait écrémé supplémenté et non- supplémenté après la production et après 28 jours de stockage. Au 28ème jour, l'UFC du lait probiotique fermenté et supplémenté avec 3% de farine de pois était le plus élevé suivi par les échantillons préparés avec SM 3-2% et 1-2% PF. Le pH moyen dans tous les échantillons a diminué de 4,5 à 4,04 sur 28 jours de stockage. La synérèse des préparations probiotiques avec 1-3% de farine de pois a été significativement plus faible que dans tous les autres échantillons. En ce qui concerne la couleur, la farine de pois a légèrement changé la couleur qui n'était pas alors aussi pâle que pour les échantillons de lait écrémé supplémenté et ils se sont avérés plus jaune dans le produit final après la production et de stockage. Le lait fermenté probiotique à base de farine de pois 1-3% a exprimé des résultats rhéologiques de stockage (G) et de perte (G˝) plus élevés en comparaison avec des échantillons supplémentés avec 1-3% lait écrémé en poudre et les échantillons témoins.

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Contribution of Authotrs

Part of the thesis research has been used to prepare several presentations at conferences as well as prepare manuscripts for publications. This research has been conducted by Fatemeh Zare and supervised by Dr. Valerie Orsat, Dr. Joyce Boye, Dr. Claude P. Champagne and Dr. Benjamin K. Simpson.

The author of this thesis was responsible for the design of experiments, experimental work, and manuscripts preparation under the guidance of the supervisors who helped in defining the problem and providing direct advisory input as the research work progressed. Details of papers presented, published and prepared are provided below:

List of publications and scientific presentations:

Boye, J.I., **Zare**, F., & Pletch, A., (2009). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*(2), pp. 414-431.

Zare, F., Boye, J.I., Orsat, V., Champagne, C.P, & Simpson, B.K., (2011). Microbial, physical and sensory properties of yogurt supplemented with lentil flour, *Food Research International (article in press).*

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Champagne, C.P, **Zare**, F., (2011), Biochemistry and Probiotics, as a chapter in the 2nd edition of the book: ``Food Biochemistry *and Food Processing``, Y. H. Hui, Wil, Wiley-Blackwell (Under revision)*.

Zare, F., Boye, J.I., Orsat, V., Champagne, C.P. & Simpson, B.K., (2009), Development of novel fermented yogurt and probiotics supplemented with pulse ingredients, Pulse Day, Sastatoon, SK, Canada (poster presentation).

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Zare, F., Boye, J.I., Champagne, C.P., Orsat, V., & Simpson, B.K. (2010), Physical and rheological properties of yogurt supplemented with lentil flour, CIFST, May 30th –June 1st, Winnipeg, MB, Canada, (poster presentation).

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There is also one manuscript soon to be submitted as below:

Zare et al., Functional properties and sensory evaluation of beverages supplemented with pulse ingredients.

Contribution to knowledge

The present work contributes to the expansion of the scientific knowledge base in the general area of food supplementation with pulses and its influence on the physical, microbial and sensory properties; mainly attributes of yogurt and probiotic supplementation. This research has contributed to the development of applications of pulses as food ingredients with both techno-functional and nutritional potential. The specific contributions to knowledge of this thesis work are described below:

1- Development of supplemented yogurt and probiotic fermented milk with pulse ingredients is a novel field of studies since previous work on dairy supplementation has mainly focussed on either pure sources of protein such as skim milk powder, whey powder or sources of fiber and carbohydrates such as inulin and resistance starch, etc.

2- Lentil flour and pea flour improved acid production during the fermentation of yogurt starter cultures. Lentil flour and pea flour improved the growth of *S. thermophilus* and *L. bulgaricus* in yogurt and provided the yogurt with high CFU counts (at least 10^8 CFU per serving) after production and following 28 days storage. Additionally, 2% and 3% pea flour increased the viable cell count of *L. bulgaricus* even more significantly than 1-3% skim milk powder in supplemented yogurt.

3- Lentil flour and pea flour shortened the fermentation time by *L. rhamnosus* in supplemented fermented milk. This finding is highly significant for its industrial application potential since it would result in huge time and energy savings. Lentil flour and pea flour increased the viable cell count of *L. rhamnosus* in probiotic supplemented milk after production and following 28 days storage in comparison with non-supplemented probiotic. Moreover, *L. rhamnosus* showed the highest stability in 3% pea flour supplemented probiotic, after production and after 28 days storage. All the supplemented probiotics contained at least 10^8 CFU per serving of *L. rhamnosus*, which is the minimum requirement of a probiotic type product.

4- Application of lentil flour and pea flour as a hydrocolloid component in yogurt formulation is also a novel field of study. Supplementation with lentil flour and pea flour increased the visco-elasticity of yogurt as well as probiotic fermented milk products and it resulted in stronger gel systems. This finding also has economical significance for commercial and industrial applications of pulses as techno-functional food ingredients.

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List of abbreviations

AAFC	Agriculture and Agri-Food Canada
CDC	Canadian Dairy Commission
CDIC	Canadian Dairy Information Center
CFU	Colony Forming Unit
CPF	Chickpea Flour
EA	Emulsifying Activity
EAI	Emulsifying activity indices
EFSA	European Food Safety Authority
Eh	Redox Potential
EMP	Emden–Meyerhof–Parnas
EPS	Exo-polysaccharide
ES	Emulsifying Stability
ESI	Emulsifying Stability Index
FAC	Fate Absorption Capacity
FACS	Fermentation Acquisition and Control System
FAO	Food and Agriculture Organization
FE	Foaming Expansion

G’	Storage Modulus
G”	Loss Modulus
GI	Glycemic Index
h	Hour(s)
L	Liter
LAB	Lactic Acid Bacteria
LCS	Loss of Cloud Stability
LF	Lentil Flour
min	Minute
NPC	Nano-powdered chitosan
PEP	Phosphoenolpyruvate
PF	Pea flour
PME	pectinmethylesterase
PP	Pea Protein
PteGlu	pteroylglutamic acid
PTS	Phosphotransferase System
rpm	Revolutions Per Minute
SM	Skim Milk

SMB	Skim Milk Base
SMP	Skim Milk Powder
UF	Ultra-filtered
USDA	United State Department of Agriculture
VSI	Visual Stability Index
WHC	Water Holding Capacity
WHO	World Health Organization
WP	Whey Powder
WPC	Whey Protein Concentrate
β-gal	β- galactosidase
β-Pgal	β- phosphogalactosidase

Chapter 1: General Introduction

Food supplementation and more precisely yogurt supplementation is not a new topic per se. However, yogurt and probiotic supplementation with pulse ingredients is a very novel idea and in addressing the justification behind this topic, a brief introduction on yogurt, probiotic, and pulses as well as their properties and importance in food trade and the food industry is presented here.

1.1 Yogurt, probiotic and prebiotic

According to ancient Persian tradition, yogurt was the secret of wealth and longevity of Abraham. More recently, Emperor Francis I of France was said to have been cured from a devastating illness by yogurt consumption in the 15th century (Tamime & Robinson, 1999). Although the origin of yogurt can be traced back to the Middle East, today, fermented milk products such as yogurt and probiotic products are manufactured in many countries around the world (Tamime & Robinson, 1999; AAFC, 2011; IDF, 2010). Yogurt is produced by fermenting milk with *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* which produce lactic acid (Damin, Alcantara, Nunes & Oliveira, 2009). Under Canadian law, these two bacteria must be present for a product to carry the name "yogurt". Yogurt is widely popular and marketed in various forms such as firm yogurt, stirred yogurt, drinking yogurt and frozen yogurt (AAFC, 2011).

Probiotic food products have been consumed by human beings in the form of fermented foods, for many years (Heller, 2001; Mahasneh and Abbas, 2010; Ranadheera, Baines, & Adams, 2010). According to the report by FAO/WHO, probiotics are: "Live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (Araya, Morelli, Reid, Sanders, Stanton, Pineiro, & Ben Embarek, 2002). The most common types of probiotics are Lactic acid bacteria (LAB) and include species from the *Lactobacillus, Pediococcus* and *Bifidobacterium* genera. Various species including *Lactobacillus rhamnosus* and *Bifidobacterium* have mainly been used as probiotics over the years (Tamime & Robinson, 1999; Ranadheera et al., 2010). An important characteristic of probiotic bacteria is that they need to survive through

the gastro intestinal track of the host. As S*. thermophilus* and *L. bulgaricus* are not expected to survive and grow in the host's intestinal tract, they are not categorized as probiotics by most scientists and are therefore considered as yogurt cultures (Senok, Ismaeel, & Botta, 2005). Hence a probiotic yogurt will typically contain conventional yogurt starters and additional probiotic bacteria so as to provide health benefits in humans by creating an improved balance in the flora of the intestines (AAFC, 2011).

Prebiotics on the other hand, are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon or in a given medium. This definition overlaps with the definition of dietary fiber, with the exception of the selectivity of probiotics for certain bacteria species. Peptides, proteins and lipids possess prebiotics characteristics, but some carbohydrates in particular have received the most attention, including lactulose, inulin, and a range of oligosaccharides that supply a source of fermentable carbohydrates for the growth of beneficial bacteria in the colon (Prado, Parada, Pandey, & Soccol, 2008).

1.2 Probiotic health benefits

It is generally accepted that daily intake of probiotics can contribute to improving human health (Lavermicocca, 2006). The health benefits of probiotics were first explained by the Russian scientist, Elie Metchnikoff who in 1907 observed the prolonged life in people who consumed a lot of fermented milk regularly and attributed it to the beneficial effect of lactic acid bacteria (Rasic, 2003). Probiotics help in maintaining a well balanced composition of the intestinal flora which improves the body's ability to tolerate certain disorders due to pathogens while maintaining the well being of the host (D'Aimmo, Modesto, & Biavati, 2007; Lourens-Hattingh & Viljoen, 2001). Some of the probiotic related benefits may result from the growth and metabolites produced by the probiotics in the media during the fermentation in cultured foods, such as probiotic yogurt or probiotic fermented milk; while some may result from the growth of certain species of probiotics in the intestinal tract following ingestion of foods containing bacteria (Lourens- Hattingh & Viljoen, 2001; Rasic, 2003). Several evidences support the

potential clinical applications of probiotics in the prevention and treatment of diseases such as gastrointestinal, respiratory and uro-genital tract diseases (Lavermicocca, 2006), improvement of the immune system, reduction of lactose intolerance (Gilliland, 1990; Kim & Gilliland, 1983), reduction of serum cholesterol level and blood pressure (Rasic, 2003), anti-carcinogenic activity (Gilliland, 1990; Ouwehand, Kirjavainen, Shortt, & Salminen, 1999; Rasic, 2003), improved utilization of nutrients and so improved nutritional value of food (Lourens-Hattingh & Viljoen, 2001).

1.3 Canadian yogurt production and consumption

Canadian yogurt production and consumption has risen significantly over the last decade. Canada's yogurt production in 2000 was 149,850 tonnes and it rose to 300,718 tonnes in 2010. Dairy products represented 6.43% (with a value of $ 988.2 billion) of the world trade market of agri-food products in the year 2009. In terms of Global Dairy trade, Canada represents 0.42% (with a value of $ 63.6 billion) by exporting dairy products mostly to the U.S. (CDIC, 2009a; AAFC, 2011). Yogurt consumption was 3.21 L (liter) per capita in 1988 and it dramatically increased to 7.7 L (liter) in 2009 in the Canadian diet (CDIC, 2009b, 2010).

1.4 Pulses and health benefits

Pulses are the dry seeds of legumes; including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and Lupins (*Lupinus perennis).* They are an excellent food source with numerous health-promoting benefits. Their nutritional composition includes complex carbohydrates (e.g. resistant starch and oligosaccharides), protein (20-23%), important vitamins and minerals (e.g., folate and iron) as well as antioxidants. They are low in fat content with a low glycemic index (GI) which all supports a healthy lifestyle. Pulses may help reduce the risks of coronary heart disease, diabetes and obesity, and can significantly lower serum cholesterol concentrations (Geil & Anderson, 1994; Pulse Canada, 2011). The Dietary Guidelines of the United States Department of Agriculture recommend eating 3 cups of legumes per week, including beans, peas, lentils and chickpeas (USDA, 2005). Canada's Food Guide to Healthy Living also recommends that consumers have meat alternatives such as beans,

lentils and tofu often and it further suggests that regular consumption of beans and other meat alternatives such as lentils can help lower the amount of saturated fat in the diet (Health Canada, 2007).

1.5 Canadian pulse production and consumption

Canada has the greatest leadership in the world for pulse production and export. In 1991, the Canadian total pulse production was 752,500 tonnes and in 2006 this amount increased to 3,685,000 tonnes which was mostly dry peas (2,519,900 tonnes) and lentils (629,000 tonnes). Canadian pulse production steadily rose to 5,185,300 tonnes in 2009 with the highest worldwide production of dry peas (3,379,400 tonnes) and lentils (1,510,200 tonnes). Canada is ranked the world's top producer of dry peas, the second largest producer of lentils, and one of the top ten producers of chickpeas and dry beans. Canada's pulse export, mostly in lentil (86%) and dry peas (13%); grew over the years 2006 to 2009. The total pulse export rose by 24% and 123% in terms of quantity and value, respectively. In 2009, around 75 % of Canadian pulse production was exported to over 150 different markets, which share nearly 40% of the global pulse trade (AAFC, 2011).

According to Statistics Canada, the total pulse disappearance, which is an approximation of pulse consumption; rose from 4.01 kg per capita to 4.10 kg per capita from 1998 to 2002 in the Canadian diet. According to a market research conducted by IPSOS REID (2010), in Canada the estimated average weekly cooked pulse consumption among Canadians who report having consumed pulses in the past six months is 1.3 cups and the median is 0.9. In regards to individual seeds, 66% of consumers indicated that they had consumed beans in the past six months. This amount dropped to just over half with regard to chickpeas (53%) and peas (52%), while consumption of lentils was limited to four-in-ten (41%) Canadians. So, in spite of this positive trend in pulse consumption in recent years, average pulse consumption in Canada is still low (Statistics Canada, 2011; IPSOS REID, 2010).

According to IPSOS REID, non adequate information about cooking or preparing the pulses and also not liking the taste or texture of pulses are the most frequent reasons for low consumption

of pulses in Canada. Canadians also may not naturally think about including pulses in their meal which is the biggest limitation to consumption (IPSOS REID, 2010, Pulse Canada, 2011).

1.6 Yogurt and probiotic supplementation to increase pulse consumption

Yogurt and probiotic fermented products are very popular in Canada as they are known to have numerous health benefits. There is great potential to develop new supplemented yogurt and probiotic products using pulses. Pulse ingredients, which suffer from under-consumption in the Canadian diet, could serve as a valuable nutritional source of prebiotic for yogurt starters and probiotics, since they contain a high amount of dietary fiber (around 15 g per cup), minerals and vitamin. In order to address the challenge of increasing pulse consumption in the Canadian diet and considering the fact that Canada has great leadership in pulse production and export, a project was initiated with the objective of developing novel food products such as pulse supplemented yogurt and pulse supplemented fermented milk.

There is an obvious potential for a synergistic effect when probiotics and prebiotics are combined appropriately, as prebiotics promote the growth and activities of probiotics (Ranadheera et al., 2010). Ingredients in food products could naturally contain prebiotics that facilitate improvement of the functionality of probiotic bacteria. Several research studies have been done to enrich food matrices with prebiotic components such as fortification in dairy products, fortification of meat products, cereals, beverages supplementation and infant formula supplementation. Most of these were aimed at increasing probiotic efficacy (Gibson, Probert, Rastall & Roberfroid, 2004). It is highly important that probiotic bacteria survive and grow in the food matrix; thus it is important to know the physico-chemical properties of the carriers used for probiotic delivery (e.g; buffering capacity and pH) as they are significant factors that influence survival of the probiotic and the subsequent potential probiotic effects during gastric transit (Ranadheera et al., 2010).

In this study, formulation of novel foods based on pulse supplementation has been considered. Initially, some food formulation designed based on pulse ingredients as supplement and fruit juice and dairy products as the food matrix (e.g; orange juice, apple juice, yogurt and probiotic

fermented milk). Preliminary studies on physico-chemical and microbial properties of our first formulations helped us to focus and develop our studies on yogurt and probiotic supplementation with some selected pulse ingredients. Thus, the main and specific objectives of this thesis are presented below.

1.7 Objectives

1.7.1 Main objective

The principal objective was the supplementation of food products with pulse ingredients and the study of the microbial, physical and sensory properties of the final food products.

The results of the preliminary studies on functional properties of pulse ingredients, beverage and fermented milk supplemented with pulse ingredients (specific objectives 1 and 2) lead our following studies (specific objectives 3 to 10). Hence, the specific objectives of this study are listed below along with the related chapters that describe each study in details.

1.7.2 Specific objectives

1. To characterize the functional properties of selected pulse ingredients to be used for this study (preliminary studies; chapter 3)
2. Evaluate the physical and sensory properties of supplemented beverages with pulse ingredients (preliminary studies; chapter 3)
3. Evaluate the effect of pulse ingredients added to milk on acid production by probiotic and yogurt starter cultures (preliminary studies; chapter 4)
4. To monitor the acidification rate due to microbial growth (*S. thermophilus* and *L. delbrueckii subsp. bulgaricus*) during fermentation in yogurt supplemented with lentil flour and evaluate the stability of yogurt starters in the final product and during storage (chapter 5)
5. Evaluate the physical, rheological and sensory properties of supplemented yogurt with lentil flour (chapter 5)

6. To monitor the acidification rate due to microbial growth (*L. rhamnosus*) during fermentation in probiotic supplemented milk with lentil flour and evaluate the stability of probiotic bacteria in the final product and during storage (chapter 6)
7. Evaluate the physical and rheological properties of supplemented probiotic with lentil flour ingredients (chapter 6)
8. To monitor the acidification rate due to microbial growth (*S. thermophilus* and *L. delbrueckii subsp. bulgaricus*) during fermentation in yogurt supplemented with pea flour and evaluate the stability of yogurt starters in final product and during storage (chapter 7)
9. Evaluate the physical, rheological and sensory properties of supplemented yogurt with pea flour (chapter 7)
10. To monitor the acidification rate due to microbial growth (*L. rhamnosus*) during fermentation in probiotic supplemented milk with pea flour and evaluate the stability of probiotic bacteria in the final product and during storage (chapter 8)
11. Evaluate the physical and rheological properties of supplemented probiotic with pea flour ingredients (chapter 8)

Chapter 2: Literature Review

In this study, fermentation of yogurt starters and probiotic (*L. rhamnosus*) has been studied in supplemented milk. Previous studies on these two categories have been reviewed separately.

2.1 Yogurt starter:

2.1.1 Yogurt starter's species

There are two typical yogurt starter microorganisms: *Streptococcus salivarius,* subsp. *thermophilus* and *Lactobacilus delbrueckii,* subsp. *bulgaricus.*

- ***Streptococcus salivarius* subsp. *thermophilus***

The taxonomic status of *Streptococcus thermophilus* has varied since the 1980s, due to the close relationship between *S. thermophilus* and *Streptococcus salivarius.* As a consequence, this microorganism was denoted as a subspecies (e.g. *S. salivarius* subsp. *thermophilus*). In 1991, a separate species status was proposed on the basis of both genetic and phenetic criteria. The proposal resulted in the restoration of *S. thermophilus* as a full separate species; it is no longer a subspecies of *S. salivarius* (Tamime & Robinson, 1999; Dellaglio, de Roissard, Torriani, Curk, & Janssens, 1994). Some characteristics of *S. thermophilus are*:

• Gram-positive, anaerobic, homo-fermentative lactic acid bacteria that produce l (+) lactate, acetaldehyde and diacetyl from lactose in milk.

• Growth at 45°C; absence of growth at 15°C may give rise to irregular cells and segments at 45°C.

• Most strains are able to grow at 50°C or survive heating for 30 min at 60°C.

• Spherical or ovoid cell morphology, <1mm in diameter and forming chains or occurring in pairs.

• Some strains produce exo-polysaccharide (EPS), and require B vitamins and some amino acids for enhanced growth rates.

• Absence of growth in methylene blue (0.1 g 100 mL^{-1}).

• The cell wall peptidoglycan type is Lys-Ala2–3, and 16S rRNA sequence data have demonstrated close association between *S. thermophilus*, *S. salivarius* and *Streptococcus vestibularis*.

- ***Lactobacillus bulgaricus***

L. bulgaricus is currently known as *L. delbrueckii,* subsp. *bulgaricus*. Some characteristics of *L. delbrueckii,* subsp. *bulgaricus* are:

• This organism ferments fewer sugars, produces d (+) lactate and acetaldehyde from lactose in milk, and some strains produce EPS.

• Slight growth occurs at <10°C and most strains are able to grow at 50–55°C.

• It is represented in Group I or Aa – the obligately homofermentative lactobacilli; the letter a indicates the affiliation to the *L. delbrueckii* group.

• The cells are rods with rounded ends, of 0.5–0.8 \ 2–9mm, and occur singly or in short chains.

• The cell wall peptidoglycan type is Lys-dAsp (Tamime & Robinson, 1999).

2.1.2 Typical growth of yogurt starters in milk; associative growth or symbiosis

Milk is a nutritionally rich medium that supports the growth of yogurt starters. The nutritional requirements of lactic acid bacteria are very complex. *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus* and many other lactic acid bacteria are unable to synthesize a full complement of amino acids; the required amino acid for their growth could be supplied from milk. Acid production and development of acidity is one of the most popular tests for monitoring starter

cultures in the growth medium and it is the metabolic activity of the organisms that indicates their growth rate. Yogurt starters follow different patterns of acidification in milk, in terms of incubation time and optimum temperature. The rate of acid development of *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus* increases when incubation temperatures increase up to maxima of 40°C and 45°C, respectively; the former organism is initially more active than *L. delbrueckii,* subsp. *bulgaricus* regarding acid production. For example, while *S. thermophilus* produces the maximum lactic acid at 40 ^{o}C after 2 hours lag phase, in *L. bulgaricus* lactic acid, production starts after 3 hours lag phase, but at an optimum temperature of 45^{o}C. There is also a noticeable difference in the rate of acid production by *S. thermophilus* and *L. bulgaricus* together, in comparison with the single strains, since the mixed starters show a significantly higher acid production rate after a one hour lag phase at an optimum temperature of 45^{o}C. Even though the activity of mixed strains is optimum at 45°C, since the ratio between *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus* should be maintain and/or achieve of 1:1; to have the desirable acidification and so the best quality of yogurt, it is suggested that the yogurt organisms should be propagated together at 42°C using a 2 mL 100 mL^{-1} inoculation rate (Tamime & Robinson, 1999).

Symbiosis is the phenomenon of growth association between two organisms *(S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus)* in a yogurt starter culture. This association is described as each organism providing compounds that benefit the other microorganism. This can be simply explained as the streptococci benefiting from the stronger proteolytic activity of the lactobacilli; in return, it provides certain compounds that stimulate the growth of *L. delbrueckii,* subsp. *bulgaricus*. Since both *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus,* as single cultures, can grow in milk, the term symbiosis should be replaced by “associative growth”. Symbiosis thus explains the fact that the acidification rate is greater when mixed yogurt cultures of *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus* are used in comparison with single strains (Pette and Lolkema, 1950; Tamime & Robinson, 1999; Rajagopal & Sandine, 1990).

2.1.3 Stimulatory factors by *L. delbrueckii,* subsp. *bulgaricus*

L. delbrueckii, subsp. *bulgaricus* stimulates *S. thermophilus'*s growth by releasing certain amino acids. Much research has been done on the essential amino acids of importance to *S. thermophilus* that are supplied by *L. bulgaricus.* According to Pette & Lolkema (1950), the associative growth between two yogurt starter organisms is mainly dependent on the production of valine by *L. delbrueckii,* subsp. *bulgaricus.* However, due to variations in the chemical composition of milk during the year, other amino acids may also be deficient and hence their research suggests that during the spring months, *S. thermophilus* requires amino acids leucine, lysine, cystine, aspartic acid, histidine and valine. During the autumn/winter months, glycine, isoleucine, tyrosine, glutamic acid, methionine, as well as the six amino acids mentioned above, were essential. Bautistae, Dahiyar, & Speck (1966), reported that *L. delbrueckii,* subsp. *bulgaricus* stimulates *S. thermophilus* by releasing glycine and histidine into the growth medium suggesting that histidine rather than valine is the most important requirement. The mechanism of this association is due to the more proteolytic activity of *L. delbrueckii,* subsp. *bulgaricus* rather than *S. thermophilus*, since the crude cell-free extracts of the yogurt lactobacilli stimulated the growth of *S. thermophilus.* It is also suggested that the addition of peptone, amino acids and, to a lesser extent, water-soluble vitamins, purines and pyridines improved the acid production in yogurt (Tamime & Robinson, 1999).

2.1.4 Stimulatory factors by *S. thermophilus*

Galesloot, Hasing, & Veringa (1968) reported that under anaerobic conditions *S. thermophilus* produces a stimulatory factor for *L. delbrueckii,* subsp. *bulgaricus* which is equal to or can be replaced by formic acid. According to their research findings, in severely heated milk (i.e. autoclaved and UHT), the stimulation was masked by a compound that could be replaced by formic acid. However, after the heat treatment that normally is used for a yogurt process (e.g. 85–90°C), *L. delbrueckii,* subsp. b*ulgaricus* needs the stimulatory factor produced by *S. thermophilus.* It is also shown that the presence of acid in milk at concentrations between 30 and 50 g mL^{-1}, increases the ratio of rods to cocci (Veringa, Galesloot, & Davelaar, 1986;

Bottazzi, Ledda, & Arrizza, 1971). Another stimulatory factor by *S. thermophilus* could be CO_2 (Ascon-Reyes, Asconcabrera, Cochet & Lebeaulti, 1995). According to their research, part of the CO_2 produced by the streptococci disappears during mixed growth with the lactobacilli. This phenomenon suggests that carbon dioxide produced by *S. thermophilus* stimulates the growth of *L. delbrueckii,* subsp. *bulgaricus.* In their study, Louaileche, Braquart, Saulnier, Desmazeaud, & Linden (1993) reported that CO_2 and sodium bicarbonate stimulated the growth of *S. thermophilus*. The source of CO_2 was apparently urea. Pyruvate and HCO_3 are other compounds that are produced by *S. thermophilus* and stimulated the growth of *L. delbrueckii,* subsp. *bulgaricus.* Other compounds that stimulated the growth of the lactobacilli are purine, adenine, guanine, uracil and adenosine, monosodium orthophosphate and sodium tripolyphosphate, oxaloacetic and fumaric acid and cysteine (Higashio, Yoshioka, & Kikuchi, 1977; Juillard, Spinnler, Desmazeaud, & Boquien, 1987).

2.1.5 Uncoupling phenomenon

Most experts agree that a balanced relationship between cocci and bacilli in yogurt manufacturing helps in improving the quality of the product, particularly in aroma and flavor emission. Because of the growth rate of each strain, the desirable stability may be altered during fermentation. Some other factors may affect this equilibrium, such as the acid production capacity of bacteria, time and temperature of incubation, inoculums ratio, the cooling rate of the product, and the inhibitors existing in milk and thermal processing conditions of milk prior to incubation (Larsen & Añón, 1990). As indicated earlier with regard to the typical growth of yogurt starters, in a mixed culture, the rate of acid development of *S. thermophilus and L. delbrueckii,* subsp. *bulgaricus* increases with increases in incubation temperature, up to a maxima of 40°C and 45°C, respectively, but the growth rate does not follow the same pattern as the acidification curve since lactic acid production continues even after microbial growth has stopped. The relationship between the acidification trend in yogurt and the growth of *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus* could be described as an uncoupling phenomenon. There are four main factors which can generate “uncoupling: pH, temperature, a_w and nutrition. These are described next.

Larsen & Añón (1990) studied acid production and growth rates in pure cultures of *Streptococcus thermophilus* and *L. bulgaricus*, and also cocci/bacilli ratios in mixed cultures of both species, using milk with modified water activity as the culture medium. They reported that the maximal growth rates were always at water activity lower than 0.992 for all strains in which water activity was adjusted with glycerol, while maximal acid production rates were at water activity between 0.992 and 0.983. These findings also demonstrate that while acid production is increased as a function of fermentation time, the final counts of bacteria may not increase and so growth and acid production were not necessarily parallel. All strains of *S. thermophilus* exhibited an "uncoupling" of growth from acid production. The optimal temperature for *S. thermophilus* and *L. bulgaricus* growth is 2°C to 8°C below the optimal temperature for acid production. In the study in question, the water activity with the greatest growth rate for the majority of *L. bulgaricus* and *S. thermophilus* strains was slightly lower than the one that had the major acidification rate. It is noticeable that this effect was produced when water activity was adjusted with glycerol, not with glucose (Larsen & Añón, 1990 and Radke-Mitchell & Sandine, 1986).

Seo, Lee, Chang, & Kwak (2009), studied the physicochemical, microbial, and sensory properties of yogurt supplemented with nano-powdered chitosan (NPC) during storage. In this study, the bacterial count of yogurt starters (CFU) in supplemented and non-supplemented samples was measured, as well as the acid production trend, during 20 days of storage. The addition of 0.3 to 0.7 w/v of NPC, as a nutritious supplement to the yogurt, resulted in varying the pH values ranging from 4.19 to 4.41 and the mean lactic acid bacteria counts ranging from 4.75×10^8 to 9.70×10^8 CFU/mL, respectively, when stored at 4°C for 20 days; this suggests the possibility of prolonging the shelf life of yogurt. On the other hand, the acidity increased during storage in each individual sample, but it was not parallel to the CFU count. For instance, in the control sample (non-supplemented yogurt), the microbial count at day 5 was 1.45×10^{10} and the pH measured was 4.1.While at day 15, pH value was 4.0 and the CFU count was 1.68×10^9.

Beal & Corrieu (1991), reported that the growth and acidification trends in a mixed yogurt culture are directly related to experimental conditions such as pH and temperature. They

quantified the simultaneous effect of pH and temperature on growth, acidification, and population of organisms. According to these authors, there are several studies supporting the idea that different optimum conditions are involved in “Uncoupling” according to the yogurt microbial strains.

Luedecking & Piret (1959) expressed the lactic acid production rate as a function of the biomass production rate according to the following equation [1]:

$$\frac{dP}{dt} = \alpha \cdot X + \beta \cdot \frac{dX}{dt} \qquad [1]$$

In this equation, *P* represents the lactic acid concentration, X the biomass concentration, *t* the time, α the specific non-growth-associated product formation rate, and β a constant. According to this equation, the uncoupling between growth and lactic acid production rates is probably due to the first term $\alpha * X$, i.e., the effect of non-growth-associated product formation rate. This factor becomes important especially in the stationary phase, since in that phase the biomass concentration is important. This phenomenon was further confirmed by Radke-Mitchell & Sandine (1986). Kailasapathy, Supriadi, & Hourigan (1996) studied the effects of replacing skim milk powder (SMP) with whey protein concentrate (WPC) on the buffering capacity of yogurt in both acidic and alkaline conditions. Buffering capacity is the degree to which a solution can resist a change in pH when either acid or alkali is added. Higher buffering capacity may increase the survival of live culture bacteria and their enzyme activity not only in the yogurt system during storage but also in the vivo system. It could help people with lactose mal-digestion to more readily consume dairy products. It is reported that in a similar amount of total solids content (18%) and protein content (8.3%), control yogurt with SMP and no added WPC showed less buffering capacity than the yogurt containing 20% WPC replaced with SMP. It is noted that partial replacement of SMP with WPC causes high buffering capacity at low pH and low buffering capacity at high pH. During three weeks of storage, the pH of control samples did not change significantly ($P<0.05$); therefore the supplementation of yogurt with WPC could produce enhanced buffered yogurt (Kailasapathy et al., 1996).

According to Mistry & Kosikowski (1985), the buffering capacity could affect the microbial count of lactic acid bacteria. They studied the buffering capacity and microbial growth of lactic acid bacteria in skim milk and highly concentrated ultra-filtered (UF) skim milk. In concentrated skim milk, the lactic acid production continued for 11 h, but pH slowed reduction significantly after the first 5 h. With increasing total protein content, more lactic acid was needed to change the pH, mainly below pH 5.5. Growth continued exponentially for approximately 4 h; subsequently, populations remained steady for the entire 11-h fermentation time. They reported that more concentrated skimmed milk resulted in a higher microbial population, while doubling times for lactic acid production increased slightly with higher protein concentration. Maximum lactic acid production averaged 1.2×10^{-6} (µmol lactic acid/CFU/min) and did not differ among different concentrates or starters. Considering the pH and starter growth, it was observed that maximum lactic starter organism population in skim milk and concentrated skim milk was observed at approximately pH 5.2 and it was not related to the total protein concentration. Lactic acid production, however, continued beyond this point at a slower rate with a doubling time of more than 3 h. Acid production in the absence of bacterial growth continued until pH 4.6. According to this study, lactic acid bacteria in milk can metabolize lactose to drop the pH to 4.4-4.2 which corresponds to about 0.7 % lactic acid concentration. Beyond this point, the cytoplasmic pH drops below its optimum and the cell stops multiplying. It is reported by Mistry & Kosikowski (1985) that at pH 5.0 to 5.2, bacterial growth slows significantly, but lactic acid production continues.

Streptococcus thermophilus and *Lactobacillus bulgaricus,* during associative growth, are dependent on an optimum growth temperature which is between 35-42^{o}C for S. *thermophilus* and 43-46^{o}C for L. *bulgaricus.* It is evident that nutritional components, more than temperature, are essential for an organism's growth; nutritional limitations could arrest microbial growth in a given medium *(*Radke-Mitchell & Sandine, 1986).

Mercade, Duperray & Loubiere (2003) studied the inhibitory factors in one industrial strain of *Lactobacillus delbrueckii,* subsp. *bulgaricus* during fermentation. Since lactic acid production is a procedure resulting from microbial growth, several factors such as pH, temperature and

availability of nutrients are essential factors for microbial growth as well as acid production. Regarding the uncoupling phenomenon; which is simply acid production raise while bacterial growth is slowed down and it happens as a consequence of lactic acid concentration causing an inhibitory effect on microbial cells, growth limitation factors are noticeable as well and should be considered in lactic acid production. Various factors, such as nutritional limitations that may arrest the microbial growth during fermentation, have been investigated by Mercade et al. (2003). Fresh cells were complemented with a solution of amino acids, salts, or vitamins to provide fresh nutrients, so they were able to grow in supernatants collected during the first stationary phase. However, cells in the fermenter without those nutrients were unable to grow. Also it has been observed that some components, due to *L. bulgaricus'*s growth, disappeared during fermentation which is suspected of being an inhibitor and so it is reported that both the culture medium and the intracellular content of the cells were responsible for the termination of the microbial growth. Growth of *L. bulgaricus* was also tested in non-fat milk as a culture medium that had the same features as the synthetic medium. A similar growth profile, characterized by a primary growth phase and then followed by a transient stationary phase and a second growth phase, was observed in this medium. Not only was the profile similar, but the growth limitation also appeared at the same time of cultivation for a similar biomass concentration (Mercade et al., 2003).

2.1.6 Biochemistry of yogurt starter's growth in milk

Several different metabolic pathways explain the biosynthetic functions of micro-organisms which could support their life-cycles. Each metabolic pathway consists of many reactions that are regulated by different enzyme systems; the levels of enzymes and their activities sustain and control the functions of the microbial cell (Stanier, Ingram, Wheelis, & Painter, 1987). Smaller molecules are the result of the breakdown of nutrients such as carbohydrates, proteins, lipids and other minor constituents that are present in the growth medium. These are consumed by the microbial cells and provide the energy requirements for their growth and survival. In lactic acid bacteria (i.e. the *lactococci, leuconostoc, lactobacilli, streptococci and bifidobacteria*), energy can only be supplied by the fermentation of carbohydrates (Lawrence,

Thomas, & Terzaghi, 1976). In yogurt, derived components from carbohydrates, proteins, lipids and other nutrients affect the metabolism and growth of *Streptococcus thermophilus* and *Lactobacillus delbrueckii,* subsp. *bulgaricus;* these can affect the characteristics and properties of the final yogurt. Two of the most important biochemical reactions during milk fermentation are "lactase" and "protease" activities by yogurt starters, which are explained in the following sections (Tamime & Robinson, 1999).

2.1.6.1 Metabolism of Carbohydrate

The main sugar in milk is lactose and it could be metabolized either through the homo-fermentative or hetero-fermentative metabolic pathways. *S. thermophilus*, *L. delbrueckii,* subsp. *bulgaricus* and *Lactobacillus acidophilus* ferment lactose homofermentatively, while *Bifidobacterium* spp. ferments the same sugar heterofermentatively (Tamime & Robinson, 1999). Lactose present in milk should enter the cytoplasm of the cell to be catabolized. According to Kanatani & Oshimura, (1994) and Marshall & Tamime, (1997) there is a specific system that is involved in lactose transport in the lactococci and certain strains of *L. acidophilus.* In this system, sugar (lactose) is phosphorylated by phosphoenolpyruvate (PEP) during translocation by the PEP dependent phosphotransferase system (PTS). This mechanism is known as PEP:PTS. This can be explained simply by the fact that β-phosphogalactosidase (β-Pgal) hydrolyses lactose-6-phosphate to its monosaccharide components. The galactose and glucose are then catabolised via Tagatose and Emden–Meyerhof–Parnas (EMP) pathways, respectively (Monnet, Condon, Cogan, & Gripon, 1996; Marshall & Tamime, 1997). However, dephosphorylation of galactose may take place and it will remain un-metabolized and sent out of the cell, but in both pathways the glucose and galactose converge at dihydroxyacetone phosphate and glyceraldehyde-3-phosphate where the three-carbon sugars become further oxidized to phosphoenolpyruvate and then produce lactic acid (Zourari, Accolas, & Desmazeaud, 1992; Tamime & Robinson, 1999).

S. thermophilus, *L. delbrueckii,* subsp. *bulgaricus* and *L. acidophilus* follow homolactic fermentation via the EMP pathway mainly for glucose catabolism. However, some organisms

including *Bifidobacterium* spp. have an alternative system for lactose transport into the cells that involves cytoplasmic proteins (permeases). This is for the translocation of the lactose without chemical modification and the mechanism could be similar to the lactose permease system in *Escherichia coli*. After the lactose enters the cell, it is hydrolysed by β-galactosidase (β-gal) to non-phosphorylated glucose and galactose. Glucose is catabolysed to pyruvate and the galactose is secreted from the cell. *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* utilize the galactose via the Leloir pathway with galactokinase as the first enzyme of the metabolic pathway, after all the glucose is depleted (Tamime & Robinson, 1999). Recalling that the growth of *L. delbrueckii,* subsp. *bulgaricus* could be stimulated by the presence of CO_2 during the fermentation, this CO_2 could be either produced from galactose metabolism by *S. thermophilus* via the Leloir pathway or from hydrolysis of urea. Lactate dehydrogenase is another enzyme that is also important in the control of carbohydrate metabolism. This enzyme in *Lactococcus* spp. is activated by fructose 1,6-bisphosphate aldolase and by tagtose 1,2-bisphosphate aldolase. The homolactic fermentation of *Lactobacillus* spp. may be different, as the enzyme from many species has been found to have innately high levels of activity that are independent of the presence of fructose 1,6-bisphosphate aldolase (Tamime & Robinson, 1999).

2.1.6.2 Lactase activity

Lactose catabolism mainly occurs by β-galactosidase (β-gal) derived from the yogurt starters in fermented milk processing. The optimum activity of streptococcal β-gal occurs at neutral pH, a temperature of 55°C in presence of buffer. The activity of β-gal is stimulated in the presence of Mg^{2+} and oxgall (0.15 mL100 mL-1), while EDTA can block the Mg^{2+} and may cause inhibition. It was also reported that the activity of the enzyme was greater in heated (63°C or 85°C for 30 min) milk than in raw milk, while the activity in a buffered system was greater than in whey or milk (Greenberg & Mahoney, 1984). Thermal denaturation occurs at 60°C, but stability can be enhanced by the addition of bovine serum albumin (Chang & Mahoney, 1994).

2.1.6.3 Production of lactic acid

Lactose catabolism may result in lactic acid or acetic acid production. *S. thermophilus*, *L. delbrueckii,* subsp. *bulgaricus* and *L. acidophilus* mainly hydrolyze lactose to lactic acid, while bifidobacteria produce acetic acid. Although the conversion process consists of many different biochemical reactions, it can be simplified by the following equation:

Lactose + Water = Lactic acid

Lactic acid is important during the manufacture of yogurt because it destabilizes the casein micelles and forms a yogurt gel. The destabilization reaction can be summarized as follows:

Ca-caseinate-phosphate + lactic acid = Casein complex + Ca-lactate + Ca-phosphate

Moreover, lactic acid gives yogurt its distinctive and acidic taste. It can also enhance or contribute to the aromatic flavor of the product. Different forms of lactic acid can be produced by lactic acid bacteria; (e.g. l (+), d (-) or d (±). In yogurt starter cultures, *S. thermophilus* produces mainly l (+) lactic acid and d (-) lactic acid is produced by the *L. delbrueckii* subsp. Since *S. thermophilus* grows faster than *L. delbrueckii,* subsp. *bulgaricus* in the fermentation process, l (+) lactic acid is produced first followed by d (-) lactic acid. Considering the fact that different isomers of lactic acid are found in yogurt, the predominance of each starter organism could be indicated. For example if yogurt starters consist mostly of *S. thermophilus*, the final yogurt will contain more than 70% of l (+) lactic acid. Also, if the fermentation carries out or the final yogurt is stored at a temperature below 40°C, the yogurt would contain around 0.8 g 100 mL-1 or less lactic acid. When yogurt contains more d (-) lactic acid than l (+) lactic acid, it has been incubated at high temperature, i.e. 45°C or more. This also may happen if yogurt was stored for a long time or the starter inoculation rate was more than 3%, or the starter contained more rods than cocci (Tamime & Robinson, 1999).

2.1.6.4 Protein metabolism

Since lactic acid bacteria cannot synthesize essential amino acids, proteolytic activity is greatly involved in both nutrition and interactions of yogurt bacteria. The protein fraction in milk is composed of casein and whey proteins and the basic constituents of a protein molecule are compounds from 21 amino acids (Tamime & Robinson, 1999). Yogurt starters require an exogenous nitrogen source and utilize peptides and proteins in their growth medium (milk) by more or less complete enzyme systems. *S. thermophilus* primarily requires glutamic acid, histidine and methionine, as well as cystine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine for growth (Zourari et al., 1992). Besides the free amino acids, the enzymatic hydrolysis of milk proteins results in the liberation of peptides of varying sizes and soluble nitrogenous compounds. These possible changes may be involved during the formation of the gel, a process that can affect the physical structure of yogurt and also play a role as functional peptides. Free amino acids may also affect the development of flavor components in yogurt (Tamime & Robinson, 1999); depending on the type of milk (animal species, season), such as the substrate, peptides, free amino acids and other released components in yogurt will be different. Furthermore, the type of proteolytic enzyme and bacterial strains, heat treatment, manufacturing techniques and storage conditions could affect the peptides and free amino acid type. The content of amino acids in cow's milk generally does not exceed 10 mg·100 mL^{-1} and it generally increases in yogurt in comparison to milk. Studies show that mainly *L. bulgaricus* is the species responsible for increasing free amino acids in yogurt (Zourari et al., 1992; Tamime & Robinson, 1999).

2.1.6.5 Proteolysis activities of yogurt starters

Studies on the proteolytic activity of *S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus* indicate that both organisms possess different exopeptidases and peptidases. While *S. thermophilus* is considered to have more exopeptidase activity than *L. delbrueckii,* subsp. *bulgaricus*, and it has some limited endopeptidase activity, *L. delbrueckii,* subsp. *bulgaricus* is able to hydrolyze casein due to its endopeptidase activity. Recalling the associative growth

between these two starters, the pattern of peptide hydrolysis in the yogurt organisms (*S. thermophilus* and *L. delbrueckii,* subsp. *bulgaricus)* can be explained by this mechanism: the endopeptidase activity of *L. delbrueckii,* subsp. *bulgaricus* hydrolyses the casein to yield polypeptides, and so polypeptides are broken down by the exopeptidases of *S. thermophilus* with the liberation of amino acids (Tamime & Robinson, 1999). The proteinase of *L. bulgaricus* is more active on β-casein than on whey proteins (Zourari et al., 1992). According to Argyle, Mathison & Chandan (1976), *L. bulgaricus* possesses a firmly cell-bound proteinase with optimum activity between 45 °C and 50 °C and pH values ranging from 5.2 to 5.8, while the partially purified cell wall-associated proteinase studied by Ezzat, Zevaco, El Soda, & Gripon, (1987), has a maximum activity at 35 °C and pH 5.5. Besides proteins, the low molecular weight peptide fractions of milk are also important nitrogen sources for yogurt bacteria. The importance of peptides for their growth inspiration and their acidification is now well established, especially for *S. thermophilus* (Zourari et al., 1992). *S. thermophilus* generally possesses a leucine-aminopeptidase activity and some strains have an arginine-amino-peptidase activity which is usually inactive against dipeptides (Bouillanne & Desmazeaud, 1980).

2.1.7 Viability of lactic acid bacteria in yogurt during storage

Several factors in fermented milk have been recognized as ones that can affect cell viability, such as pH, the presence of other microorganisms, temperature and the presence of oxygen. The stability of viable yogurt starters is an important factor contributing to claims that sustained yogurt consumption to yield health benefits. However, there is no universally accepted number of cells required to obtain a health benefit from probiotic microorganisms (Reid, 2008). However, according to the European Food Safety Authority, a minimum of 10^8 CFU per serving of probiotics is required in order to provide health benefits towards lactose digestion (EFSA, 2010). Also, it is generally recommended that yogurt or fermented milk should contain at least one million viable cells per gram at the time of consumption. To maintain these numbers, it is important to test the colony- forming unit (CFU) of bacteria as a growth and viability index during cold storage (Damin et al., 2009).

Dave & Shah (1997a), studied the viability of yogurt and probiotic bacteria assessed during fermentation and 35 days storage in yogurt with four commercial starter cultures. The changes in the viable counts of *S. thermophilus* and *L. delbrueckii,* ssp. *bulgaricus* during manufacture and storage of yogurt showed that the primary counts of *S. thermophilus* were 6 to 18 x 10^6 at 0 h, rising to 5.3-6.7 x 10^8 for cultures C1, C2 and C3 and to 23x 10^8 for C4 at a storage temperature of 4 °C at 0 day. This variation may be caused by strain differences and the different fermentation times needed by these cultures to reach 4.5 pH. According to this result, after fermentation was completed and at day 0 of the storage period, the counts of *S. thermophilus* increased by 15-20% in all samples, which could be due to residual activity during this period. For other storage periods, the *S. thermophilus* counts showed a decline of 30-70%; however, the *L. delbrueckii,* ssp. *bulgaricus* count was less than 10^5 after 20 days. This observation indicated the greater stability of *S. thermophilus* under the conditions employed in this study. Lower counts of *L. delbrueckii,* ssp. *bulgaricus* have been claimed to be advantageous for the viability of probiotic organisms. It is also reported that the increase of acid content- and subsequent decrease in pH- were higher for the samples stored at 10°C in comparison with those at 4°C, indicating continued remaining fermentation at this temperature. However, the drop in pH was not high enough to lower the pH of yogurt to less than 4, which has been reported to be more detrimental for the survival of probiotic organisms. Dave and Shah (1997b) also studied the effect of oxygen content and temperature on the viability of microbial cells. According to their results, hydrogen peroxide production was not dependent on either the temperature or oxygen content; rather, it depended on the associative yogurt organisms. For up to 10 days of storage, the difference in the oxygen content was higher; the differences were less after 20 days storage at 4°C. Therefore, any variations in microbial counts were due to the changes in oxygen content, since other factors such as acidity, pH, and hydrogen peroxide remained almost the same in all products stored at 4°C.

Damin, Minowa, Alcântara & Oliveira (2006), examined the effect of storage at 4˚C for 35 days on the viability of *Lactobacillus delbrueckii,* ssp. *bulgaricus* (LB), *Lactobacillus acidophilus* (LA) and *Bifidobacterium animalis,* ssp. *lactis* (BL) in co-culture with *Streptococcus thermophilus* (ST) in fermented milk. The average initial microbial count for each of the activated cultures was

measured as 10^7 CFU/mL. After 24 hours of fermentation, these counts were log 8.52, 7.96 and 9.15 for LB, LA and BL, respectively. During 35 days of storage, the CFU of *Lactobacillus bulgaricus* and *Bifidobacterium lactis* counts decreased around 2.0 log cycles. The LA counts reduced 2.34 log cycles by 28 days of storage, indicating that with these CFU, LA can perform the minimum requirements for beneficial properties for up to only 3 weeks of storage. The ST counts were higher in comparison with those of the other strains, and they were stable throughout the tested storage period.

2.2 Probiotic, *Lactobacillus rhamnosus*

L. rhamnosus, formerly called *Lactobacillus casei rhamnosus,* is an aerotolerant, homofermentative (facultatively heterofermentative (lactobacilli Group 2), according to Valik-Alzbeta & Liptakova (2008). It is a non-motile, Gram-positive, non-endo-spore forming rod bacterium originally isolated from the gut flora of healthy children (Coudeyras, Marchandin, Fajon & Forestier, 2008). It is a mesophilic organism, but depending on the strain, it may grow at temperatures lower than 15 °C or higher than 40 °C. It requires a lot of vitamins including folic acid, riboflavin, niacin, pantothenic acid and calcium. The optimal initial growth pH is between 6.4 and 4.5, and it grows as single rods or in short chain rods. The dimension of the cell is from 0.8 to 1.0 μm in width and from 2.0 to 4.0 μm in length. It converts hexoses into L (+)-lactic acid, according to the Embden-Meyerhof pathway, and due to aldolase and phosphoketolase, the pentoses are also fermented. Lactic acid is usually produced up to 1.5% in the glucose medium. In the absence of glucose, it produces lactic acid, acetic acid, formic acid and ethanol (Valik-Alzbeta & Liptakova, 2008). *L. rhamnosus* is very resistant to technological treatments such as freeze-drying and it is able to stick on intestinal epithelial cells in vitro. So it may inhibit the growth and adherence of several pathogens, and may survive and persist within the gastrointestinal tract (Coudeyras et al., 2008).

2.2.1 The growth of probiotic bacteria in milk in association with yogurt starters

Recalling the health benefits of probiotic microorganisms and according to several studies, the probiotics used for the production of fermented milks are claimed to impart nutritional and

health benefits to consumers (Mital & Garg, 1992; Klaver & van der Meer, 1993; Rasic, 2003). For example, *Lactobacillus acidophilus* is characterized by its capacity to colonize in the intestine even under low surface tension caused by the presence of bile salts. Milk inoculated with *L. acidophilus* has been found to improve lactose tolerance among lactose mal-absorbers and inhibit enteric and food borne microbial pathogens, and has thus been considered as a growth media for probiotic organisms (Shah, 2007). However, several lactic acid bacteria (LAB), including *L. acidophilus* strains are very fussy about growing in milk and survive poorly in fermented products (Shah, 2007; Gaudreau, Champagnea & Jelen, 2005). The growth requirements of probiotics are complex especially in terms of amino acids requirements (Stanier, Adelberg & Ingraham, 1976). The free amino acids and peptides that exist in milk are not sufficient to ensure optimal bacterial growth in this substrate and so hydrolysis of proteins must be done. The growth of LAB microorganisms on lactose requires the synthesis of specific enzymes to digest and convert the disaccharide into glucose and galactose. So it could be suggested that the ability of LAB to grow extensively in milk depends, at least partially, on their ability to hydrolyse lactose and proteins (Mills & Thomas, 1981; Amoroso & Manca de Nadra, 1991). Shah, Lankaputhra, Britz & Kyle, (1995), suggested that the number of viable *L. acidophilus* cells should be greater than 5 log CFU g^{-1} if they are to have any therapeutic benefits. However, Health Canada (2009) has recently recommended that foods contain 10^9 CFU of probiotic bacteria per portion. Therefore, the use of probiotic cultures that provide high viable counts during the storage of the product are essential. At the same time, it is important to make sure that the probiotic organisms can survive and remain stable in milk (Gardini, Lanciotti, Guerzoni & Torriani, 1999).

According to Gardini et al. (1999), survival of the *L. acidophilus* strain during storage was higher at a low concentration of non-fat milk solids in comparison with fat containing milk; Gardini et al., (1991), also said that the proper selection of strains, on the basis of the desired features, is essential in order to reach the minimum number of probiotic organisms during storage- a number that should be much greater than 5 log CFU g^{-1}. To promote the development of the probiotic strains, addition of supplements to milk, especially peptones or yeast extracts, have been proposed (Gaudreau et al., 2005). Shah, Warnakulsuriya & Lankaputhra (1997) reported

that ruptured cells of yogurt bacteria could improve viable counts and the viability of *bifidobacteria* or *Lactobacillus acidophilus* in milk. However, this increased the time required to complete the fermentation. In another study however, Dave & Shah (1997a), suggested that increasing the microbial count of probiotics during manufacture and the viability of these organisms during storage is dependent on the species and strain of associative yogurt organisms. The presence of *L. delbrueckii,* ssp. *bulgaricus,* affects the viability of *L. acidophilus,* and *bifidobacteria,* whereas *bifidobacteria* showed better stability in the yogurt in the culture containing *L. delbrueckii,* ssp. *bulgaricus.* The viability of both probiotic organisms was higher in the product with lower dissolved oxygen concentrations. The storage temperature of the yogurt affected the viability of *bifidobacteria*, but not *L. acidophilus.*

According to Shah (2000), acid and hydrogen peroxide produced by yogurt bacteria as well as oxygen content in the product, and oxygen permeation through the package, all affect the viability of probiotic bacteria in yogurt. Since yogurt starters produce acid and reduce the pH, probiotic stability is affected by yogurt starters. It is reported that *L. acidophilus* and *bifidobacteria* tolerate acid, but a rapid decline in their numbers in yogurt has been observed. *Bifidobacteria* are not as acid tolerant as *L. acidophilus* and although the growth of *L. acidophilus* ceases below pH 4.0, the growth of the *Bifidobacterium* spp. ceases below pH 5.0. So, during yogurt manufacturing, *L. acidophilus* and *Bifidobacterium* spp. grow slowly in milk; the usual production practice is to incorporate yogurt cultures along with probiotic cultures as co-cultures. Gaudreau et al. (2005) also studied milk supplementation with extract of *L. bulgaricus* to simulate growth of *L. rhamnosus.* According to this study, three components of the ruptured yogurt cells could potentially stimulate the growth of probiotic cultures such as galactosidase from supplemented extract, since yogurt bacteria are one of the highest lactase producers in LAB bacteria. The second component would be proteases that could provide peptides and amino acids for growth of *L. rhamnosus*. The final component would be cell lysates that contain many growth factors; using cell lysates as supplements could thus provide multiple stimulatory actions. It is reported that the extract of yogurt cells containing essential enzymes and growth factors stimulates the growth of *L. rhamnosus* in milk.

Almeida, Tamime & Oliveira (2008), studied the acidification rates of probiotic bacteria: *Lactobacillus delbrueckii, subsp. bulgaricus* (Lb), *Lactobacillus acidophilus* (La), *Lactobacillus rhamnosus* (Lr), and *Bifidobacterium animali,s subsp. lactis* (Bl) in co-culture with *Streptococcus thermophilus* (St) in Minas frescal cheese whey. Some other parameters such as post-acidification and counts of health- promoting micro-organisms due to co-culture composition and the final pH values, were also studied. The results indicated that comparing different co-culture combinations, the fermentation time to reach pH 4.5 was longer when St–Lr was used, while St–Lb had the shortest fermentation time. During the storage period, post-acidification occurred in all products and the lowest values had been observed in St–Bl co-culture combination. It was observed that the acidifying rates of the probiotic bacteria in Minas frescal cheese whey was affected by co-culture composition and the final pH level at which the fermentation was stopped. The fermentation time required to reach pH 4.5 was the longest when the co-culture St–Lr was used and the shortest when St–Lb and St–Bl co-cultures were used. For other co-cultures, the fermentation time ranged between 3 and 12.4 h. Counts of the bacteria varied significantly; *B. animalis subsp. lactis* had the highest counts (around 8.43 $\log_{10}$ CFU mL^{-1}), while *L. rhamnosus* had a much lower growth rate with counts that do not provide the requirements for a probiotic product. This study concluded that the combination of microorganisms in a co-culture starter and the pH level at which the fermentation was stopped are the most important factors to be controlled if improvement of the fermentation process of whey- based probiotic beverages is desired. In another study where *L. helveticus* or *B. longum* were combined with *S. thermophilus (*Champagne, Green-Johnson, Raymond, Barrette & Buckley, 2009), the counts of probiotic bacteria, particularly the *bifidobacteria*, were much lower in the fermented products when the yogurt starter was included. So, lower starter content in *L. Bulgaricus* results in higher probiotic production. According to Hekmat & McMahon (1992), there is a poor viability of *L. acidophilus* and *B. bifidum* in yogurt; this is due to its sensitivity to low pH. Adding *L. acidophilus* to yogurt, after production, causes 90–99% losses after three to five days of storage.

As a result, various strategies have been proposed with respect to the management of starter inoculation and composition and they are listed as: compatible starters, fermentation time, pre-

inoculation of probiotic, inoculation level of starter or probiotic (CFU/mL) and fermentation temperature. It is reported that probiotic starters are assimilated by galactose faster than by lactose and combining them with strains producing galactose would improve their growth. Also, using starters that do not produce antimicrobials such as H_2O_2 and bacteriocins, or the strains which consume oxygen, could help probiotic growth. In terms of fermentation time, since probiotics generally take longer to lower the pH rather than yogurt starters, choosing strains (co-culture) with longer fermentation times and sufficient growth factors in the medium would favour the development of probiotics. Also, if the probiotic is not dependent on proteolysis or oxygen consumption by the culture, reducing the inoculation level of the co-culture will favour the probiotic starter. Considering the optimum temperature for probiotics, lowering the temperature below 40˚C will be unfavourable to *L. bulgaricus*, while further lowering of the temperature (i.e. 30˚C) can be beneficial to some probiotics such as *L. rhamnosus, L. casei,* and *L. plantarum* (Kailasapathy and Champagne, 2010).

2.2.2 Effect of pH, redox level, antioxidant and vitamin C

In yogurt production, since *L. delbrueckii,* ssp. *bulgaricus* produces lactic acid during fermentation and refrigerated storage, the pH drops lower than the pH of fermentation (4.5), a process known in the industry as ”post-acidification.” In probiotic yogurt, post acidification is found to cause a loss of viability of the probiotic bacteria showing that probiotic are sensitive to lactic acid (Shah, 2000). Favaro-Trindade, Bernardi, Bodini, Balieiro & Almeida (2006), studied the effect of culture, pH and fat concentration on the probiotic population and sensory characteristics of probiotic fermented yellow Mombin (*Spondias mombin L*) ice cream. Twelve different ice cream formulas were provided with different starter cultures (*Lactobacillus acidophilus 74-2, L. acidophilus LAC 4* and yogurt starter culture). According to this study, in terms of probiotic viability, the lower pH of the final product resulted in a higher population of *Lactobacillus acidophilus.* It is also reported that higher concentrations of fat in ice cream formula did not provide greater protection for the probiotic micro-organisms.

The electrical potential (Eh), is associated with the oxidation and reduction potential of a substance (redox potential). Microorganisms, classified as aerobic, anaerobic, or facultative, are classified based on the Eh required for multiplication and metabolism. Aerobes require positive Eh values, while anaerobes require negative Eh values; facultative organisms grow in either positive or negative Eh values. Recalling the fact that oxygen content in the media affects the viability of probiotics such as *L. acidophilus* and *bifidobacteria*, it is important to know the Eh of the media. The medium redox potential (Eh) influences the folate-limited growth of *Lactobacillus casei var. rhamnosus*. This effect was studied by Tennant (1976) who used ascorbic acid to prevent oxidation of the folate activity of serum during assay procedures using *Lactobacillus casei.* According to that study, ascorbic acid stimulates the growth of *L. casei* cultures containing pteroylglutamic acid (PteGlu) as the folate source. It is reported that microbial growth was maximal at an Eh of +120 mV at pH 6.35. Dave and Shah (1997b) also studied the viability of yogurt and probiotic bacteria during manufacture and storage with four concentrations of ascorbic acid (0, 50, 150, or 250 mg/kg). Since milk and dairy products can be fortified up to 10-15% of the daily requirement in ascorbic acid (vitamin C), this fortification can be used as an oxygen scavenger. They studied the viability of supplemented yogurt and probiotic bacteria during manufacture and 35 days of cold storage. Four levels of ascorbic acid with four commercial starter cultures were assayed and oxygen content and redox levels were measured. Results indicated that while fortification with ascorbic acid would increase the nutritive value of yogurt, there were no marked differences in titratable acidity and pH of all samples. At the same time, there was a difference in oxygen content and redox potential in the yogurt samples. During the storage of yogurt, increase in oxygen content and redox potential and percentage retention of ascorbic acid were identical for all four starter cultures; however, the increase was dependent on the levels of ascorbic acid. The oxygen content and redox potential gradually increased during storage in plastic cups and with higher levels of ascorbic acid it stayed lower. During the manufacture and storage of yogurt, loss of ascorbic acid happened and its retention was only 15-20% (approximately) after 35 days storage at 4°C. In terms of microbial viability in yogurt; with increasing concentration of ascorbic acid, *Streptococcus thermophilus* were lower, whereas those of *L. delbrueckii,* ssp. *bulgaricus* were

higher. It is reported that microbial counts of probiotic bacteria such as *Lactobacillus acidophilus* decreased less rapidly with increasing concentrations of ascorbic acid for all four starter cultures, whereas the counts of *bifidobacteria* remained unchanged. Ibrahim & Carr (2006), reported that yogurt and probiotic products containing a high concentration of antioxidants and proteins enhances the viability of *Lactobacillus* and *Bifidobacteria* during cold storage.

2.2.3 Effect of amino acids

The effect of supplementation with amino acids on growth of probiotic and yogurt starter culture has been considered in several studies. Dave & Shah (1997a), reported that two factors; amino nitrogen and reduction of redox potential, might help the growth of anaerobic *bifidobacteria* species. Amino nitrogen as a growth factor could be provided by cysteine as a sulfur-containing amino acid. Dave & Shah (1998), also studied the effect of different amino acid sources such as whey protein (WP), two different brands of whey protein concentrate (WPC1 and 2), acid protein hydrolysates (ACH) or tryptone, on the viability of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacteria*. Changes in pH, titratable acidity (TA), redox potential, and viability of bacteria were monitored during 24 hours of fermentation and refrigerated storage of yogurt for 35 days. They reported that the required time to reach pH 4.5 was significantly affected by the ingredients. The time to reach pH 4.5 increased considerably on the addition of 250 and 500 mg of cysteine/L, but the incubation time decreased in yogurt mixes supplemented with WPC, ACH, or tryptone. Also, the drop in pH or the increase in acidity and redox potential was dependent on the added ingredients. The drop in pH or rise in TA in yogurt supplemented with cysteine, WPC, ACH, or tryptone was more than double that observed in the control yogurt. Yogurt supplemented with WP showed a similar trend of decrease in pH or increase in TA compared to that of the control yogurt. It is reported that because of the availability of an amino nitrogen source through the supplementation, approximately up to 0.3-unit drop in pH and 0.1% increase in TA were observed during 35 days of storage for yogurt supplemented with ACH or tryptone. Therefore, the pH or TA of all yogurt samples remained stable within the mentioned range and the drop in pH or increase in TA did

not seem to have an effect on the viability of the *bifidobacteria* during refrigerated storage of supplemented yogurt. Changes in the pH and TA of yogurt supplemented with 50 mg of cysteine/L were similar to that of the control yogurt during storage while the changes in pH of yogurt supplemented with 250 mg of cysteine/L or WPC1 were similar to those of yogurt supplemented with 500 mg of cysteine/L or WPC2, respectively. Yogurt supplemented with 500 mg of ACH or tryptone/L gave results similar to yogurt supplemented with 250 mg of ACH or tryptone/L, respectively. The redox potential in yogurt supplemented with 50 or 250 mg of cysteine/L stayed negative for 10 and 25 days, respectively. This factor remained negative throughout the 35-d storage period at 500 mg of cysteine/L supplemented yogurt. The redox potential, in the control and other yogurts also, increased with WP, WPC, ACH, or tryptone supplementation during all the storage period. In terms of the viability of probiotic and yogurt starters: the count of *S. thermophilus* was adversely affected by cysteine, but the viability of *L. acidophilus* was improved on addition of this ingredient. The counts of *L. acidophilus* remained $>10^5$ CFU/g throughout the storage in all yogurts. Counts of *bifidobacteria were* observed to be reduced by more than 3 log cycles when the pH reached 4.5 in the control yogurt as well as in the yogurt supplemented with WP. The viability of *bifidobacteria* improved to a variable extent in yogurt supplemented with cysteine, WPC, ACH, or tryptone. In this study, SDS-PAGE and amino acid analyses also confirmed that the nitrogen source in the form of peptides and amino acids correlated with improved viability of *bifidobacteria* in yogurt made with a commercial starter culture, which demonstrated a significant decrease in the counts of this organism (Dave & Shah, 1998).

Collins & Hall (1984), reported an improved viability of some *bifidobacteria* species in 10-12% reconstituted skim milk containing 0.05% cysteine. They studied the effect of yogurt supplementation with 0, 50, 250 and 500 mg of L-cysteine on the growth and viability of yogurt and probiotic bacteria made with four commercial yogurt starters. They also monitored changes in pH, redox potential and viable counts of S. *thermophilus, L. delbrueckii,* ssp. *bulgaricus, L. acidophilus* and *bifidobacteria* during manufacture and cold storage for 35 days. Results indicated that the incubation time required to reach a pH of 4.5 was highly affected by cysteine supplementation. During refrigerated storage, the relative drop in pH was higher in

yogurts containing 250 and 500 mg L^{-1} cysteine, but it did not differ in yogurts prepared with 0 and 50 mg L^{-1} cysteine. The redox potential stayed negative for 25-30 days in yogurt supplemented with 500 mg L^{-1} cysteine, but it stayed positive throughout storage in non-supplemented control yogurt. In all yogurt samples, the redox potential gradually increased to positive values; however, the rate of increase was different as the level of cysteine and the type of starter culture varied. Supplementation with 50 mg L^{-1} of cysteine improved the growth of yogurt bacteria in yogurts made with all the four commercial starter cultures. The growth of *S. thermophilus* was unfavorably affected by cysteine when it was more than 50 mg L^{-1}. Counts of *L. acidophilus* increased during production and storage when the cysteine level was 250 mg L^{-1}, but the viability of *bifidobacteria* in supplemented yogurts made with starter cultures that contained both yogurt bacteria decreased. At the same time, its viability was improved in yogurts made with starter cultures containing *S. thermophilus* only. Ibrahim & Carr (2006) also reported that amino acids, casein hydrolysate or peptides increased the populations of *lactobacillus* and *bifidobacteria* in yogurt products. One important concern with cysteine, however, is its effect on flavor.

2.3 Pulse characteristics and food supplementation

2.3.1 Functional properties of pulse protein, pulse flours, protein concentrates and isolates:

Plant proteins have a variety of functional properties that can be exploited in food formulations. In addition to nutritional benefits, functional properties of pulse ingredients could also play an important role in food systems. Functional properties are mostly defined as solubility, water binding, fat binding, emulsification, foaming, gelation, thickening and flavour- binding capacity, which are largely affected by the protein content and their properties. These physico-chemical properties, referred to as functional properties, play an important role during processing, storage, preparation and consumption (Kinsella, 1982). Amino acid composition, structure and conformation and interactions between proteins and other food components (e.g., salts, fats, carbohydrates and phenolics compounds) as well as pH, temperature and other process specifications, all affect the functional properties of pulse ingredients (Boye, Zare, & Pletch,

2009). Several studies have been done on different functional properties of pulse ingredients. These are selected and summarized here.

Water binding capacity, water absorption capacity or water holding capacity (WHC), is defined as the amount of water that can be absorbed per gram of material and this is very important for the application of food ingredients in food formulations and food processing systems. Water absorption in food material decreases the fragility and increases the tendency for higher moisture content in food products (Bencini, 1986). Water holding capacity is reported to be affected by the percentage of protein, cultivar and processing treatments. For instance, chickpea protein isolates have higher WHC compared to chickpea flour. In addition to the protein content, different varieties showed different WHC; for example, desi chickpea has lower WHC compared to kabuli chickpea (Kaur & Singh, 2007). Heat treatment also results in an increase of the WHC of pulse flours, as reported by Obatolu, Fasoyiro, & Ogunsunmi (2007), who evaluated WHC in raw, processed, boiled, fermented, roasted and malted yam bean. Their results showed that WHC ranges from 1.32 g/g to 2.19 g/g for raw flour and boiled flour, respectively.

Protein Solubility of pulse ingredients is directly related to the isoelectric point of the protein components. The isoelectric point (IP) is the pH at which protein has the least solubility where it precipitates. The solubility of pulse protein also varies in each type of pulse; in most pulse proteins, the solubility is highest at low acidic and high alkaline pH values. The isoelectric point is generally between pH 4 and pH 6 for most pulses (Torki, Shabana, Attia & El-Alim, 1987; Fernandez-Quintela, Macarulla, Del Barrio & Martinez, 1997). Solubility is affected by protein composition as well as processing treatments. According to Swanson (1990), at pH 6.4, pea isolate exhibits greater nitrogen solubility in comparison with soy isolate, while its solubility is comparable with lentil isolate. He also reported that drum drying decreases nitrogen solubility in pulse ingredients. According to Torki et al. (1987), protein fractions have different IP and solubility over the pH range. They reported the solubility of albumin in chickpea: 79% at pH 2, and 94% at pH 9.5. Also the solubility of lentil albumin was reported to be 95% at pH 7.8 and 77% at pH 1.8, while the solubility of globulin in lentil was 89% at pH 8.9. Interaction between

protein and polysaccharides may also affect the solubility, as reported by Braudo, Plashchina & Schwenke (2001), and the interactions of faba bean legumin protein and hydrolysed legumin with polysaccharides (chitosan) could increase their solubility at the isoelectric point and at higher pH values.

Fat binding capacity (FBC), is calculated as the weight of oil absorbed per weight of food ingredient (Achouri, Boye, Yaylayan & Yeboah, 2005). FBC varies in different pulses and pulse fractions and it is affected by protein content, variety and cultivar and processing treatments. Fernandez-Quintela et al. (1997) reported the FBC of faba bean (*var. Muchamiel*) protein isolate to be 1.6 g/g, a figure that is greater than for pea and soy protein isolates with 1.2 g/g and 1.1 g/g, respectively. According to Kaur & Singh (2007), the fat binding capacity in chickpea isolate with higher protein content is higher than those with lower protein content (2.08 g/g vs 3.96 g/g). They also reported higher FBC for the kabuli chickpea protein isolate rather than that of the desi protein isolates which is probably due to the presence of more non- polar amino acids in kabuli chickpea protein. Furthermore, the method of protein isolates preparation and extraction could affect the fat binding capacity as reported by Paredes-Lopez, Ordorica-Falomir & Olivares-Vazquez, (1991).

Emulsifying properties of food ingredients are expressed by their emulsifying activity (EA) and emulsifying stability (ES). Emulsifying properties are affected by hydrophobicity and hydrophilicity properties of protein and amino acids that contain the structure of proteins. Proteins can form a thin layer or protein film around oil droplets in a food system to make an emulsion. EA could simply be defined as the amount of oil that can be emulsified per unit of protein while ES shows the ability of the emulsion to oppose changes to the structure of emulsion over a period of time (Pearce & Kinsella, 1978; Boye et al., 2009). Paredes-Lopez et al. (1991) reported that EA is higher (72.9 vs 63.7) in chickpea protein isolate with higher protein content (87% vs 84%). They also measured a higher ES with higher protein content. Protein type as well as protein content affects the emulsifying properties, and it has been reported that vicilin proteins in pea generally have better emulsifying properties rather than vicilin-legumin (Dagorn-Scaviner, Gueguen & Lefebvre, 1987) and albumins from Great Northern bean are

better emulsifiers in comparison with globulin proteins (Sathe & Salunkhe, 1981). Some other studies also show that processing such as hydrolysis and interaction between carbohydrates (chitosan) and proteins could improve emulsifying properties in pulse ingredients (Boye et al., 2009).

***Foaming properties**:* In food processing and food formulations, such as beverages, mousse and desserts, foam formation is very important and practical. Proteins can help to keep the foam from collapsing which is related to its foaming properties. The protein in pulses can provide foaming capacity when the proteins form a fragile film layer holding air inside a bubble to form a suspension of air in liquid. Foam expansion (FE) or foam capacity (FC) and foam stability (FS) are the most frequently used indices for measuring foaming properties (Baniel, Fains & Popineau, 1997). FC or FE is expressed as the volume (%) of foam which increases due to whipping while foam stability is defined as the change in the volume of foam over a time period (Boye et al., 2009). Foaming properties also are related to protein content, protein structure and processing treatments. Paredes-Lopez et al. (1991), measured higher FE and FS for chickpea protein isolate with higher protein content. Furthermore, ultra-filtration could improve the foaming properties in yellow pea (*Pisum sativum L. cv Trapper*) and faba bean (*Vicia faba equina L cv. Diana*) in comparison with skim milk powder which was not ultrafiltered (Vose, 1980). According to Obatolu et al. (2007), foaming capacities in raw yam bean (40.20%) were significantly greater than boiled yam bean (1.98%), further highlighting the effect of processing treatments.

2.4 Food Supplementation

2.4.1 Beverages supplementation

The purpose of food supplementation is either to improve the nutritional or physico-chemical and sensory properties of the food or to make new foods which satisfy the different needs and appetites among consumers. In general, the supplemented food should meet the physicochemical, microbial, sensory and marketability standards of quality foods systems.

Beverage supplementation, especially fruit juices, has been considered in recent years. Clark & Johnson (2002) studied the supplementation of orange juice, bread, muffins, pasta and breakfast bars with Lupin (*Lupinus angustifolius*) kernel fiber. According to an untrained panel of 44 people, fiber enrichment did not change the overall acceptability of the bread and pasta ($p < 0.05$); however, it reduced the overall acceptability of the muffin, orange juice and breakfast bar ($p < 0.05$). Overall, the flavor of fiber-enriched products was the attribute most highly correlated with the overall acceptability.

Temelli, Bansema, & Stobbe (2004), studied beverage supplementation with ß –glucan to enhance the nutritional properties of the beverage. The beverage was formulated using water, sucrose, pectin, citric acid, corn syrup, ascorbic acid, orange essence and ß-carotene. Two main ingredients, namely mixed linkage (1.3), (1.4)-ß-D-glucan (ß -glucan) in amounts of 0.5 % (w/w) from barley and whey protein isolate (WPI) from milk in concentrations of 0 %, 0.5 %, 1.0% and 1.5 % (w/w) were used as supplements. For the sensory analysis work, both trained and consumer panels were used to evaluate the beverage quality and acceptability. The trained panelists found the beverages to be similar in sweetness ($P > 0.05$). Sourness and orange-flavor intensity decreased ($P \leq 0.05$) as the WPI concentration increased, while cloudiness and viscosity increased ($P \leq 0.05$). The consumer panel reported no difference ($P > 0.05$) in the degree of liking for all the attributes studied. This study concluded that barley ß-glucan had great potential as a functional beverage ingredient.

The development of whey-based beverages using fruit components have been studied by Djurić, Carić, Milanović, Tekić, Panić (2004). The beverages were comprised of whey, fruit components (orange, pear, peach and apple), citric acid and sucrose. Sensory characteristics including flavor, odor, color, sediment, appearance and total quality (sum of the previous factors) were determined on prepared and pasteurized samples. Apart from pH, which changed within a narrow range, none of the characteristic functions studied changed significantly. Results also showed that the quality of formulated beverages with orange and pear mostly depended on the sucrose content, while that of blends prepared with peach and apple depended on the dry matter of the fruit. Interaction of dry matter and sucrose was most

significant for the blend with pear, while the balance between sucrose and pH strongly depended on the quality of all the other products. The study concluded that the peach–whey beverage, with a pH of 3.6 containing 6 % of dry matter and 2 % of sucrose, had the best quality in comparison with other formulations.

Vojnovic, Ritz & Vahcic (1993), also studied the sensory properties of whey-based fruit beverages. Samples were made from pasteurized whey permeate and other ingredients including five kinds of fruit juices in amounts of 20-40 % (w/w). This study showed the good sensory qualities of prepared whey beverages; it was also found that whey permeates obtained as a by-product during cheese- making can be used to produce permeate-based beverages.

The nutritional, physical and sensory characteristics of chocolate-flavored, peanut-soy beverages have been studied by Deshpande, Chinnan & McWatters (2005). Twenty-eight formulations were evaluated for nutritional (lysine content) and physical (viscosity [h], visual stability index [VSI]) attributes as well as sensory properties. According to this study, the lysine contents (44.1–57.1 mg/g protein) were close to the reference of essential amino acid consumption (NAP, 2002) which is 51.0 mg/g protein, and in the desirable range observed for other peanut-based beverages. The viscosity affected consumers' likeliness of beverages. In this formula, increasing the viscosity of the product resulted in lower rates of appearance and color acceptability; lowering the viscosity produced a lower VSI, which means there needs to be a balance between viscosity, VSI, appearance and color. A formulation containing 43.9 % peanuts, 36.3 % soy protein isolates (SPI) and 19.8 % chocolate syrup exhibited the highest consumer liking and the highest balance of physical properties, as compared to commercial chocolate milk. Two formulations made with SPI and one with a soy flour formulation obtained the highest scores for aroma, color and flavor.

2.4.2 Yogurt and probiotic supplementation

The concept of functional foods has become focused on food additives which have a positive effect on the gut microbiota composition. The microbiota is composed microorganisms that naturally live in the region or organ. For instance, the gut microbiota or gut microflora consists

of microorganisms that live in the digestive tracts of animals, the largest reservoir of the host. These microorganisms have been mainly determined to be probiotics. Recently, interest in probiotic and the probiotic's food (ie. prebiotic) has increased. Recalling that "prebiotics" are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon, it should be mentioned that this definition overlaps with the definition of dietary fiber, with the exception of its selectivity for certain species. Peptides, proteins and lipids contain prebiotic characteristics, but carbohydrates have received the most attention; these include lactulose, inulin, and a range of oligosaccharides that supply a source of fermentable carbohydrates for use by the beneficial bacteria in the colon (Prado et al., 2008). Synbiotics is a combination of pre-and probiotics in a single product and their applications have received increased interest in recent years (Helland, Wicklund & Narvhus (2004). Because of the nutritional benefits associated with microflora management approaches, foods are the main vehicle for probiotic, prebiotic and synbiotics. Amino acids, carbohydrates, minerals and vitamins are essential requirements of probiotic organisms as well as yogurt culture microorganisms and could be provided for optimal growth by supplementation. Numerous studies show that enriching milk with supplements enhances the growth of probiotics and yogurt starters (Dave & Shah, 1997a, b; 1998; Shah, 2000). Examples of potential supplements are extracts or juices from yeast (Kim, Baick & Yu, 1995), citrus (Sendra, Fayos, Lario, Fernandez-Lopez, Sayas-Barbera & Perez-Alvarez, 2008), soy (Drake, Chen, Tamarapu, & Leenanon, 2000), cereals (Kyung & Young, 1993; Vasiljevic, Kealy & Mishra, 2007), and whey (Christopher, Padmanabha-Reddy & Venkateswarlu, 2006). A noteworthy trend is the addition of prebiotics, which include fructooligosaccharides (FOS) (Bruno, Lankaputhra & Shah, 2002) such as chicory's inulin (Aryana, Plauche, Rao, McGrew & Shah, 2007; Juhkam, Elias, Roasto & Tamme, 2007), lactulose (Bruno et al., 2002), oat and barley glucans (Vasiljevic et al., 2007), galactooligosaccharides (GOS) (Shin, Lee, Pestka & Ustonol, 2000), starch/maltodextrins (Bruno et al., 2002) and raffinose (Martinez-Villaluenga, Frias, Gomez & Vidal-Valverde, 2006).

The effect of milk base and starter culture on acidification, texture and probiotic cell counts in fermented milk processing was also studied by Sodini, Lucas, Oliveira, Remeuf & Corrieu (2002).

Two strains of probiotic bacteria (*Lactobacillus acidophilus LA5* and *L. rhamnosus LR35)* along with two different yogurt starter cultures were used. One starter culture consisted only of *Streptococcus thermophilus ST7* (single starter culture) and the other one was a mixed culture of (*S. thermophilus ST7* and *L. bulgaricus LB12)*. They used four commercial dairy ingredients as supplements, including two milk protein concentrates and two casein hydrolysates and the experiment allowed the comparison of the supplemented fermented milks with those obtained with non-fortified control milk and milk fortified with 2 % skim milk powder, in terms of probiotic growth and stability. This study showed that *L. acidophilus* LA5 grew well on milk but showed a poor stability during storage. *L. rhamnosus* LR35 grew weakly on milk but was remarkably stable during storage. The best probiotic cell counts were obtained with the use of a single starter culture with the addition of casein hydrolysate. The fermentation time was about 11 hours and the number of probiotics after five weeks of storage was higher than 10^6 CFU/mL for *L. acidophilus* LA5 and 10^7 CFU/mL for *L. rhamnosus* LR35. In order to improve texture and flavour, further studies are required for the optimization of the level of casein hydrolysate added to the milk base.

Helland et al., (2004) studied the growth and metabolism of selected strains of probiotic bacteria in milk- and water-based cereal puddings. The growth of four probiotic strains, (*Lactobacillus acidophilus* La5 and 1748, *Bifidobacterium animalis* Bb12 and *Lactobacillus rhamnosus* GG), were studied in milk and water based puddings. Viable cell count, pH and metabolites during fermentation (12 h, 37 °C) and after refrigerated storage (21 d, 4–6 °C) were analyzed. All strains showed good growth and survival in milk-based puddings (8–9.1 log CFU g^{-1}), but *L. rhamnosus* GG was the only strain with an acceptable survival in water-based puddings (8 log CFU g^{-1}). After storage, 560–9800 mg kg^{-1} lactic acid was produced, with a significant increase in milk-based puddings (P<0.05). The pH levels in the products were reduced to 3.4–4.4. High concentrations of diacetyl were detected in puddings inoculated with *L. rhamnosus* GG, reaching 18 mg kg^{-1} in milk-based puddings.

The effects of dried dairy ingredients on physical and sensory properties of non-fat yogurt were studied by Isleten & Karagul-Yuceerl (2006). In their study, sensory and texture properties of

non-fat yogurts made from reconstituted skim milk powder (SMP) fortified with SMP as a control, whey protein isolate (WPI), a milk protein based component as a yogurt texture improver (TI) and sodium caseinate (NaCAS) were investigated over a 12 day storage period. They reported that addition of WPI improved the physical properties of yogurts, so that the highest viscosity and the lowest syneresis resulted. They also reported that yogurt supplemented with WPI did not have desirable sensory properties and the descriptive panel rated this formula with the lowest score for fermented flavor. In general, yogurts fortified with NaCAS and TI displayed better physical and sensory properties than the control sample and WPI-fortified yogurts. In terms of flavour acceptability, consumer testing showed that yogurts with NaCAS and TI were not different from the control. Yogurts fortified with NaCAS and TI was the most preferred.

Physical and rheological properties of yogurt supplemented with various commercial whey protein concentrates (WPC) were studied by Sodini, Montella & Tong (2005). The difference between whey proteins was basically in the protein and fat contents; the samples came from different cheese manufacturers of cheddar, mozzarella and other cheeses. Two commercial starters were used that are blends of standard yogurt starters and probiotic and they are characterized as ropy and non-ropy. Generally ropy cultures are microorganisms that can produce exopolysaccharide while non-ropy cannot. According to this study, the water holding capacity of the supplemented yogurt with whey protein increased in comparison with the control sample. Rheological properties were different and independent of the starter used in supplemented samples in comparison with control samples. Whey protein produced yogurt with lower firmness, lower Brookfield viscosity (6 Pa s compared with 9 Pa s), lower yield stress (2 Pa compared with 4 Pa), lower complex viscosity (13 Pa s compared with 26 Pa s) and lower apparent viscosity (0.4 Pa s compared with 1 Pa s) in comparison with the control yogurt. The yogurts with the lowest firmness and viscosities were produced with the concentrates containing the highest amount of non-protein nitrogen.

Drake et al., (2000) reported that soy protein supplementation (1, 2.5, or 5 %) did not affect the microbiological properties of dairy yogurts after production and during a one month storage at

5 °C. However, viscosity, darkness and chalkiness increased in soy- supplemented yogurt in comparison with non-supplemented yogurt. They also reported that soy supplementation added a soy aroma which was constant over the storage time.

The effect of supplementation of yogurt with calcium and fibre on its physicochemical and rheological properties has been studied by Velez-Ruiz, Sosa-Morales, & Alatriste-Montiel (2003). Two levels of calcium (20 and 40 mg/100 mL of milk), three concentrations of fibre (0.75, 1.0 and 1.25 %) from coconut and fig were added to the fortified yogurt. Results of the acidification rate showed that additions of fibre and calcium caused the pH to decrease, ranging from 4.6 to 4.3, showing a reverse relationship to acidity that increased from 0.72 to 1.18 depending on the specific formulation. Calcium content, density, fibre and moisture contents of both yogurt types did not change during the storage period. Water activity had values from 0.954-0.993; syneresis decreased with fiber supplementation and increased with calcium supplementation. Considering the rheological properties, all samples showed a shear-thinning behavior, when the apparent viscosity of fortified settled yogurt (SEY) was higher than stirred yogurt (STY). A similar study was done by Aportela-Palacois, Sosa-Morales, & Velez-Ruiz, (2005). They reported that due to fibre and calcium supplementation, yogurt consistency increased, syneresis decreased (20 to 48 %) and final pH decreased from 4.5 to 4.2 during 3 weeks of storage.

In view of the fact that many probiotic organisms are poor users of lactose, the addition of a more readily available carbohydrate can selectively enhance the growth of probiotics during yogurt fermentation. The addition of 5% lactulose, GOS or inulin reduced the doubling time of bifidobacteria in milk by 25 to 50% (Shin et al., 2000; Bruno et al., 2002). As well as probiotics, yogurt fortification has been considered as a means to increase the nutritional properties of yogurt and develop novel products. Hekmat and Reid (2007), studied the growth and survival of *Lactobacillus reuteri RC-14* and *Lactobacillus rhamnosus GR-1* in yogurt supplemented with inulin. Inulin is a complex carbohydrate that is not directly metabolized by us; rather, it provides an energy source for intestinal microbes. In this study, four different formulations were made based on milk (1% fat) supplemented with 0.33% yeast extract (T1); 0.4% inulin (T2); 0.33%

yeast extract and 0.4% inulin (T3), as well as non-supplemented control sample (T4). Survival of *L. reuteri RC-14* and *L. rhamnosus GR-1* was monitored after 1, 7, 14, 21, and 28 days of storage at 4 °C. It is reported that both bacteria were able to grow and survive in all samples; however, they showed a higher survival rate in inulin supplemented treatment (T3) ($\boldsymbol{P} < 0.05$). The total colony counts for the inulin samples (T3), however, decreased by 1 log cycle for both bacteria after 28 days of storage, indicating a maximum storage life. In yogurt samples containing 0.4% inulin only (T2), the viability or maintenance of *L. rhamnosus GR-1* and *L. reuteri RC-14* did not improve significantly ($P<0.05$) but using 0.4% inulin and 0.33% yeast extract resulted in the highest counts for *L. reuteri RC-14* ($P<0.05$).

Physicochemical and microbial properties of inulin- supplemented yogurt have been studied by Aryana et al., (2007). Short, medium and long-chain inulins (1.5 % w/w) were added to probiotic fat-free plain yogurt and their effect on yogurt starters and *Lactobacillus acidophilus* were studied as well as the effect of supplementation on the physicochemical properties of yogurt. Results showed that the addition of short chain inulin decreases the pH and raises the flavour score in comparison with other inulin- supplemented yogurt. Inulin supplementation improved *Lactobacillus acidophilus* growth in comparison with the control sample; however, it did not affect viscosity, color, and the appearance of the product. The yogurts containing long chain inulin showed less syneresis compared with the control yogurt and had better body and texture when compared with other inulin -supplemented yogurts.

There are discrepancies in the literature on this subject, however, and Donkor, Nilmini, Stolic, Vasiljevic & Shah (2007) observed an increase in viscosity upon inulin addition. Presumably the discrepancies are due to yogurt formulation, the types of microorganisms used, inulin and/or milk ingredients. These data indicate that inulin can be incorporated in yogurt for combined health benefits, but changes in texture can also be expected under certain circumstances.

2.4.3 Potential components of the pulses that can enhance growth of yogurt starters and probiotic

Some previous studies have shown that enriching milk with prebiotic supplements enhances the growth of probiotics and yogurt starter's microorganisms; however, there are very few studies that have been conducted with pulses as the prebiotic source. At the same time, pulses including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*) chickpea (*Cicer arietinum*) and lupins (*Lupinus perennis)* are excellent food sources with numerous health-promoting benefits. Pulses constitute an important source of energy, dietary protein, carbohydrate, dietary fibre, minerals and vitamins required for human health. The nutritional composition of pulses such as complex carbohydrates (i.e., resistant starch and oligosaccharides), minerals (e.g., folate, calcium, manganese, magnesium, iron, copper, phosphate, potassium and zinc) as well as antioxidants, and only very small amounts of unsaturated fats, have made this ingredient a very good source of prebiotic components for yogurt and probiotics fortification. In Tables 2.1 and 2.2, the nutritional composition and carbohydrates content of some pulses are summarized (Boye et al*.,* 2009).

Table 2.1- Proximate composition of various pulses (Boye et al., 2009).

Pulse		Composition (g/100 g of sample)				
	Variety	Protein[1]	Fat	Fiber	Ash	Carbohydrate
Pea	Pigeon	19.39	3.24[5]	5.56[3]	4.05	-
	Cowpea	22.53	1.60[5]	5.33[3]	3.81	-
	Lencolen	34.7	2.4	4.25	3.93	54.72
Chickpea	Garbanzo beans,	19.30	6.04	17.4[2]	2.48	60.65
	Kabuli (Iraq)	23.6	4.9	4.4	3.1	-
	Kabuli (India)	20.6	6.6	3.0	3.5	-
	Kabuli (Canada)	22.1	6.5	7.8	2.6	-
	Desi (India)	18.4	5.8	6.2	3.4	-
	Desi (Canada)	25.1	6.1	8.4	2.8	-
	Surutato	21.7	5.6	4.3[4]	3.0	65.4
	Surutato	26.2	6.0	5.5[3]	2.8	59.5
	Kabuli	29.0	6.0	6.0	3.0	-
	Desi	25.0	4.5	9.0	3.2	-
Lentil	Giza	27.5	1.16	-	4.03	63.4
	Family 91	26.7	1.24	-	3.41	64.6
	Pakistani	26.4	1.25	-	1.46	64.5
	Giza 9	31.4	1.15	6.75	4.16	56.53
Bean	Kidney	23.58	0.83	24.9	3.83	60.01
	Red kidney	16.89	1.64[5]	30.34	1.14	-
	V.C 2010	26.40	1.75	6.15	4.50	61.20

[1]N x 6.25; [2]Total dietary; [3]Crude; [4]Acid detergent; [5]Ether extract

Table 2.2- Average carbohydrate content in different Canadian pulses and their digestibility in human body (Wang and Daun, 2004).

Sample	Mean of carbohydrates content in pulses (g/100 g dry matter)				
	Sucrose	**Raffinose**	**Stachyose**	**Verbascose**	**Oligosaccharides**
Digestibility in human body	+	-	-	-	-
Chickpea (desi)	2.03	0.54	1.64	ND[a]	2.18
Chickpea (kabuli)	3.84	0.61	2.20	ND[a]	2.81
Green lentil	2.01	0.43	2.09	0.56	3.07
Red lentil	1.80	0.42	1.94	0.52	2.87
Field pea	2.8	0.7	2.7	1.0	4.4
Navy bean	3.2	0.5	4.0	ND[a]	4.6
Black turtle bean	3.93	0.57	3.50	0.07	4.14
Cranberry bean	4.14	0.23	3.13	0.21	3.58
Dutch brown bean	2.85	0.34	2.97	0.17	3.47
Dark red kidney bean	3.45	0.26	3.80	0.14	4.20
Great Northern bean	5.14	0.54	3.42	0.02	3.98
Light red kidney bean	4.69	0.26	3.44	0.16	3.85
Pink bean	4.54	0.31	3.65	0.02	4.02
Pinto bean	4.40	0.37	3.65	0.04	4.07
Small red bean	4.74	0.45	3.48	0.09	4.02
White kidney bean	3.67	0.22	3.53	0.18	3.93

a: Not detectable

Recalling the nutritional requirements of lactobacilli bacteria, it is hypothesized that pulse ingredients could be used as beneficial sources of prebiotic as well as protein, peptide and amino acids for growth and stability of yogurt starters and probiotic microorganisms. According to the literature, the protein type and ingredients strongly affect the microbial, physical and sensory properties of the yogurt and probiotics. Meanwhile, pulses can bring fibers, minerals and protein to the yogurt blend. This constitutes an opportunity for innovation and according to the literature, little has been done with pulses. An examination of the effects of pulse

ingredients on these prospects is therefore warranted. In preliminary studies of this project, we planned to examine the effect of supplementation with pulse ingredients including lentil flour, chickpea flour, pea protein and pea fiber, in comparison with some common protein sources such as skim milk and soy protein. We also planned to look at the acidification trend in yogurt starter and probiotic microorganisms. First we examined two commercial yogurt starters and two probiotics (*L. rhamnosus and L. acidophilus*). Later on, based on the results, we narrowed our research to selected pulse ingredients as well as microbial cultures.

Connecting Statement to Chapter 3

Canada has great leadership in pulse production and export, however in spite of the high nutritional value of pulses; they do not represent a significant share of the Canadian diet. The importance of functional properties of pulse ingredients and food supplementation was described in two previous chapters. Previous studies on food supplementation have rarely focused on the use of pulse ingredients in food systems. Therefore, to address the challenge of increasing pulse consumption in the Canadian diet, food supplementation with pulse ingredients was considered.

Preliminary studies are focused on studying the functional properties of pea protein, pea fiber, lentil flour and chickpea flour. These results are presented in chapter 3. Furthermore, to determine the stability of these ingredients in acidic beverage systems, apple juice and orange juice supplemented using the selected pulse ingredients were formulated. The physical and sensory properties of the supplemented beverages were studied and the results are also presented. Parts of this study were presented at the following conference:

Zare, F., Boye, J.I., Orsat, V. & Simpson, B.K. (2009), Development of food products supplemented with pulse ingredients. IFT, June 6^{th}-10^{th}, Anaheim, CA, USA, (poster presentation).

Chapter 3: Functional properties and sensory evaluation of beverages supplemented with pulse ingredients

Abstract

The objective of this study was to explore the use of pulse ingredients in the development of orange juice and apple juice supplemented beverages. Pulse fractions including pea protein, chickpea flour, lentil flour and pea fibre were selected and initially characterized with respect to specific functional properties (i.e., water holding capacity, fat absorption capacity, protein solubility, emulsifying and foaming properties). These properties were selected because of their importance in beverage applications. Apple juice was supplemented with 1-4 % pulse ingredients, whereas a supplementation level of 1-2% was used for orange juice. The physical and sensory properties of the supplemented beverages were measured after production and during 4 weeks refrigerated storage. Beverage supplementation with 1% and 2% pulse fractions gave similar results in terms of sensory attributes for both orange and apple juice in comparison with their relative control and control with pectin added samples. In terms of turbidity apple juice and orange juice supplementation with 2 CPF, 1 PF, and 2 PF either increased or altered slightly the turbidity of beverages in comparison with control and pectin-added control sample. However, in terms of the cloud stability, visual stability and color, 1-4% supplementation negatively affected the beverages especially for orange juice beverages in comparison with their relative control and control with pectin added samples. The results suggest that pulse fractions such as 1-2% of pea protein, pea fiber, chickpea flour and lentil flour could serve as good value added ingredient for some beverage applications due to their physical and sensory properties, while more studies are recommended to improve the stability of the final production especially during storage.

3.1 Introduction

Pulses are the dry seeds of low fat legumes including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*). They are highly nutritional and

contain high or good amounts of complex carbohydrates (e.g., resistant starch and oligosaccharides), protein, vitamins and minerals (e.g., folate and iron) as well as antioxidants, and only very small amounts of unsaturated fats (Ofuya & Akhidue, 2005).

Various research studies have suggested that regular dietary intake of pulses may reduce the risk of developing chronic health problems such as obesity, diabetes, heart disease and cancer (Hu, 2003; Jacobs & Gallaher, 2004; Kelly, Frost, Whittaker, & Summerbell, 2004; Williams, Gafenauer, & O'Shea, 2004; Schatzkin & Mouw, 2007). Furthermore, regular consumption of pulses may assist with weight management by increasing the feeling of satiety and also controlling blood sugar and appetite due to their low glycemic index (Koh-Banerjee, Franz, Sampson, Liu, Jacobs, Spiegelman, Willett, & Rimm, 2004). According to the United State Department of Agriculture (USDA, 2005), "Diets including beans may reduce risks of heart disease and certain cancers**"**. Canada's 2007 Food Guide to Healthy Living also recommends taking meat alternatives such as beans, lentils and tofu on a regular basis and further suggests that regular consumption of beans and other meat alternatives such as lentils can help to lower the amount of saturated fat in the diet (Health Canada, 2007).

In addition to nutritional benefits, functional properties of pulse ingredients could play an important role in food systems. The techno-functional properties of interest in food formulation include solubility, water binding, fat binding, emulsification, foaming, gelation, thickening and flavour binding capacity. These physico-chemical properties play an important role during food processing, storage, preparation and consumption (Kinsella, 1982). Amino acid composition, structure and conformation and interactions between proteins and other food components (e.g., salts, fats, carbohydrates and phenolics) as well as pH, temperature and other process specifications all affect to some extent the quality and functionality of food ingredients (Boye, Zare, & Pletch, 2009).

Food supplementation with pulse ingredients could offer a promising opportunity to improve the nutritional properties or the functional properties of formulated food products. Beverage supplementation with nutraceutical components and traditional nutritional ingredients has

been shown to improve the nutritional and rheological quality of beverages (Renuka, Kulkarni, Vijayanand, & Prapulla, 2009). Several studies have reported beverage supplementation with different food ingredients such as fiber (Beristain, Cruz-Sosa, Lobato-Calleros, Pedroza-Islas, Rodriguez-Huezo, Verde-Calvo, 2006; Dahl, Whiting, Isaac, Weeks, & Arnold, 2005), whey and whey protein (Kazmierski, Agboola & Corredigi, 2003; Pescuma, Hébert, Mozzi, Valdez, 2010; Vojnovic, Ritz , & Vahcic, 1993), soy flour and soy protein (Jasentuliyana, Toma, Klavons, & Medora, 1998; Kent & Harper, 2003; Tiziani, & Vodovotz, 2005), peanut (Deshpande, Chinnan, McWatters, 2008), fructooligosaccharide (FOS)(Renuka et al., 2009), β-Glucan (Din, Anjum, Zahoor & Nawaz, 2009; Temelli, Bansema, & Stobbe, 2004) and more.

To ensure market acceptability, supplemented beverages will need to be comparable to or better than non-supplemented beverages in terms of quality, marketability and shelf life. Only very few studies have been done on beverage supplementation using pulse ingredients such as chickpea (Luz-Fernandez de Tonella, & Berry, 1987) and pea (Jackman & Yada, 1989). In general, in spite of their high nutritional value and accessibility, pulses have not represented a significant share of the Canadian diet. In order to offer Canadians with options to increase their pulse consumption, preliminary studies were conducted to explore the stability of selected pulse ingredients in acidic beverage systems using apple juice and orange juice as model food systems. The pulse ingredients selected included pea protein, chickpea flour, lentil flour and pea fiber. The functional properties of the selected pulse ingredients were initially studied. Subsequently the physical and sensory properties of beverages supplemented with the pulse ingredients (in the presence and absence of pectin which was used as a stabilizer) were studied and compared with soy supplemented beverage as well as non-supplemented control beverages (i.e., apple and orange juices with or without pectin).

3.2 Materials and methods

3.2.1 Sample preparation and supplementation

Pulse fractions used in this study were as follows: Chickpea flour from Diefenbaker Seeds Company (Elbow**,** SK, Canada), lentil flour from K2 Milling Company (Tottenham**,** ON, Canada),

pea fiber from Best Cooking Pulses Inc (Rowatt**,** SK, Canada) and pea protein from Nutri Pea Company (Portage La Prairie**,** MB, Canada). Soy protein concentrate from Oleanergie F2001 Company (St. Hyacinthe, QC, Canada) and soy flour from ADM Company (Decatur**,** IL, USA) were used. Unfiltered and unpasteurized apple juice prepared from the McIntosh variety, from Quinn farm (Ile Perrot; QC, Canada) and fresh oranges from the retail market (Navel Orange variety) were obtained. Low-methoxy pectin was purchased from TIC Gum Company (PA, USA).

Apple juice was stored in the refrigerator at 4 °C before use. Oranges were washed with tap water and the juice was extracted with a household juice extractor model E415 (Presse-Agrumes, France) and stored in a refrigerator prior to supplementation with the pulse fractions. For comparison, apple juice and orange juice supplemented with 2% soy protein concentrate were also prepared. Plain juice (with and without 2% pectin) were also prepared and used as controls. Figure 3.1 presents a schematic diagram of the process used for juice supplementation. All samples were subsequently analyzed to determine their physico-chemical properties such as pH, turbidity, loss of cloud stability, visual stability index, and color as well as sensory properties such as flavour, mouth feel and overall acceptance. Table 3.1 shows the composition and coding used for the different samples.

Table 3.1. Composition and coding used for the different beverage samples studied

Sample	Code	Ingredient used as supplement and concentration (w/v)	Pectin concentration	Beverage Matrix(mL)
			Pectin (% w)	Apple juice / Orange juice
Non-supplemented control apple juice/ orange juice	AJ Control/ OJ Control	-----	0	100
Non-supplemented control fruit juice with pectin	Control-pectin	-----	2	100
2% soy protein concentrate	SPC	2% soy protein concentrate	2	100
1% pea protein	1 PP	1 % pea protein	2	100
2% pea protein	2 PP	2 % pea protein	2	100
3% pea protein	3 PP	3 % pea protein	2	100
4 % pea protein	4 PP	4 % pea protein	2	100
1% chickpea flour	1 CPF	1 % chickpea flour	2	100
2% chickpea flour	2 CPF	2 % chickpea flour	2	100
3% chickpea flour	3 CPF	3 % chickpea flour	2	100
4% chickpea flour	4 CPF	4 % chickpea flour	2	100
1% lentil flour	1 LF	1 % lentil flour	2	100
2 % lentil flour	2 LF	2 % lentil flour	2	100
3 % lentil flour	3 LF	3 % lentil flour	2	100
4 % lentil flour	4 LF	4 % lentil flour	2	100
0.6% pea fiber	PF1	0.6 % pea fiber	2	100
1.25% pea fiber	PF2	1.25 % pea fiber	2	100

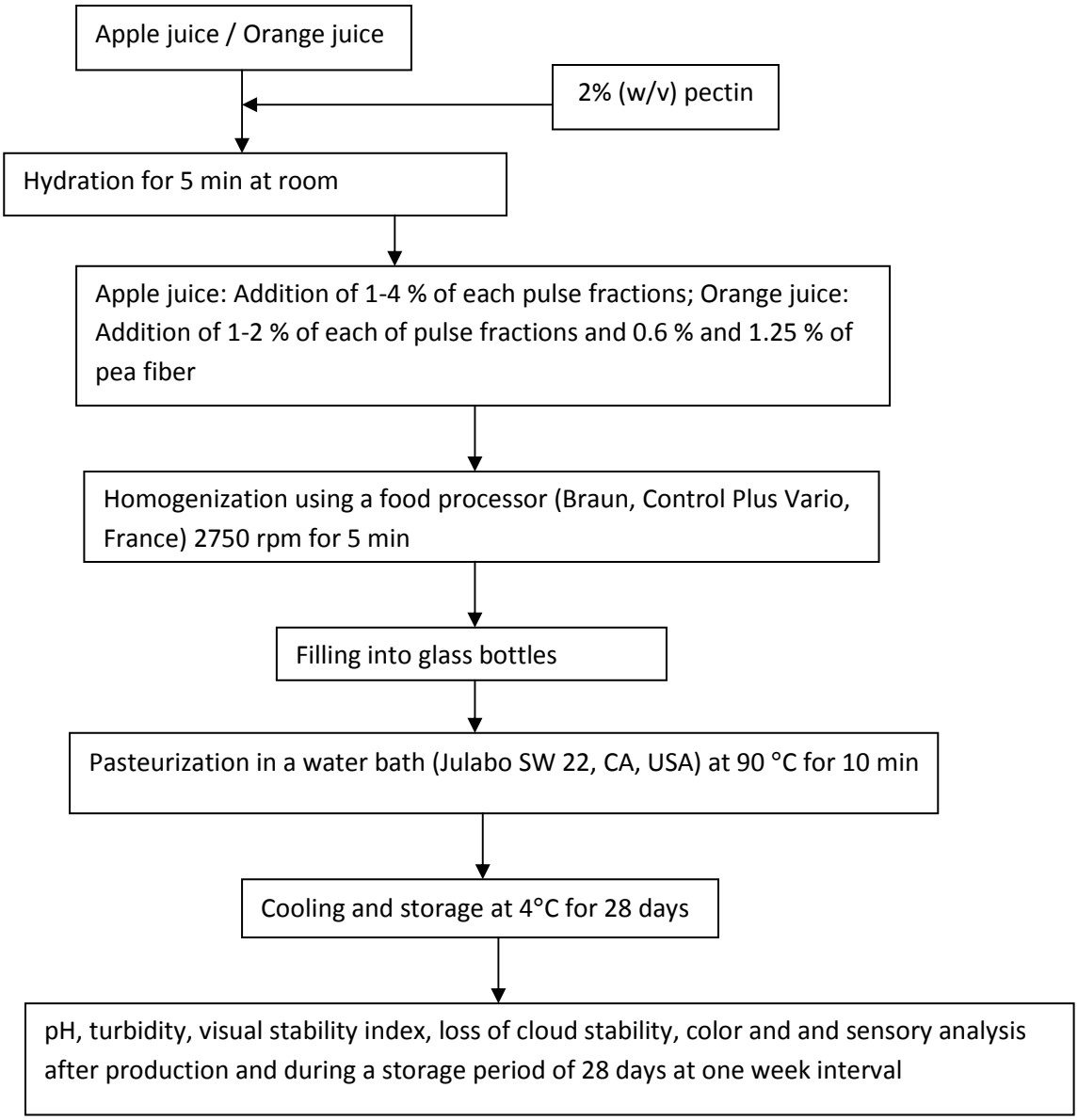


Figure 3.1- Schematic presentation of the process used for the preparation of the fruit juices

3.2.2 Functional properties of pulse ingredients

Proximate analysis of pulse fractions including protein, moisture, fat and ash measurements were done using standard AOAC methods (AOAC, 1990). pH was measured using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada). Functional properties studied included water holding capacity using AACC method 88-04 (AACC, 1990); fat absorption capacity according to the method described by Lin, Humber and Sosalki, (1974); protein solubility with the Bradford method (1976); emulsifying properties with the method described by Pearce and Kinsella (1978); and foaming capacity using the method described by Waniska and Kinsella (1979).

3.2.3 Beverage characterization

Turbidity of the beverages was measured according to the method described by Stähle-Hamatschek and Gierschner (1989); with some modifications. Briefly, the transparency of 100 mL of sample was measured (Ts) using a UV-Visible spectrophotometer (Cary 300 Bi, Varian, Canada) at 695 nm. Samples were then centrifuged at 20 °C for 20 min at 4200 rpm (SARSTEDT centrifuge, AG & Co., Germany). The transparency of the supernatant was measured (Tc) and the percentage turbidity (% T) was calculated as (Tc/Ts)*100. Cloud stability in fortified juices was measured by the method described by Kazmierski et al., (2003). Transparency of the supernatant of the centrifuged samples (20 °C /15 min/3000 rpm, SARSTEDT centrifuge, AG & Co., Germany) was measured at 659 nm using a UV-Visible spectrophotometer (Cary 300 Bi, Varian, Canada). An increase in transparency was considered as an indication of loss of cloud stability. This measurement was carried out at 7-day intervals during the storage period. Visual stability index (VSI) was measured according to Jasentuliyana et al., (1998); from the height of the cloudy phase and the total height of the beverage measured in a graduated glass cylinder: % VSF was calculated as (height of the cloudy phase or solid particulates /total height of suspension)*100. This measurement was carried out at 7-day intervals during the storage period. The color of the beverages was also measured using a Labscan II colorimeter (Hunter Associate Laboratory, Inc., Restone, VA). Beverage pH was measured with an Accumet pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada).

3.2.4 Sensory analyses of beverages

Sensory analyses (flavor, mouth feel and overall acceptance) of the supplemented samples and controls were evaluated by 25 untrained panelists using a 9- point hedonic scale. Each panelist was provided with a maximum of 3 samples at a time and they were asked to score samples from extremely like (1) to extremely dislike (9). The sensory room was equipped with red light to blind the panelists to the color of the beverages.

3.2.5 Statistical analysis

Excel 2007 was used for the calculation of means and standard deviation. Statistical analysis was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two sample t-test for comparison of two means. All experiments were done in three separate independent trials.

3.3 Results and Discussion

3.3.1 Proximate analysis of pulse ingredients

The proximate composition of the pulse ingredients are summarized in Table 3.2. The pea protein concentrate contained the highest protein content (79.96% w/w) whereas pea fibre contained the lowest (7.20% w/w). Fat content of the pulse ingredients ranged between 0.06% (w/w) for lentil flour to 7.38% (w/w) for chickpea flour. Moisture content varied from 3.18% to 9.99% for the different pulse ingredients.

Table 3.2- Proximate composition of pulse fractions

Sample	Protein % (w/w)	Moisture % (w/w)	Fat % (w/w)	Ash% (w/w)	Carbohydrate % (w/w)
	Average ± SD	Average ± SD	Average ± SD	Average± SD	Average
Pea protein	79.97 ± 0.13 a	3.18 ± 0.07 b	0.53 ± 0.86 b	4.79 ± 0.42 a	11.53
Chick pea flour	23.52 ± 0.09 b	9.99 ± 0.01 a	7.39 ± 12.77 a	3.16 ± 0.36 b	53.94
Lentil flour	24.83 ± 0.12 b	9.45 ± 0.14 a	0.06 ± 0.10 b	2.68 ± 0.27 b	62.98
Pea fibre	7.21 ± 0.17 c	5.29 ± 0.04 b	0.38 ± 0.14 b	1.95 ± 0.29 c	85.17

Means with the same letters are not significantly different, for a given column (P<0.05)

3.3.2 Functional properties of pulse ingredients

Results for the functional properties are summarised in Table 3.3. The water holding capacity (WHC) of pulse fractions ranged between 0.8-3.1 (mL/g). The highest WHC was obtained for pea protein followed by pea fiber and the lowest for chickpea flour. Water holding capacity is affected by percentage of protein, cultivar and processing treatments (Kaur & Singh, 2007). WHC is also attributed to the hydrophilic sites of the protein molecules (Lin et al., 1974) as well as the fiber content (Heller & Hackler, 1977). The higher protein content in pea protein in comparison with other pulse ingredients explains its higher water holding capacity. Also pea fiber ranked as the second for water holding capacity which is due to higher fiber content in comparison with other samples (Heller & Hackler, 1977). According to the report by L'Hocine, Boye, & Arcand (2006), soy protein concentrate (SPC) and soy protein isolate (SPI) had WHCs ranging between 3.9- 4.3 (mL/g). Comparing these results with WHC of pea protein sample (containing about 80% protein) it could be suggested that pea protein has a similar WHC to soy fractions.

Solubility profile of pulse ingredients at pH ranging from 1-11 are presented in Figure 3.2. In most pulse proteins, the solubility is highest at low acidic and high alkaline pH values. The Isoelectric point is generally between pH 4 and pH 6 for most pulses (Torki et al., 1987; Fernandez-Quintela et al., 1997). According to our study, the isoelectric point of pea protein

and lentil flour ranged between pH 3.5 to 4.5 (region of lowest solubility), while that of chickpea flour was between pH 2.5 to 4.5. Interestingly, the solubility of both the chickpea flour and lentil flour (at the region of highest solubility) was higher (60 – 80%) than for pea protein concentrate (20-25%). The processing treatment used for the production of our pulse ingredients is unfortunately not known; however, it is reported that the processing method affects the functional properties of pulses (Obatolu et al., 2007; Swanson, 1990). Production method such as precooking and drum drying also could reduce the nitrogen solubility of pulse flour (Carcea, 1986). Therefore, the lower solubility of pea protein in spite of higher protein content could be attributed to protein denaturation and deformation of molecular structure against the solubility in proteins which probably resulted from the processing treatments (L'Hocine et al., 2006). In our studies the protein solubility in pea protein is the lowest while its water holding capacity is the highest among other pulse ingredients. This is in accordance with what was reported by Lin et al., (1974), for sunflower protein. However, L'Hocine et al., (2006), reported that there is not always a correlation between the water holding capacity and the solubility. Interaction between protein and polysaccharides may also affect the solubility, as reported by Braudo et al., (2001), however, this interaction is not studied in our research

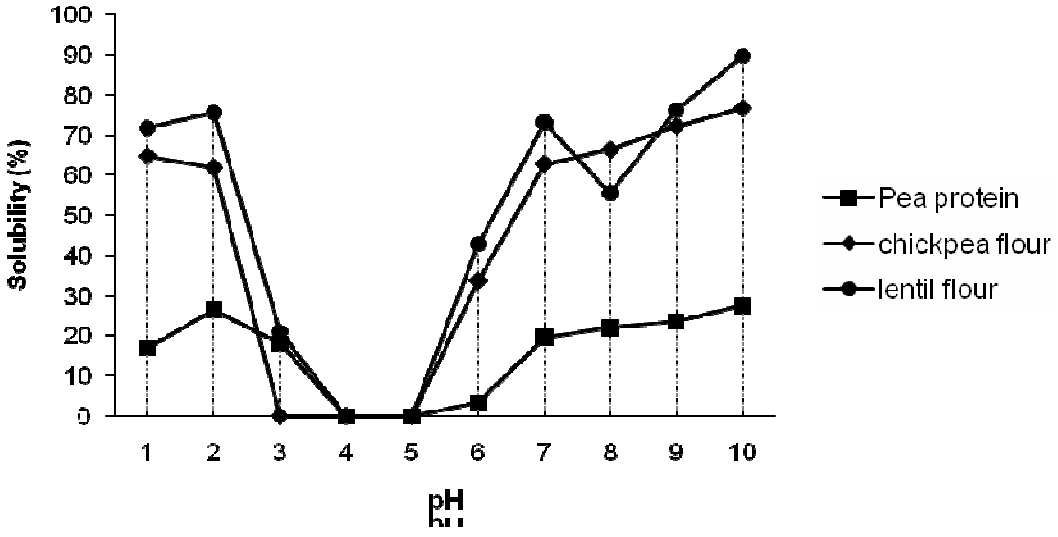


Figure 3.2- Protein solubility profile of pulse ingredients

Fat absorption capacities (FAC) of all samples ranged from 76 % to 116 % (w/w). The lowest FAC was found for lentil flour and highest for pea fibre. FAC of pea protein and chickpea flour were 79% and 87% (w/w) respectively, which are lower than what Fernandez-Quintela et al. (1997) reported. According to Kinsella (1976), the FAC mechanism is mostly attributed to physical entrapment of oil, but it may be also affected by hydrophobicity of protein molecules and with our results, the highest FAC indicates the physical oil entrapment in the pea fiber. All the FACs measured in this study were lower than for SPC or SPI (FAC= 218-251 % (L'Hocine et al., 2006)).

Emulsifying activity indices (EAI) of the pulse fractions ranged between 11-14 m^2/g, while the emulsifying stability index (ESI) varied between 26-33 min. Emulsifying properties are affected by hydrophobicity and hydrophilicity properties of proteins and amino acids that are contained in the structure of proteins. Proteins can form a thin layer or protein film around oil droplets in a food system to make an emulsion. EC could simply be defined as the amount of oil that can be emulsified per unit of protein while ES shows the ability of the emulsion to oppose changes to the structure of emulsion over a period of time (Pearce & Kinsella, 1978; Boye et al., 2009). Paredes-Lopez et al. (1991) reported that EA is higher (72.9 vs 63.7) in chickpea protein isolate with higher protein content (87% vs 84%). Some other studies also have showed that processing such as hydrolysis and interaction between carbohydrates (chitosan) and proteins could improve emulsifying properties in pulse ingredients (Boye et al., 2009). According to L'Hocine et al., (2006), higher emulsion stability could be due to the presence of more native proteins. In our studies, lentil flour and chickpea flour had the lowest while pea protein had the highest emulsifying properties among all the samples, which can be attributed to the higher protein content in pea protein. However there was no significant difference between the measured EAI and ESI of all samples.

Foaming capacity (FC) or foaming expansion (FE) is expressed as the volume (%) of foam which increases due to whipping while foam stability (FS) is defined as the change in the volume of foam over a time period (Boye et al., 2009). Foaming expansion of our pulse fractions ranged between 400-1500 %. The highest value was found for chickpea flour and the lowest for lentil flour. Foaming properties of pea protein and lentil flour however, were not significantly

different (P<0.05). Foaming properties are related to protein content, protein structure and processing treatments. Paredes-Lopez et al., (1991), measured higher FE and FS for chickpea protein isolate with higher protein content. According to Obatolu et al., (2007), foaming capacities in raw yam bean (40.20%) were significantly greater than boiled yam bean (1.98%), further highlighting the effect of processing treatments. Again, since the processing treatment of our pulse fractions is not known to us, the difference in foaming properties could be attributed to protein content and processing treatments. In comparison with soy protein isolates which gave foaming expansion (FE) values ranging from 400-550, pulse fractions such as chickpea flour may have better foaming properties (L'Hocine et al., 2006). Solubility has an important influence on foaming behaviour as well as the ionic strength (Lawal, 2005), although we did not test the ionic strength of our samples, the lower foaming capacity of pea protein can be attributed to its lowest solubility.

Table 3.3- Functional properties of pulse fractions

Sample	Water Holding Capacity (mL/g)	Fat Absorption Capacity % (w/w)	Emulsifying Properties		Foam Expansion (FE %)
			EAI (m^2/g)	ESI (min)	
	Average ± SD	Average ± SD	Average ± SD	Average ± SD	Average ± SD
Pea protein	3.13 ± 0.02 a	79.70 ± 4.85 c	13.3755 ± 0.00 a	32.7530 ± 0.30 a	514.97 ± 49.50 b
Chickpea flour	0.83 ± 0.01 d	87.69 ± 5.18 b	11.9363 ± 0.01 a	25.7969 ± 4.46 a	1348.20±14.94 a
Lentil flour	0.88 ± 0.01 c	76.70 ± 2.71 c	12.9838 ± 0.02 a	26.1138 ± 4.18 a	478.26 ± 7.62 b
Pea fibre	2.73 ± 0.00 b	116.28 ± 3.67 a	ND	ND	ND

Means with the same letters are not significantly different (P<0.05); ND: not defined

3.3.3 Physico-chemical properties of beverages

3.3.3.1 pH

pH values of all the supplemented apple juice were significantly different from control and pectin-added control samples (P<0.05) while the pH was also different for orange juice supplemented samples except for certain supplementations (1 CPF, 1 LF, PF1 and PF2) (Table 3.4). Pulse supplementation increased the pH in what appeared to be a dose dependent manner (i.e., the greater the level of supplementation, the higher the pH value in both the

apple juice and orange juice samples), however the difference is not significant (Table 3.4). Pectin did not alter the pH in the pectin-added control in comparison with the control samples, while the pH of the control apple juice and pectin-added apple juice was 3.53 and 3.50, respectively, pH of the supplemented apple juice samples ranged between 3.6 (apple juice with 1 % CPF) to 4.21 (apple juice with 4% PP). Similarly, the pH of the control orange juice samples (with and without pectin) was 3.78 and 3.75, respectively, whereas pH of the supplemented orange juice samples ranged between 3.78 (orange juice with 0.6 and 1.25 % PF) to 4.02 (orange juice with 2 LF and 2 PP) (Table 3.4)). Hence, the addition of the pulse ingredients had the general effect to reduce the acidity of the supplemented juices.

3.3.3.2 Turbidity

Supplementation had a significant effect on turbidity of apple juice, and all supplemented samples except 2 CPF, had higher turbidity in comparison with both controls (Table 3.4). Increasing the level of orange juice supplementation significantly increased turbidity (P<0.05), except for samples 3 CPF and 4 CPF and also 2PP and 3PP, in comparison with control and control pectin-added sample, where the differences were not significant (P<0.05). Additionally, the results showed that the turbidity of the supplemented orange juice samples had greater variability and the supplemented samples also showed significantly greater turbidity than the control samples. Addition of pectin did not alter the turbidity of both apple juice and orange juice in comparison with the control sample. According to Benitez, Genovese, & Lozano (2007), the turbidity of fruit juices can be affected by several factors such as pH, ionic strength and particle size. Although we did not test the particle size or ionic strength of our sample, changes in pH value are in accordance with turbidity increase and can be solely attributed to the supplementation. Since the apple juice and orange juice used as the beverage matrices for this study were not filtered and hence contributing already to the turbidity, it resulted that apple juice and orange juice with lowest supplementation with 2 CPF, 1 PF, and 2 PF provided the most satisfactory turbidity results in comparison with control and pectin-added control samples.

Table 3.4- Turbidity and pH of apple and orange juice and control and supplemented samples (In each beverage matrix, means with the same letter are not significantly different (P<0.05)

Sample	% Turbidity (Average ± SD)	pH
Apple juice (AJ)/ Orange juice (OJ)		
AJ control	1785.85 ± 20.59 i	3.53 d
AJ Control-pectin	1458.91 ± 3.50 i	3.50 d
AJ SPC	12961.04 ± 332.50 b	3.92 b
AJ 1 PP	12274.47 ± 55.44 c	3.72 bc
AJ 2 PP	15670.02 ± 99.48 a	3.98 b
AJ 3 PP	15900.76 ± 309.62 a	4.11 a
AJ 4 PP	12530.21 ± 193.73 c	4.21 a
AJ 1 CPF	5590.67 ± 63.17 g	3.60 c
AJ 2 CPF	1648.60 ± 14.86 i	3.67 c
AJ 3 CPF	712.24 ± 83.07 j	3.75 bc
AJ 4 CPF	801.87 ± 13.32 j	3.82 b
AJ 1 LF	7971.23 ± 37.21 d	3.60 c
AJ 2 LF	7392.69 ± 34.66 e	3.66 c
AJ 3 LF	4885.11 ± 74.80 h	3.72 bc
AJ 4 LF	6576.81 ± 366.59 f	3.79 b
OJ Control	3743.49 ± 10.23 g	3.78 c
OJ Control-pectin	3910.72 ± 8.09 fg	3.75 c
OJ SPC	9298.72 ± 142.41 b	3.95 ab
OJ 1 PP	7294.28 ± 92.99 c	3.91 ab
OJ 2 PP	12184.99 ± 174.68 a	4.02 a
OJ 1 CPF	3088.00 ± 24.20 h	3.82 bc
OJ 2 CPF	1418.99 ± 390.62 i	3.90 b
OJ 1 LF	5068.77 ± 46.48 d	3.80 bc
OJ 2 LF	5269.79 ± 138.36 d	4.02 a
OJ PF1	4156.55 ± 55.98 f	3.78 c
OJ PF2	4290.14 ± 69.17 e	3.78 c

3.3.3.3 Loss of cloud stability

Loss of cloud stability (L.C.S) in supplemented apple juice and orange juice after 1 week and during 3 weeks refrigerated storage are presented in Figures 3.3 and 3.4. Our results showed greater transparency of the centrifuged samples as a function of time. This indicates that the cloudy appearance of the supplemented apple juice and orange juice was not stable during the 3 weeks storage period in comparison to the control and pectin-added control samples. Except for pea protein in apple juice, the level of supplementation appeared to influence cloud stability (i.e., as the percentage of pulse ingredients increased, loss of cloud stability decreased after 3 weeks) (Figure 3.3).

In supplemented orange juice, loss of cloud stability varied for different samples. Supplementation with pea fiber had a great effect on L.C.S., while cloudiness of 2 CPF, 1 LF and SPC supplemented sample remained stable after 3 weeks and L.C.S. of 2 PP sample was close to the control samples.

Cloudiness of fruit juice is basically due to presence of pulp particles and pectin naturally present in the apple and orange juices (Kazmierski et al., 2003). It is expected that, with the effect of gravity, the settling of the pulp in both apple and orange juices would increase over the storage period. Our results showed that loss of cloud stability is affected by the supplementation level in both orange and apple juices. According to Kazmierski et al. (2003), the most important cause of cloud de-stabilization and clarification of fresh juice is the activity of the enzyme pectinmethylesterase (PME). It is therefore important to heat inactivate the PME enzyme to minimize its activity on the juice pectins, activity which was not an issue in our study since the pasteurization process of our juices would have inactivated the PME enzyme. However, even in enzyme-inactivated, cloud-stable juice, the introduction of proteins could influence the stability due to possible interactions between pulse protein and juice components such as pectins and other pulp components (Kazmierski et al., 2003). In our study, the pectin-added control samples showed higher L.C.S values in both apple and orange juices which is supporting Kazmierski et al. (2003) report. Electrical charge of juice particles, pH and

temperature may also affect cloud stability of fruit juice. Kazmierski et al. (2003), reported that whey protein supplemented orange juice was more stable at pH 3 and 4 rather than pH 5. Regarding the pH of supplemented apple juice and orange juice, our data does not support this hypothesis except for PF1 and PF2 supplemented orange juice.

3.3.3.4 Visual stability index

Figures 3.5 and 3.6 present the visual stability index (VSI) of the supplemented apple juice and orange juice respectively after 1 week and 4 weeks of storage. Visual stability index was measured one day following production and at 7-day intervals during storage. After preparation, all samples were poured into a calibrated cylinder for observation. The results showed that on Day 1, practically all of the samples had an acceptable appearance (i.e., not much sedimentation was observed). One week after production, all samples still maintained the cloudy appearance except for apple juice supplemented with 4% chickpea flour which showed greater transparency in the upper layers indicative of greater sedimentation and loss of cloud stability. Significant sedimentation (i.e., more diluted appearance in the upper layers) was observed for most of the samples two weeks after production. Visual stability index (VSI) for all supplemented apple juice decreased moderately (between 13 - 40% after four weeks) whereas in apple juice supplemented with 4% chickpea, the VSI dramatically declined about 85% after one week. In supplemented orange juice the VSI varied between 16-40% after three weeks of storage and reduction was affected significantly by supplementation; however VSI of all supplemented orange juice were almost similar after 4 weeks. The results of VSI in pectin-added control samples were not different from control samples in apple juice while it showed higher stability in orange juice. According to Deshpande et al. (2005), there should be a balance between the visual stability, viscosity and color in a formulated beverage. An increase in the viscosity of a beverage would increase the VSI. So with the higher level of supplementation a higher VSI is expected. The results of VSI in our supplemented samples support this statement, especially for orange juice supplemented samples.

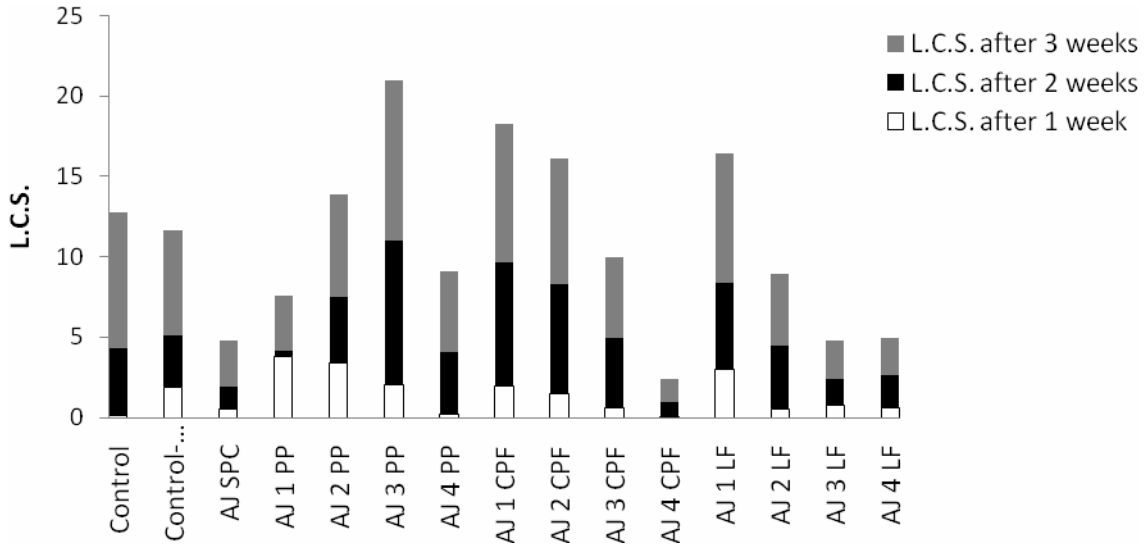


Figure 3.3- Loss of cloud stability of supplemented apple juice and control sample after 1 week and 3 weeks storage

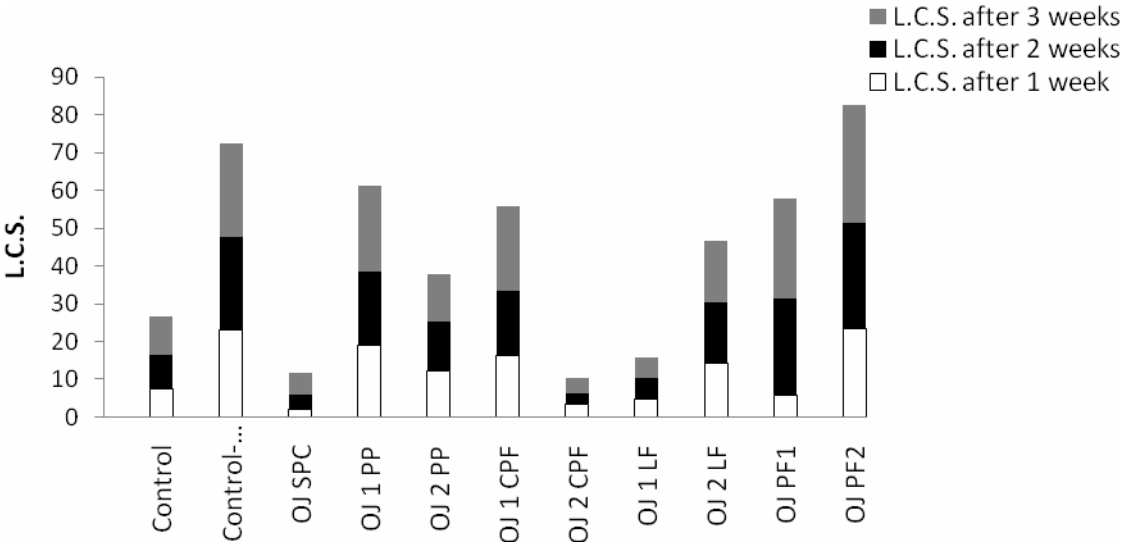


Figure 3.4- Loss of cloud stability of supplemented orange juice and control sample after 1 week and 3 weeks storage

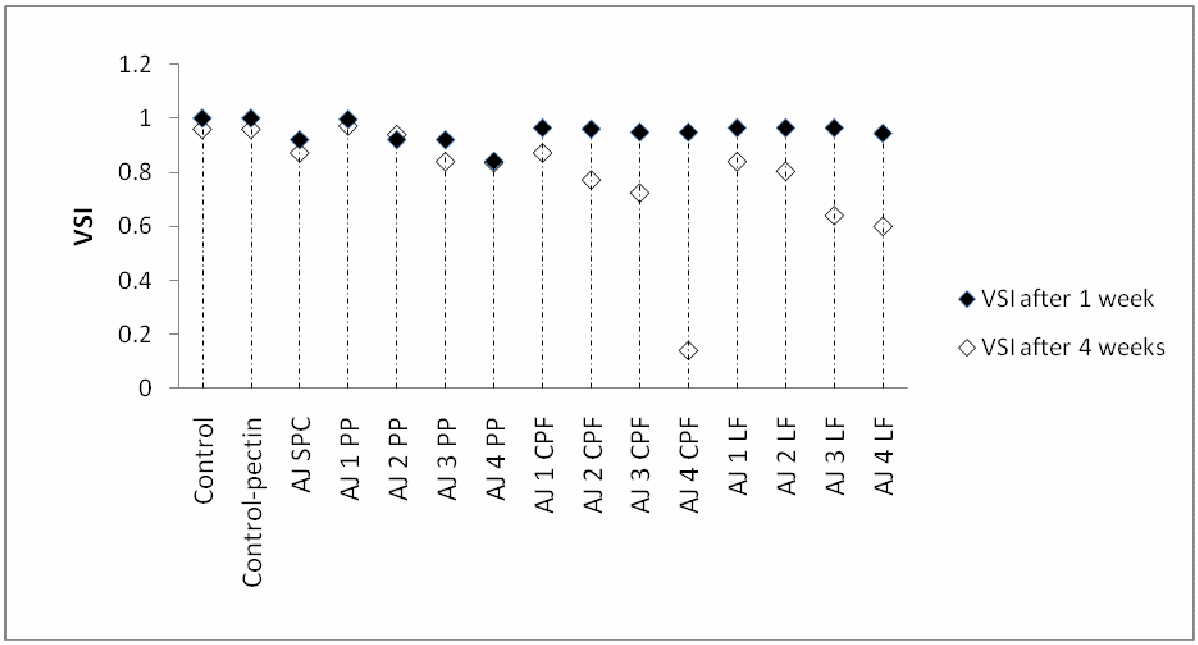


Figure 3.5- Visual stability index of supplemented apple juice and control sample after 1 week and 4 weeks storage

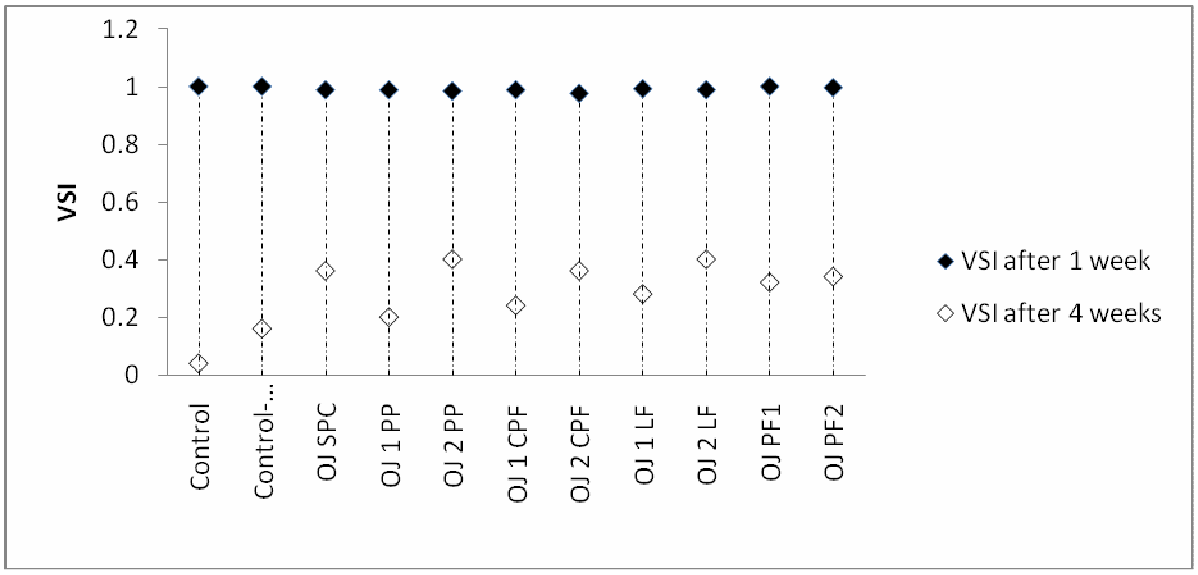


Figure 3.6- Visual stability index of supplemented orange juice and control sample after 1 week and 4 weeks storage

3.3.3.5 Colour

Colour measurements obtained for the supplemented apple juice, orange juice and control samples are presented in Table 3.5. In apple juice the "L" factor (representing lightness, 0 = black to 100 = white) remained in the range of 22.31- 42.34 in all supplemented samples as well as the control. The "a" value (negative values indicate green whereas positive values indicate red/magenta) increased in all samples supplemented with chickpea flour and pea protein but it was almost equal to the control sample for apple juice supplemented with the lentil flour. The "b" value (negative values indicate blue and positive values indicate yellow) dramatically increased in all samples except for apple juice with 3 CPF in comparison with the control and pectin-added control samples. Results for ΔE (color difference between supplemented apple juices and control samples) showed that addition of the pulse fractions changed the color of the juices. Pea protein had the largest effect compared to chickpea and lentil flours, however, there were no significant differences between 1-3% pea protein supplemented samples (P<0.05). There was also, no notable color difference between 2 CPF and 2 LF supplemented apple juice sampled compared to the control sample with pectin.

For supplemented orange juice the results indicated that supplementation affected "b" and "L" compared to the control sample (i.e., "b" was 42.9 - 57.23 in comparison with 48.90 for control and "L" was 26.25 - 48.93 in comparison with 36.35 for the control sample). "a" increased for all supplemented samples with pea fibre (16.3 and 17.3) but it remained in the same range (between 5.6-8.6) for all the other supplemented and control samples. ΔE, which represents the color difference between the supplemented orange juice and control samples, varied between 6.7 and 18.25. ΔE values generally tended to be higher for samples supplemented with higher amounts of the pulse ingredients, but there was no significant difference between ΔE of 1 CPF, 2 CPF, 2 PP and PF 1 and PF2 samples. Besides, 1 LF and SPC supplemented orange juice had no significant color difference when compared with control sample with pectin (P<0.05). The lowest color difference was observed in pectin added control orange juice, in comparison with control sample which indicated that pectin did not affect the color of both apple and orange juices (P<0.05).

Table 3.5- Color parameters (L), (a), (b) and color difference in supplemented apple and orange juice and control samples

Sample	L(Average ± SD)	a (Average ± SD)	b (Average ± SD)	ΔE (Average ± SD)
Apple juice				
AJ control	31.40 ± 2.64	3.98 ± 0.22	0.13 ± 2.71	----------
AJ Control-pectin	22.31 ± 1.56	3.19 ± 0.10	11.76 ± 3.65	14.81 ± 3.8 f
AJ SPC	31.36 ± 0.48	3.36 ± 0.04	21.44 ± 1.37	21.32 ± 1.36 cd
AJ 1 PP	26.17 ± 0.06	5.16 ± 0.01	32.66 ± 0.06	32.97 ± 0.07 b
AJ 2 PP	34.16 ± 0.15	6.12 ± 0.03	32.62 ± 0.01	32.68 ± 0.02 b
AJ 3 PP	35.97 ± 0.05	6.33 ± 0.01	32.40 ± 0.08	32.68 ± 0.08 b
AJ 4 PP	37.89 ± 0.01	7.60 ± 0.02	36.43 ± 0.16	37.05 ± 0.16 a
AJ 1 CPF	30.8 ± 0.21	4.29 ± 0.04	26.55 ± 0.49	11.40 ± 0.16 g
AJ 2 CPF	34.68 ± 0.14	5.16 ± 0.07	31.18 ± 0.30	16.29 ± 0.03 ef
AJ 3 CPF	37.07 ± 0.00	6.89 ± 0.00	25.25 ± 0.00	20.46 ± 3.38 d
AJ 4 CPF	42.38 ± 0.07	4.99 ± 0.05	24.73 ± 0.17	22.92 ± 0.06 c
AJ 1 LF	28.03 ± 0.05	2.87 ± 0.02	21.97 ± 0.08	9.36 ± 0.07 h
AJ 2 LF	34.84 ± 0.01	2.86 ± 0.03	21.48 ± 0.09	15.94 ± 0.03 ef
AJ 3 LF	37.05 ± 0.13	3.13 ± 0.06	21.18 ± 0.12	18.13 ± 0.16 e
AJ 4 LF	42.27 ± 0.12	3.68 ± 0.02	23.61 ± 0.08	22.88 ± 0.13 c
Orange juice				
OJ Control	36.35 ± 0.30	5.64 ± 0.20	48.90 ± 1.54	---------
OJ Control-pectin	35.33 ± 0.21	7.1 ± 0.27	55.42 ± 0.93	6.76 ± 0.998 d
OJ SPC	44.02 ± 1.48	7.49 ± 0.71	47.83 ± 6.36	9.32 ± 2.80 cd
OJ 1 PP	43.03 ± 0.81	8.17 ± 0.35	55.38 ± 3.79	10.05 ± 1.75 c
OJ 2 PP	46.49 ± 0.17	9.42 ± 0.05	57.24 ± 0.34	13.66 ± 0.12 b
OJ 1 CPF	48.39 ± 0.07	5.93 ± 0.09	43.0 ± 0.65	13.43 ± 0.34 b
OJ 2 CPF	47.96 ± 0.03	7.05 ± 0.05	46.22 ± 0.66	12.01 ± 0.13 bc
OJ 1 LF	38.24 ± 0.08	7.60 ± 0.24	57.54 ± 0.14	9.06 ± 0.17 cd
OJ 2 LF	43.52 ± 0.97	8.69 ± 1.58	65.28 ± 3.03	18.24 ± 2.59 a
OJ PF1	26.26 ± 0.07	16.30 ± 0.06	45.09 ± 0.126	15.17 ± 0.04 b
OJ PF2	28.42 ± 0.61	17.46 ± 0.20	48.78 ± 1.07	14.26 ± 0.47 b

3.3.4 Sensory properties of beverages

Results of sensory evaluation for flavour, mouth feel and overall acceptance of the supplemented apple juice and orange juice samples are presented in Table 3.6. Samples were ranked from extremely like (1) to extremely dislike (9). Overall, the results showed that apple juice supplemented with 1% of all the pulse ingredients and also SPC were acceptable in terms of flavor in comparison with control and control-pectin samples (P<0.05). For mouth feel and overall acceptance, all supplemented samples were ranked significantly higher (i.e., less acceptable) in comparison with both control samples. Apple juice supplemented with pectin only, was also found to be as good as the control apple juice in terms of flavour, mouth feel and overall acceptance.

For orange juice, in terms of flavour, 1 CPF, 2 CPF, 1 LF and 2 LF samples were ranked as acceptable in comparison to the control or pectin-added sontrol samples (P<0.05). For mouth feel, all samples except 2 PP ranked similar to the control sample. For overall acceptance, only SPC was ranked significantly non-desirable from control or control-pectin sample (P<0.05).

It is also notable that apple juice or orange juice supplementation with 1% of each of pulse ingredients resulted in overall acceptance score higher than 5 (i.e., neither like nor dislike or better scores). This may suggest that a 1% supplementation level could be a promising target for the creation of innovative products using pulse ingredients. Future studies and further formulation development work could therefore start by exploring this supplementation level.

Results of sensory evaluation did not show any improvement due to presence of pectin in both apple juice and orange juice supplemented beverage, in comparison with control sample (P<0.05).

Table 3.6- Sensory evaluation scores (ranged from extremely like= 1 to extremely dislike= 9) of supplemented apple and orange juice and control samples.

Sample	Flavour	Mouth feel	Overall acceptance
	Average ± SD	Average ± SD	Average ± SD
Apple Juice			
AJ control	3.64 ± 1.46 b	3.44 ± 1.26 b	3.40 ± 1.22 b
AJ Control-pectin	3.48 ± 1.66 b	3.44 ± 1.15 b	3.44 ± 1.32 b
AJ SPC	4.68 ± 1.93 ab	5.16 ± 1.77 a	5.24 ± 1.69 a
AJ 1 PP	4.64 ± 2.03 ab	5.00 ± 2.08 a	4.96 ± 2.07 a
AJ 2 PP	5.32 ± 1.93 a	5.28 ± 2.05 a	5.40 ± 1.80 a
AJ 1 CPF	4.92 ± 1.95 ab	5.20 ± 2.0 a	4.92 ± 1.77 a
AJ 2 CPF	5.48 ± 1.75 a	5.44 ± 1.29 a	5.48 ± 1.35 a
AJ 1 LF	4.32 ± 1.34 ab	4.68 ± 2.13 a	4.56 ± 1.52 a
AJ 2 LF	5.6 ± 2.08 a	5.72 ± 1.81 a	5.88 ± 1.92 a
Orange Juice			
OJ Control	4.56 ± 1.91 cd	4.24 ± 2.00 c	4.68 ± 2.05 b
OJ Control-pectin	4.88 ± 2.38 bcd	5.04 ± 2.14 bc	4.92 ± 2.28 ab
OJ SPC	6.24 ± 1.98 ab	6.28 ± 1.79 a	6.44 ± 1.73 a
OJ 1 PP	5.96 ± 1.88 abc	5.48 ± 1.80 abc	6.16 ± 1.97 ab
OJ 2 PP	6.4 ± 2.04 a	6.12 ± 2.18 ab	6.4 ± 2.10 a
OJ 1 CPF	4.48 ± 1.87 cd	4.96 ± 1.79 abc	5.00 ± 1.95 ab
OJ 2 CPF	5.48 ± 1.73 abcd	5.52 ± 1.32 abc	5.56 ± 1.41 ab
OJ 1 LF	4.32 ± 1.46 d	4.64 ± 1.70 bc	4.68 ± 1.77 b
OJ 2 LF	4.56 ± 1.35 cd	5.52 ± 1.19 abc	5.28 ± 1.36 ab
OJ PF1	4.84 ± 1.31 bcd	4.32 ± 1.46 c	4.60 ± 1.25 b
OJ PF2	5.8 ± 1.63 abcd	5.36 ± 1.57 abc	5.72 ± 1.56 ab

Means with the same letter are not significantly different (P<0.05).

3.4 Conclusion

Functional properties of food ingredients in beverage applications are affected by a variety of factors including protein content, pH and temperature. This research illustrated differences in the functional properties of the different pulse fractions studied. The physical analysis on the beverage systems showed that supplementation at all levels and in both orange and apple juice matrices increased pH. Recalling the importance of pH on a wide range of functional properties of pulses, it could be suggested to consider pH adjustment in beverage supplementation process. This study also showed that 2 CPF, 1 PF, and 2 PF supplementation either increased or altered slightly the turbidity of the beverages in comparison with control and pectin-added control samples. Meanwhile, supplementation with pulse fractions in both apple juice and orange juice decreased cloud stability and visual stability index, during storage. This is indicating that the levels of supplementation and type of supplement are important factors that could alter the physical balance of the supplemented beverages. VSI of all supplemented orange juice was also lower than for the control sample. It is apparent from these results that increasing the content of the pulse fractions lowered the stability of the beverage system and prevented the formation of a stable homogenous system. Color measurements at all levels of supplementation in apple juice indicated that pulse supplementation alters the color of apple juice tending to red and yellow rather than green and blue. Also, at all levels of supplementation the apple juice beverages tended to be whiter than the pectin-added control and control samples. In orange juice supplemented samples, pulse ingredients affected the beverage color to a more yellow and white hue and a significant increase in the “a” value due to pea fiber in orange juice, indicating a color tending to redness, in comparison with all other samples. In terms of sensory attributes (flavour, mouth feel and overall acceptance), 1% or 2% of all pulse ingredients in apple juice and orange juice supplementation gave relatively acceptable products in comparison with control and control-pectin products. This result was highlighted for chickpea flour and pea fiber supplemented orange juice.

Overall, it can be concluded that there is a great potential for beverage supplementation with pulse ingredients (especially 1-2% of supplementation) and studies aiming for new supplemented beverages such as vegetable cocktail supplementation is recommended.

Connecting Statement to Chapter 4

The study of the functional properties of selected pulse ingredients and beverage supplementation showed that overall, 1% and 2% of pulse fractions could give satisfactory results in terms of turbidity, cloud and visual stability, color and sensory attributes for both orange and apple juice in comparison with control and control with pectin added samples. The results therefore suggested that the pulse fractions could serve as a potential value added ingredient for some acidic beverage applications. To follow up on these exploratory studies, we were further interested to determine if pulse ingredients could be used to supplement acidic products such as yogurt and probiotic fermented milk beverages. As pulse ingredients are good sources of complex carbohydrates (e.g., fibre, resistant starch and oligosaccharides), protein, important vitamins and minerals (e.g., folate and iron) as well as antioxidants, it was hypothesized that they could serve as good source of nutrients for yogurt starters and probiotic cultures. In chapter 4 the effect of the addition of pulse ingredients to milk on acid production by probiotic and yogurt starter cultures was therefore studied. The results of this study have been presented at the following conference and a paper has been submitted to LWT Food Science and Technology as follows:

Zare, F., Boye, J.I., Orsat, V., Champagne, C.P. & Simpson, B.K. (2009), Development of novel fermented yogurt and probiotics supplemented with pulse ingredients, Pulse Day, Saskatoon, SK, Canada (poster presentation).

Zare, F., Champagne, C.P, Simpson, B.K., Orsat, V., Boye, J.I., (2011). Effect of the addition of pulse ingredients to milk on acid production by probiotic and yoghurt starter cultures. *LWT - Food Science and Technology,* (article in press).

Chapter 4: Effect of the addition of pulse ingredients to milk on acid production by probiotic and yogurt starter cultures

Abstract

Pulses contain amino acids, carbohydrates, minerals and vitamins which are essential requirements in the human diet and which could also serve as growth nutrients for probiotic and yogurt starter cultures. In this study, milk supplementation with pulse ingredients is examined as a means to increase the nutritional properties of yogurt and probiotic type beverages. The acid production rate of two yogurt starters (A and B) and two probiotic cultures (*Lactobacillus rhamnosus* or *Lactobacillus acidophilus*) was followed in milk supplemented with the following pulse and soy ingredients: pea protein, chickpea flour, lentil flour, pea fiber, soy protein concentrate and soy flour. The pulse ingredients had no negative effect on the acidification trends of the fermented milks. On the contrary, with yogurt culture B, pea fiber, pea protein and lentil flour significantly enhanced the acidification rate. All ingredients used for supplementation improved the acidification rate of probiotic cultures, but the highest effects were obtained with lentil and soy flour. Lentil flour supplementation had the lowest pH after 12 hours which was significantly lower than the product enriched with the same quantity of skim milk powder. The effect of ingredient supplementation on the microbial composition (ratio of cocci to bacilli) of the yogurt products was also examined. The ratio of cocci to bacilli was between 1.8 and 2.5 for all supplemented yogurt samples obtained with culture A, and these variations were not statistically significant ($P<0.05$). With yogurt products obtained from culture B, however, the proportional content of lactobacilli in all supplemented samples was higher than for the milk control and particularly for lentil flour.

4.1 Introduction

Yogurt is a dairy product fermented by a starter culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. Increasingly, these two species are

accompanied with probiotic bacteria which may enhance health benefits (Yang, Swem, & Li, 2006). Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host (Araya et al., 2002). Unfortunately, many probiotic bacteria do not grow rapidly in milk (Klaver, Kingma, & Weerkamp, 1993; Roy, 2005*)*. As a result, numerous studies have been carried out aimed at enriching milk with supplements in order to enhance the growth of probiotics which constitutes an opportunity for innovation. Examples of potential supplements to milk to favor the development of probiotics include extracts or juices of the following: yeast (Kim et al., 1995), citrus (Sendra et al., 2008), ginseng (Goh, Chae, Gang, Kwon, Choi, Lee, & Park, 1993), tomato (Babu, Mital, & Garg, 1992), peanut (Murad, Fathy, & Abdel-Ghani, 1997), soybean (Yajima, Hashimoto, Saita, & Matsuzaki, 1992), cereals (Kyung, & Young, 1993; Vasiljevic et al., 2007), honey (Ustunol, & Gandhi, 2001), berries and mango (Kailasapathy, Harmstorf, & Phillips, 2008), herbs (Ray-Chowdhury, Chakraborty, & Raychaudhuri, 2008) and whey (Christopher et al., 2006).

Since many probiotic bacteria show limited ability to assimilate lactose, the addition of a more readily available carbohydrate compound to milk could selectively enhance probiotic growth during fermentation. Addition of 5% lactulose, galactooligosaccharides (GOS) or inulin reduced the doubling time of bifidobacteria in milk by 25 to 50% (Shin, Lee, Pestka, & Ustonol, 2000; Bruno et al., 2002). Other potential prebiotics include oat and barley glucans (Vasiljevic, Kealy, & Mishra, 2007), starch/maltodextrins (Bruno et al., 2002) and raffinose (Martinez-Villaluenga et al., 2006). Milk fortification is, thus, a recognized method of improving the growth of lactic and probiotic cultures in fermented milk.

In addition to promoting probiotic growth, fortification can increase the nutritional properties of yogurt and novel food products. Fiber fortification using food sources such as fruit, nut and grains are good examples (Hashim, Khalil, & Afifi, 2009; Aportela-Palacois et al., 2005). Protein concentrates and isolate derived from milk (Sodini et al., 2002*,* Helland et al., 2004) and whey protein (Drake et al., 2000) have also been considered. However to date, little has been done with pulses in yogurt fortification.

Pulses, including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*) chickpea (*Cicer arietinum*) and lupins (*Lupinus perennis)* are good sources of food with potential to benefit health. Pulses contain complex carbohydrates (e.g., fibre, resistant starch and oligosaccharides), protein, important vitamins and minerals (e.g., folate and iron) as well as antioxidants, and only very small amounts of unsaturated fats. Pulse ingredients could therefore, constitute a very good source of growth factors and prebiotic components for yogurt and probiotic beverage supplementation (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000) and may offer the possibility of improving the formulation of fermented milk from both a nutritional and a bacterial growth enhancement perspective.

In this chapter, we report on the effect of addition of pea, chickpea and lentil ingredients to milk on the acidifying activity of starter and probiotic cultures and compare the results with a non-supplemented control, a skimmilk supplemented control and samples supplemented using soy protein concentrate and soy flour.

4.2 Materials and methods

4.2.1 Materials

Pulse fractions used were the following: Chickpea flour from Diefenbaker Seeds Company (Elbow**,** SK, Canada), lentil flour from K2 Milling Company (Tottenham**,** ON, Canada), pea fiber from Best Cooking Pulses Inc (Rowatt**,** SK, Canada) and pea protein from Nutri Pea Company (Portage La Prairie**,** MB, Canada). Soy protein concentrate (71.6% protein) from Oléanergie F2001 Company (St. Hyacinthe, QC, Canada) and soy flour from ADM Company (Decatur**,** IL, USA) were used. Skim milk used was the Quebon brand from Agropur (St. Laurent, QC, Canada). Mixed yogurt cultures containing *Lactobacillus delbrueckii* ssp *bulgaricus* and *Streptococcus thermophilus* were from the following companies: Yogourmet (culture A) was from Lyo-San Inc. (Lachute, QC, Canada) and Yogotherm M133 (culture B) was from Abiasa Inc. (St. Hyacinthe, QC, Canada). Probiotic cultures *Lactobacilus rhamnosus* AD200 and *Lactobacilus acidophilus* AD200 were also from Abiasa Inc. The cultures were obtained in freeze-dried form, packaged in laminated foils and were stored at 4 °C and only opened when used.

4.2.2 Supplementation of yogurt and probiotic cultures

A re-hydrated skim milk (RSM) solution containing 9.5 % solids (w/w) (RSM-9.5) served as control and the base for supplementation. Pulse or soy products at 20 g/L were each added to the RSM-9.5; these formulations, having approximately 11.5% solids, will subsequently be referred to as a function of the soy or pulse ingredient added. Additionally, for one series of assays, the RSM-9.5 was enriched with 20 g/L of skim milk powder in order to have a milk-only product having the same level of solids as those of the pulse- and soy-enriched yogurt formulations; this will be referred to as RSM-11.5. The RSM-9.5, RSM-11.5 and the soy/pulse supplemented products were then boiled for 1 min and cooled to 42 °C. The pH was adjusted to 6.5 and then inoculated with the bacterial cultures. The whole process is presented in Figure 4.1.

The manufacturers' instructions were followed for the inoculation of the yogurt starter cultures. This represented the addition of 10 g of the culture to 2 L (liter) of milk at 42 °C. The probiotic cultures were sold in a concentrated form. The commercial probiotic cultures had a microbial concentration of 2 x 10^{11} CFU/g. They were re-hydrated at room temperature in the pasteurized skim milk to obtain 2 X 10^{9} CFU/mL. Subsequently 2 mL of this dilution was added to 200 mL media which represented an inoculation level of approximately 2 x 10^{7} CFU/mL. The cultures were incubated at 42 °C and the pH was constantly monitored using a pH-meter via a data acquisition system.

4.2.3 Product characterization

Proximate analysis of pulse fractions including protein, moisture fat and ash measurements were done using standard AOAC methods (AOAC, 1990).

Acidification trend in yogurt and probiotic cultures were measured according to the method described by De Brabandere and De Baerdemaeker (1999) using FACS (Fermentation Acquisition and Control System - Forma Scientific, OH, US). To ensure a homogenous environment for pH readings, continuous stirring at 100 rpm was carried out using a magnetic bar in a 250 mL Pyrex vessel (De Brabandere, & De Baerdemaeker, 1999).

The buffering capacity of the different blends was estimated by acid titration and pH measurements using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada) and a 50 mL digital burette (Brinkmann Instruments Ltd., ON, Canada).

Three microbial slides were prepared from each fermented yogurt sample. Once the sample had air-dried on the slide, it was fixed by flame and dyed using methylene Blue. Microscopic examination of the slide to determine the ratio of streptococci or lactobacilli chains per field was conducted using a Nikon Eclipse microscope (model E600). Individual cells or chains of many cells were each counted as 1 in order to simulate the colony-forming unit (CFU) data which would have been obtained in a plating procedure. At least 10 separate fields per slide were examined.

4.2.4 Statistical analysis

Statistical analysis was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two sample t-test for comparison of two means. The linear regression analysis between data of strain ratios was carried out with InStat software (GraphPad, San Diego CA, USA). All experiments were done in three separate independent trials.

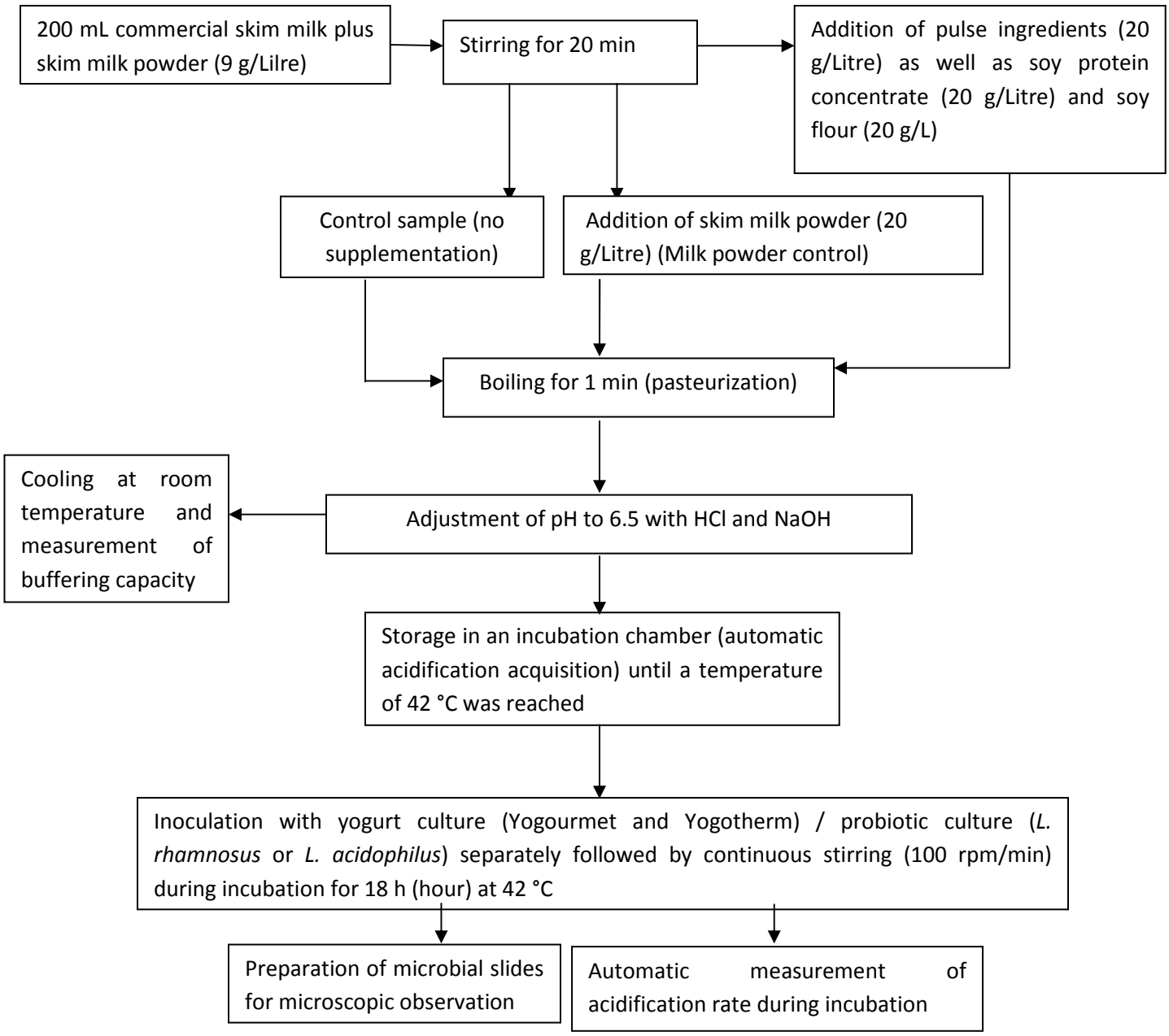


Figure 4.1- Schematic representation of the process used for the preparation of the yogurt and probiotic media supplemented with soy and pulse ingredients and control

4.3 Results and discussion

4.3.1 Proximate analysis of pulse ingredients

Proximate analysis of the pulse ingredients showed considerable variations in protein, fat, moisture and ash contents (Table 4.1). The highest mineral and protein content of the pulse fractions were found in the pea protein and soy protein concentrate. The chickpea flour was different from the other pulses in that it had a significantly higher fat content, while lentil flour had the highest moisture level (Table 4.1). The moisture content of the powders was not taken into account when the milk fortification was carried out. Thus, the addition of 20 g/L of lentil flour for example only contributed 18 g of solids. This was taken in consideration when analyzing results. The bulk of the remaining solid was assumed to consist of fibre and other carbohydrates. This would suggest that the pea protein concentrate (14% of solids), soy protein concentrate (19% of solids) and chickpea flour (36% of solids) were relatively low supplier of fibres and carbohydrates. On the other hand, lentil flour and pea fibre supplied much more fibre and carbohydrates since these powders contained 63 % and 85 % of their solids, respectively, with this fraction. Skim milk powder (0.1% fat) contains 55% carbohydrate (lactose) and minerals but it does not supply fibre.

Table 4.1 - Proximate analysis of pulse ingredients

Sample	**Protein % (g/g)** **Average ± SD**	**Moisture % (g/g)** **Average ± SD**	**Fat content % (g/g)** **Average ± SD**	**Ash % (g/g)** **Average ± SD**
Pea protein	78.0 **±** 0.1 a	3.2 ± 0.1	0.5 ± 0.9	4.8 ± 0.4
Chick pea flour	23.5 **±** 0.1 b	2.0 ± 0.0	7.4 ± 2.8	3.2 ± 0.4
Lentil flour	24.8 **±** 0.1 b	9.4 ± 0.1	0.1 ± 0.1	2.7 ± 0.3
Pea fibre	7.2 **±** 0.2 c	5.3 ± 0.1	0.4 ± 0.1	2.0 ± 0.3

4.3.2 Acid production in yogurt and probiotic fermented milks supplemented with pulse ingredients

The acid production rate was monitored in this study as it is a more important processing parameter than viable counts, especially from the perspective of yogurt production. Indeed, the acidification rate affects the time required for processing which is critical to economic viability. At the beginning of fermentation, the growth rate is generally linked to acidification. A lack of correlation between growth rate and acidification eventually occurs (Turner & Thomas, 1975) when pH drops below 5.0. Nevertheless, the acidification profiles and strain ratios noted in this study do provide a glimpse of bacterial growth patterns. The drop in pH of a medium during fermentation is affected by its buffering capacity. For identical acid production rates, increasing the buffering capacity will reduce the rate of pH reduction and help to improve biomass yields. As an example, RSM-11.5 had 20% more buffering capacity than RSM-9.5, and this effect on the solids level was statistically significant (P<0.05). Addition of pea fiber to RSM-9.5 had no significant effect on buffering capacity (P<0.05). Other than pea fibre, addition of the ingredients to RSM-9.5 increased its buffering capacity by 5.8 to 13% (Table 4.2).

Acidification trends in fortified yogurt were examined with two different commercial cultures. With respect to yogurt culture A (Figure 4.2) supplementation with pulses did not strongly alter the ability of the microbial cultures to change the pH. As yogurt starters grow, they produce acid which causes a decrease in pH. In Figure 4.2, pH generally started to decrease from 6.5 to 6.2 after 1 hour and then rapidly dropped during the next 3 hours to a pH of 4-4.5. With culture A, the pH then remained in this range until the fermentation was completed. Although the acidification trends were generally similar, there were nevertheless small differences in the pH values read at various incubation times. Acidification was generally faster in RSM-9.5, probably due to its lower buffering capacity (Table 4.2). The decrease in pH with culture A did not vary significantly for the different pulse ingredients nor soy ingredients and milk powder after 1 hour of incubation, but small differences were observed after 4 hours of fermentation (Table 4.3)

Table 4.2 - Amount of HCl (1 M) required to acidify 100 mL of control and supplemented samples from pH 6.5 to 4.0

Sample	**Titrable HCl (mL)**
	Average ± SD
RSM-9.5 supplemented with pea protein	7.8 ± 0.1 ab
RSM-9.5 supplemented with chickpea flour	7.4 ± 0.0 abc
RSM-9.5 supplemented with lentil flour	7.3 ± 0.4 abc
RSM-9.5 supplemented with pea fiber	6.7 ± 0.2 c
RSM-9.5 supplemented with soy protein concentrate	7.7 ± 0.2 ab
RSM-9.5 supplemented with soy flour	7.7 ± 0.0 ab
RSM-11.5	8.3 ± 0.1 a
RSM-9.5 control	6.9 ± 0.4 bc

Means followed by the same letter are not significantly different (P > 0.05)

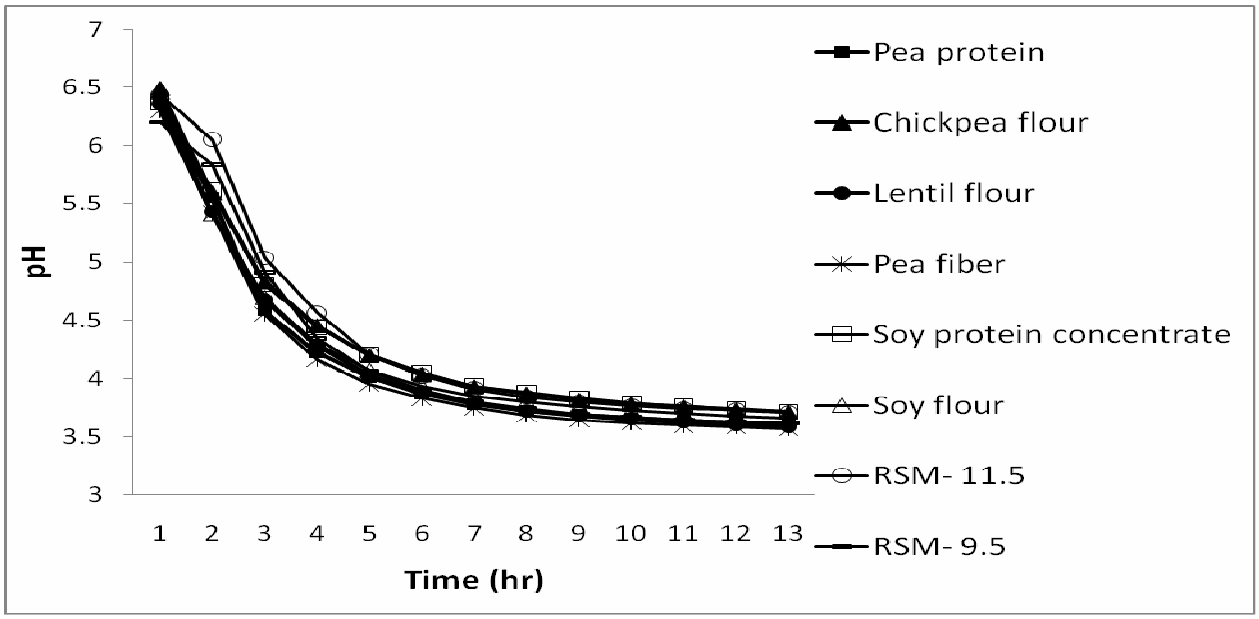


Figure 4.2- Change in pH as a function of incubation time during the acidification of yogurt supplemented with pulse fractions, soy fractions, milk powder (RSM- 11.5) and control (RSM-9.5) sample using Yogourmet (culture A)

Table 4.3 - pH decrease of the control and supplemented yogurt culture and probiotic media at different times during fermentation (PP: pea flour, CPF: chickpea flour, LF: lentil flour, PFi: pea fiber, SPC: soy protein concentrate, SF: soy flour, RSM-11.5: rehydrated skim milk 11.5% W/W, RSM-9.5: rehydrated skim milk 9.5% W/W)

Sample	**Yogurt culture A**		**Yogurt culture B**		**Probiotic (*Lactobacillus rhamnosus*)**			**Probiotic (*Lactobacillus acidophilus*)**		
	T=1 hour	T=4 hour	T=1 hour	T=4 hour	T=2 hour	T=8 hour	T=12 hour	T=2 hour	T=8 hour	T=12 hour
PP	6.4 ± 0.0 a	4.2 ± 0.1 c	6.1 ± 0.0 a	4.0 ± 0.0 bc	6.5 ± 0.0 a	6.1 ± 0.0 a	5.3 ± 0.1 ab	6.3 ± 0.0 ab	5.0 ± 0.1 b	4.1 ± 0.1 bc
CPF	6.4 ± 0.0 a	4.4 ± 0.2 ab	6.2 ± 0.1 a	4.7 ± 0.3 ab	6.3 ± 0.1 a	5.9 ± .04 a	5.1 ± 0.5 ab	6.3 ± 0.0 ab	4.9 ± 0.1 b	4.4 ± 0.1 b
LF	6.3 ± 0.1 ab	4.2 ± 0.0 bc	6.1 ± 0.1 a	4.4 ± 0.3 bc	6.2 ± 0.2 a	5.6 ± 0.4 a	4.5 ± 0.8 b	6.1 ± 0.2 b	3.9 ± 0.0 d	3.7 ± 0.0 c
PFi	6.2 ± 0.0 ab	4.1 ± 0.0 c	5.5 ± 0.1 a	4.0 ± 0.6 c	6.5 ± 0.2 a	6.1 ± 0.0 a	5.4 ± 0.6 ab	6.2 ± 0.0 ab	4.9 ± 0.0 b	4.3 ± 0.0 bb
SPC	6.3± 0.0 ab	4.4 ± 0.0 ab	5.9 ± 0.2 a	4.4 ± 0.0 bc	6.5 ± 0.0 a	6.2 ± 0.0 a	5.6 ± 0.1 ab	6.3 ± 0.0 ab	5.8 ± 0.1 a	5.1 ± 0.4 a
SF	6.4 ± 0.0 a	4.2 ± 0.0 bc	5.8 ± 0.2 a	4.3 ± 0.2 bc	6.5 ± 0.2 a	5.2 ± 0.8 a	4.4 ± 0.8 b	6.1 ± 0.0 b	4.4 ± 0.0 c	3.9 ± 0.8 bc
RSM-11.5	6.4 ± 0.0 a	4.5 ± 0.0 a	6.4 ± 0.0 a	4.6 ± 0.3 ab	6.5 ± 0.1 a	6.2 ± 0.0 a	5.8 ± 0.2 a	6.3 ± 0.0 ab	5.8 ± 0.1 a	5.5 ± 0.1 a
RSM-9.5	6.1 ± 0.1 b	4.3 ± 0.0 bc	6.2 ± 0.1 a	5.1 ± 0.0 a	6.5 ± 0.0 a	6.1 ± 0.0 a	4.9 ± 0.2 ab	6.4 ± 0.0 a	5.9 ± 0.1 a	5.4 ± 0.4 a

Means with the same letter are not significantly different, for a given column (P<0.05)

With yogurt culture B, no significant differences were observed in the pH measurements after 1 hour of incubation (Table 4.3). However, significantly lower pH values were recorded after 4 hours for the pea fiber sample compared to the RSM-9.5 and RSM-11.5 products. The same trend was also noted for yogurt culture A at the same time. It is unclear which specific component of the pea fibre contributed to the beneficial effect as this was outside the scope of the study. However, since this supplement contained the highest level of carbohydrates/fibres, these results would suggest that the pea carbohydrate fraction may be involved. In other studies, modifying the carbohydrate composition of milk increased the acidification rate of yogurt starters (Tamime & Robinson, 1999). Pea protein, soy protein concentrates and soy flour supplemented samples had significantly lower pH after 4 hours compared to the RSM-9.5 control.

There are increasingly more fermented milk products (i.e., "non-yogurt") on the market which are solely fermented by probiotic bacteria, and assays were carried out to evaluate the benefits of the supplements to improve the fermentation patterns in such probiotic beverages. Fermentation rates were overall much slower with pure cultures of the probiotic bacteria (Figures 4.4 and 4.5) than for the yogurt starter cultures (Figures 4.2 and 4.3). This confirms many previous studies which reported slow growth rates of probiotic bacteria in milk (Klaver et al., 1993; Roy, Mainville, & Mondou, 1997; Roy, 2005; Gaudreau et al., 2005; Champagne et al., 2009). However, the effects of the pulse supplements were much greater with the probiotic fermented milks. Many supplements significantly increased acidification rates as compared to both RSM-9.5 and RSM-11.5 products (Figures 4.4 and 4.5; Table 4.3). In some instances it is unclear if this may be partly or solely due to the greater buffering capacity of RSM-11.5 (Table 4.2). There are treatments where the rate of pH reduction is faster than in the RSM-9.5 which clearly indicated increased acid production rates. The greatest improvements in acidification rates were obtained with lentil and soy flour (Figures 4.4 and 4.5). Additionally, no inhibition of microbial activity seemed to have occurred.

The pH measurements with *Lactobacillus rhamnosus* AD200 after 2 hours (T=2), representing the lag phase, were not significantly different (Table 4.3). Acidification after 12 hours with *Lactobacillus rhamnosus* AD200 showed that lentil flour and soy flour had significantly lower pH values than RSM-11.5. The extent to which this is linked to buffering capacity remains to be ascertained. There was no significant difference between the other ingredients studied and the two milk controls. The greatest effects of supplementation were seen with *Lactobacillus acidophilus* (Figure 4.5). Decreases in pH of up to 0.4 units already appeared after 2 h, and 4 products including pea protein, chickpea flour, lentil flour and soy flour supplemented samples showed significantly lower pH values than both RSM treatments after 12 hours of incubation (Table 4.3). Lentil flour and soy flour again showed trends of being the most effective in accelerating the fermentation (Figure 4.5 and Table 4.3).

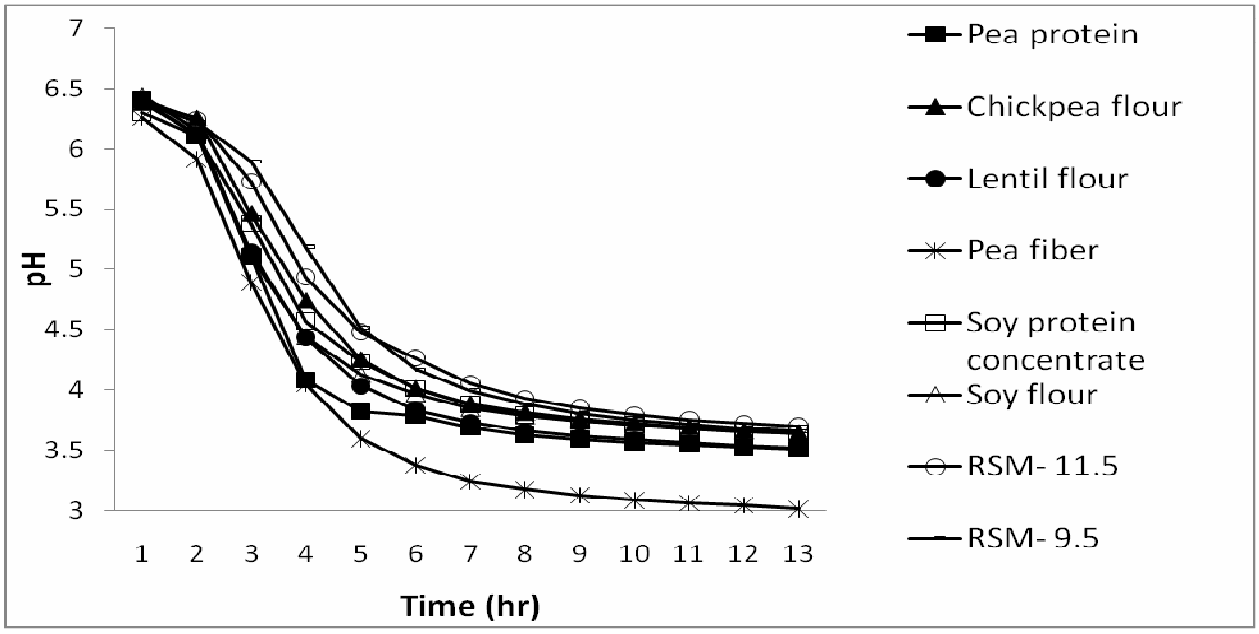


Figure 4.3- Change in pH as a function of incubation time during the acidification of yogurt supplemented with pulse fractions, soy fractions, milk powder (RSM- 11.5) and control (RSM-9.5) sample using Yogotherm M133 (culture B)

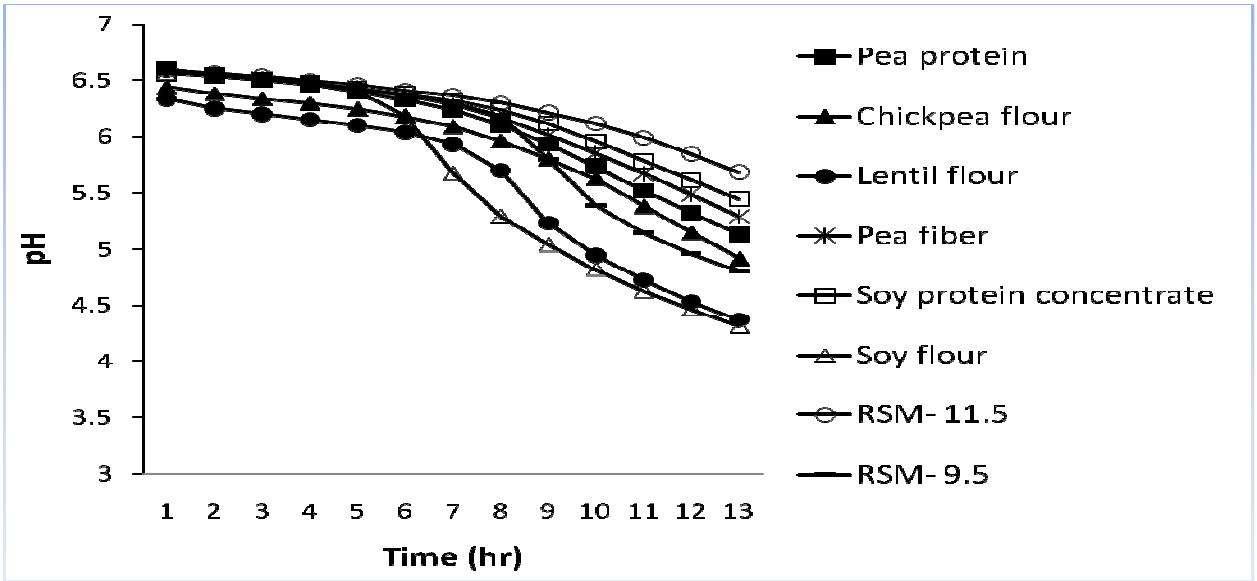


Figure 4.4- Change in pH as a function of incubation time during the acidification of probiotic media supplemented with pulse fractions, soy fractions, milk powder (RSM- 11.5) and control (RSM- 9.5) sample using *L. rhamnosus* AD200 (culture C)

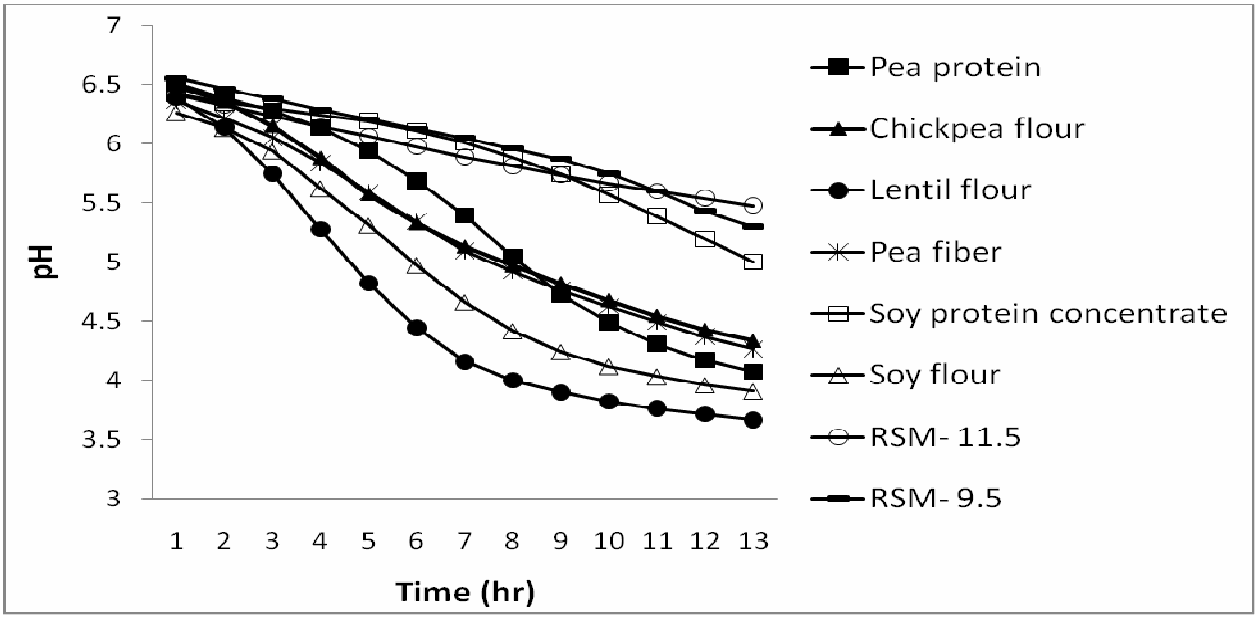


Figure 4.5- Change in pH as a function of incubation time during the acidification of probiotic media supplemented with pulse fractions, soy fractions, milk powder (RSM- 11.5) and control (RSM- 9.5) sample using *L. acidophilus* AD200 (culture D)

4.3.3 Ratio of cocci to bacilli in yogurt

In addition to the acidification rate, the ratio of cocci to lactobacilli in the fermented product is also important. Indeed, the two bacterial species in yogurt contribute to the production of different flavour compounds, and post-acidification during storage can be a problem if high levels of lactobacilli are found in the product (Tamime, & Robinson, 1999). Therefore, the cocci:bacilli ratio in the fermented products were estimated by direct microscopic examination. Although viable counts would have provided a better picture of the potential post-fermentation changes in the fermented milk products, microscopic examination of the cocci:bacilli ratio was used as this was an exploratory study designed to select ingredients having the most potential to benefit the fermentations. In fresh products, however, there is a correlation between viable counts and microscopic evaluation and these data offer valuable information on the fermentation process. With yogurt culture A, the ratio of cocci to bacilli varied between 1.2 and 2.5 and there was no significant difference between all supplemented samples (Table 4.4). In yogurt culture B, however, there was a relative increase in lactobacilli in at least 3 products, when compared to the two milk controls (Table 4.4). It is noteworthy that two of the products which showed most increase in the lactobacilli fraction in the fermented yogurt (soy and lentil flours; Table 4.4) were also those which improved acidification rates in the probiotics (Table 4.3). This result suggests that for the pulse ingredients, the lentil flour improved the growth of lactobacilli the most. The observations that the two probiotic lactobacilli strongly benefited from milk supplementation by the pulse and soy ingredients lend support to preferential benefit to the lactobacilli in the yogurt cultures. It was examined to what extent the yogurt cultures reacted similarly to the supplements. A linear regression test between yogurt cultures A and B and the ratio of cocci to bacilli was done. Data showed that there is no significant correlation in terms of the ratio of cocci to bacilli between the two starter cultures ($r = 0.15$) for the different samples exist. However, linear regression test with pH values at $t = 4$ h (hour) showed a good correlation ($r = 0.95$). Therefore, supplementation with pulses similarly affected the overall production of acid in the two yogurt cultures, but the effect on cocci:bacilli ratios differed.

Table 4.4 - Ratio of cocci to bacilli in yogurt supplemented with and without pulse and soy ingredients

Sample	Average of the ratio of cocci to bacilli	
	Yogurt culture A	Yogurt culture B
	Average ± SD	Average ± SD
Pea protein	2.1 ± 0.5 a	1.8 ± 0.0 b
Chickpea flour	2.2 ± 0.6 a	0.8 ± 0.1 c
Lentil flour	1.2 ± 0.4 a	1.0 ± 0.3 c
Pea fibre	2.5 ± 0.8 a	1.2 ± 0.0 bc
Soy protein concentrate	2.1 ± 0.8 a	1.6 ± 0.6 b
Soy flour	1.5 ± 0.2 a	0.8 ± 0.0 c
Milk powder	2.0 ± 0.1 a	1.8 ± 0.1 b
Control (no supplementation)	1.8 ± 0.4 a	2.6 ± 0.0 a

Means followed by the same letter are not significantly different, for a given column (P<0.05).

4.4 Conclusion

Yogurt is a strictly regulated fermented dairy product; however, some legislation authorizes fortification at up to 2 % of total solids (Canadian Legal Legislation Institute, 2008). This provides opportunities to enhance the health value of yogurt and of probiotic-containing fermented milks. Results from this study clearly showed that some pulse ingredients may have beneficial effects on probiotic and yogurt starter cultures. Minor benefits of milk supplementation with pea products, particularly pea fibre, were noted for the two yogurt

starter cultures. However, with probiotic bacteria, lentil flour demonstrated the greatest ability to increase the acidification rates as did soy flour. Pea protein, chickpea flour and pea fibre also showed some promise. Data particularly pointed to the stimulation of growth of lactobacilli by lentil flour. This exploratory study focused specifically on acidification rate and the cocci:bacilli ratios since they are two important parameters in yogurt and probiotic fermented milk manufacturing. Further studies to ascertain the effects of the most promising ingredients on viable counts as well as to determine the effects on flavor and texture are in progress as this information will be instrumental to help in identifying application development opportunities.

Connecting Statement to Chapter 5

Results from the previous studies showed that selected pulse ingredients have beneficial effects on yogurt starters and probiotic bacteria in fermented milk. The effect of pulse ingredients on acidification and cocci:bacilli ratios were studied, and further investigation to ascertain the effects of the most promising ingredients on viable counts as well as to determine effects on physical and sensory properties of final product is necessary. From this point two pulse ingredients comprising lentil flour and pea flour were selected to supplement milk in presence of yogurt starters and probiotic organisms.

In chapter 5, we investigate the effect of yogurt supplementation with 1-3% lentil flour on acid production during fermentation, growth of yogurt starters, pH, syneresis, color, rheological and sensory properties of the final product immediately after production and during one month of storage. For a comparison study, yogurt (skim milk as the base media for yogurt formulation), was also supplemented with 1-3% skim milk powder and analyzed for all quality aspects as well as a non-supplemented control yogurt. The results of this research have been presented as follow:

Zare, F., Boye, J.I., Champagne, C.P., Orsat, V., & Simpson, B.K. (2010), Acidification and microbial growth of yogurt and probiotic supplemented with lentil flour, IFT, July 17th-20th, Chicago, IL, USA, (poster presentation).

Zare, F., Boye, J.I., Champagne, C.P., Orsat, V., & Simpson, B.K. (2010), Physical and rheological properties of yogurt supplemented with lentil flour, CIFST, May 30th –June 1st, Winnipeg, MB, Canada.

Zare, F., Boye, J.I., Orsat, V., Champagne, C.P, & Simpson, B.K., (2011). Acidification, microbial growth, physical and rheological properties of yogurt supplemented with lentil flour, 8th Canadian pulse research workshop, November 3th -5th, Calgary, AB, (poster presentation).

Zare, F., Boye, J.I., Orsat, V., Champagne, C.P, & Simpson, B.K., (2011). Microbial, physical and sensory properties of yogurt supplemented with lentil flour, *Food Research International (*article in press).

Chapter 5: Microbial, physical and sensory properties of yogurt supplemented with lentil flour

Abstract

In this study, skim milk (9.5 % w/v solid content) was supplemented with 1-3% (w/v) lentil flour or skim milk powder, inoculated with a yogurt culture, fermented and stored at 4˚C. Acid production during the fermentation, microbial growth, physical properties (pH, syneresis, and color), rheological properties (dynamic oscillation temperature sweep test at 4-50 ˚C), during 28 days of refrigerated storage and also sensory properties (flavor, mouth feel, overall acceptance and color) after production, were studied. Milk supplementation with 1-3% lentil flour enhanced acid production during fermentation, but the microbial population (CFU) of both *S. thermophilus* and *L.delbrueckii ssp bulgaricus* were in the same range in all lentil flour and skim milk powder supplemented yogurts. The average pH of samples decreased from 4.5 to 4.1 after 28 days storage. Syneresis in 1-2% lentil flour supplemented yogurts was significantly higher than all other samples; however, greater lentil supplementation (3%) resulted in the lowest syneresis during the 28 days storage. With respect to color, ``a`` and ``L`` values did not significantly differ in all samples and remained constant after 28 days whereas, ``b`` value increased as a result of lentil supplementation. Yogurt with 3% lentil flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the non-supplemented control yogurt. Storage modulus (G′) was higher than loss modulus (G″) in all samples and at all temperatures between 4-50 ˚C and they showed a hysteresis loop over this temperature range when the samples were heated and cooled. 1-2% lentil flour supplemented yogurt showed comparable sensory properties in comparison with 1-2% skim milk powder supplemented yogurt and the control sample.

5.1. Introduction

Fermented milk products containing probiotic bacteria and yogurt beverages are some of the most popular fermented food products in the world. Fermented milk products have numerous

health benefits due to the functional properties of their viable microorganisms and a health claim for yogurt has recently been allowed towards improved lactose digestion for individuals with lactose maldigestion (EFSA, 2010). The "Canadian Dairy Commission" reported that prebiotic and probiotic yogurt were among the fastest growing food sub-sectors in 2009 (CDC, 2009). Yogurt is made with milk allowed to ferment in the presence of lactic acid bacteria (LAB), *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp*. bulgaricus* (Tamime & Robinson, 1999, Yang et al., 2006).

Besides the health benefits of yogurt due to the presence of live microorganisms (EFSA, 2010) and its nutritional content, its physical properties, appearance and texture are important aspects for consumer acceptability. Acid production by yogurt starters, microbial growth during fermentation and storage, yogurt texture and flavor can be modified by altering process conditions (e.g., milk heat treatment) or by the addition of ingredients (e.g., milk solids or stabilizers). Food ingredients addressing different nutritional and/or technical applications used for milk supplementation include dried dairy ingredients (Isleten & Karagul-Yuceer, 2006), whey protein (Sodini et al., 2005), milk protein (Peng, Serra, Horne & Lucey, 2009), prebiotics (Bruno et al., 2002; Shin et al., 2000) calcium and fibres (Aportela-Palacios et al., 2005; Vasiljevic et al., 2007; Sendra, Kuri, Fernandez-Lopez, Sayas-Barbera, Navarro & Perez-Alvarez, 2010). Lentil flour was tested in this study for similar purposes.

Various factors, e.g., quality and composition of milk and its fat and total solid content, heat treatment of milk, combination of the lactic acid bacteria used, acidification rate of milk and storage time, all affect the texture and sensory properties of yogurt (Paseephol, Small, & Sherkat, 2008). Various methods are available to assess texture properties of yogurt. Rheometry is a useful technique for measuring the textural properties of foods. Viscoelastic property measurements provide an understanding of the rhelogical properties of foods and give an estimate of the initial experience of a consumer in terms of mouth feel (Kealy, 2006). Several workers have used the oscillatory test to assess the rheological characteristics of yogurt (Ozer, Robinson, Grandison & Bell, 1997; Remeuf, Mohammed, Sodini & Tissier, 2003; Sodini,

Lucas, Tisier & Corrieu, 2005b). The evaluation of syneresis, defined as the separation of whey from the yogurt without the application of an external force (Peng et al., 2009) is also of importance, particularly during storage. As supplementation influences several physical properties of yogurt, a variety of measurements therefore need to be undertaken when different ingredients are considered for yogurt supplementation. Results reported for different ingredients used in past studies have varied probably due to the different types of supplements, methodology and equipment used for the physical analysis (Sodini et al., 2005b). Gelatin, whey protein, carrageenan, starch and pectin have been added to yoghurt in order to enhance firmness and improve texture, but no study has been carried out on the potential of lentil flour for the same purposes.

Texture properties can often be assessed with instruments, but this is insufficient in characterizing the product. Many consumers use the sensory properties of foods to judge the freshness and quality of a product (Kealy, 2006). Sensory properties including flavor, mouth feel and also color could be evaluated by trained or untrained panelists and consumer testing could provide the most meaningful and reliable information on the textural quality and acceptability of yogurt (Jaworska, Waszkiewicz-robak, Kolanowski & Swiderski, 2005). Therefore, in this study, both instrumental and panel testing procedures were carried out.

Lentil flour was not only selected as a supplement for its technological and sensory properties, but also for its nutritional benefits. Indeed, a noteworthy trend in recent times is the addition of prebiotics for the improvement of the nutritional properties of yogurt and fermented dairy products. Some plant-based matrices are very rich in prebiotic compounds and inulin-containing chicory is probably the best example of this. There is great economic interest in finding other prebiotic-rich food matrices. Preliminary data (unpublished) suggest that probiotic lactobacilli grow better in milk supplemented with lentil flour, thus suggesting a prebiotic potential. Lentil contains high amount of protein, fibre, vitamins (e.g., folate) and minerals and is low in fat. Canada's lentil production reached 674,000 tonnes in 2007, placing Canada as the second largest producer in the world. Despite this, Canadian lentil consumption remains low

(i.e., 0.6 kg/capita/year) (FAO, 2008). Lentil may serve as a good source of nutrients for the yogurt starter culture. The effect of yogurt supplementation with lentil flour on growth of the yogurt cultures has, however, not been considered previously. With its high protein and fiber content, addition of lentil flour to yogurt may alter the physical properties of the final supplemented yogurt formula and thus requires investigation.

In this study, therefore, we investigated the effect of yogurt supplementation with 1-3% lentil flour on acid production during fermentation, growth of yogurt starters, pH, syneresis, color, rheological and sensory properties of the final product immediately after production and during one month of storage. For a comparison study, the yogurt (skim milk as the base media for yogurt formulation), was also supplemented with 1-3% skim milk powder and analyzed for all quality aspects as well as a non-supplemented control yogurt.

5.2. Materials and methods

5.2.1 Production of yogurt

Non-fat skim milk powder used was from Agropur (Quebon brand; St. Laurent, QC, Canada); lentil flour was from K2 Milling Company (Tottenham, ON, Canada); mixed yogurt cultures containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus (*Yogotherm M133) was from ABIASA Inc. (St. Hyacinthe, QC, Canada); the cultures were obtained in freeze-dried form, packaged in laminated foils. They were stored at 4°C until use. The experimental protocol used for yogurt supplementation and production are shown in Figure 5.1. Skim milk powder mixed in distilled water (9.5 % w/v) served as the base for supplementation and will be referred to as the "control". In two series of experimental assays 1-3% (w/v) of lentil flour or 1-3% of skim milk powder were added separately to the skim milk base (control).

5.2.2 Product characterization

Acidification trends in yogurt were measured during yogurt fermentation according to the method described by De Brabandere & De Baerdemaeker (1999), using a FACS (Fermentation

Acquisition and Control System) installed in a Forma Scientific (OH, US) programmable incubator. The buffering capacity of the different blends was estimated by acid titration and pH measurements using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada) and a 50 mL digital burette (Brinkmann Instruments Ltd., ON, Canada).

For viable counts, two culture media - acidifed MRS agar from Difco Company (KS, USA) and M17 agar from Oxoid Company (ON, Canada) were used for quantifying the S*treptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* after production and during 28 days storage at 4°C at 7 days interval. pH, syneresis, color, rheological and sensory parameters were measured on the first day of production and during 28 days storage at 4 °C, at 7 days interval.

pH was also measured during storage using a portable pH meter, (Accumet AP61, Fisher Scientific Inc, ON, Canada). Syneresis was determined as the amount of spontaneous whey separation from yogurt according to the method described by Lucey, Munro, & Singh (1998), with some modifications. Volume of whey drained from 100 mL of undisturbed set yogurt prepared in cylindrical tube was measured and reported as percentage.

Color was determined as lightness (*L*), red/greenness (a), and yellow/blueness (*b*), using a spectrophotometer (Konica Minolta, CM-503 c, NJ, US).

Dynamic oscillation tests were conducted to determine the flow behaviour and characterize the viscoelastic properties of yogurt, using a rheometer (TA Instruments, SR-2000, DE, US) fitted with a 40-mm-diameter cone and 2 degree cone angle and plate geometry with a 4 mm gap. To ascertain the applicable stress and frequency in which storage modulus (G') and loss modulus (G") parameters of yogurt would demonstrate a linear constant rate, dynamic frequency ramp tests and dynamic stress ramp tests were conducted at 25 °C (range of frequency from 1-10 Hz and stress set as 3 Pa and range of stress from 1-10 Pa and frequency set as 2.5 Hz, respectively). Dynamic temperature ramp tests were done at stress and frequency of 1.0 Pa and 2.5 Hz, respectively, in a temperature range of 4-50 °C (heating) and 50-4 °C (cooling), at a rate of 7°C/min. Aliquots of the samples were carefully removed from the undisturbed yogurt cup

and placed on the center of the rheometer plate; the top plate was slowly lowered on the top of the sample prior to analysis.

Sensory analyses (flavor, mouth feel, overall acceptance and color) of the lentil flour and skim milk powder supplemented samples as well as control sample were evaluated by 25 untrained panelists using a 9-point hedonic scale. Panelists were asked to score samples from extremely like (1) to extremely dislike (9). The sensory evaluation room was equipped with red light to blind the panelists to the color for first 4 questions (flavor, mouth feel and overall acceptance) and also white light for the question of color evaluation.

5.2.3 Statistical analysis

Statistical test was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two sample t-test for comparison of two means.

5.3. Results and discussion

5.3.1 Acidification trend during yogurt fermentation

Acidification trend in yogurt fermentation is presented in Figure 5.2. The addition of 3% lentil flour increased the acidification level, as compared to the control, and this effect was found to be statistically significant ($P<0.05$) after 1 hour of incubation. The addition of 1 and 2% lentil flour also resulted in faster acidification but this only became significant after 2.5 hours of incubation. Milk has greater buffering capacity in comparison with lentil flour (Table 5.1). Therefore, the greater acidification rates in products supplemented with lentil flour could have been simply due to their lower buffering capacity when compared to corresponding skim-milk-supplemented yogurts. This is not the case however, when the data with lentil flour are compared to the control. The latter suggests that growth of the yogurt strains could have been stimulated by lentil flour, and viable counts were carried out on the products.

Table 5.1- Amount of HCl (1 M) required to acidify 100 mL of 1-3 % lentil flour (LF) or 1-3 % skim milk (SM) supplemented yogurt and non-supplemented control samples from pH 6.5 to 4.0 (SM: skim milk, LF: lentil flour)

Sample	Titrable HCl (mL)
	Average ± SD
1 % SM	6.84 ± 0.00 b
2 % SM	7.58 ± 0.07 c
3 % SM	9.145 ± 0.00 d
1 % LF	6.51 ± 0.01 a
2 % LF	6.830 ± 0.21 b
3 % LF	7.055 ± 0.03 b
Control	6.38 ± 0.00 a

Means with the same letter are not significantly different (P<0.05).

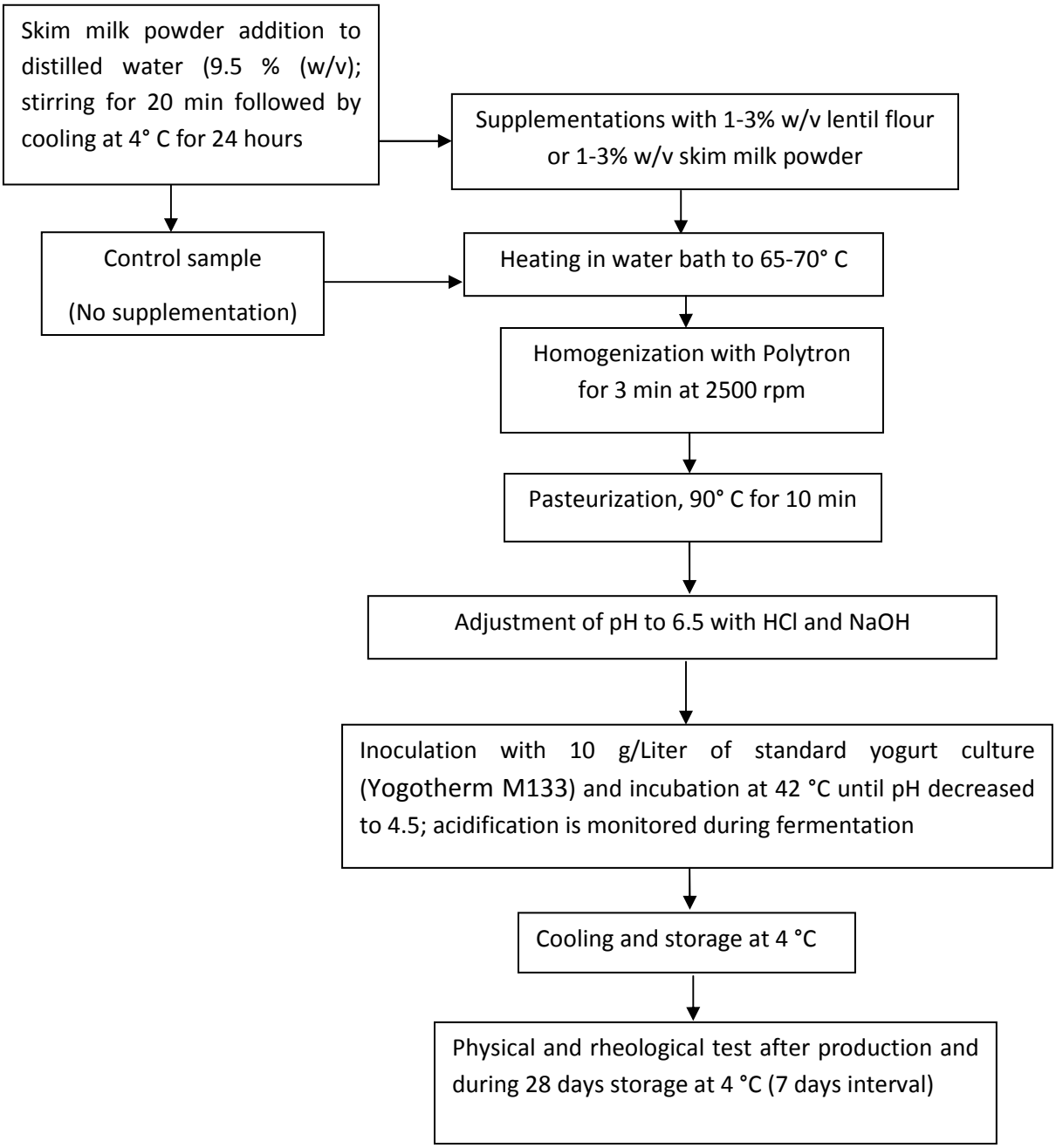


Figure 5.1- Schematic presentation of the process used for the preparation of the yogurt supplemented with skim milk powder (SM) and lentil flour (LF) and the control yogurt (skim milk base with no supplementation).

5.3.2 Microbial growth in yogurt after production and during storage

The two microorganisms, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* of the mixed yogurt starter culture have "associate growth" in yogurt (Turner & Thomas, 1975). Microbial growth continues during storage and the number of viable microorganisms is a critical factor in the final product in terms of acidification and also nutritional health benefits attributed to yogurt starters as probiotics. It is generally recommended that yogurt or fermented milk should contain at least 10^8 CFU/serving (EFSA, 2010), which represents approximately one million viable cells per gram at the time of consumption. To maintain these numbers, it is important to follow viability during cold storage (Damin et al., 2006).

Viable counts of *S. thermophilus* in lentil flour and skim milk powder supplemented yogurt, varied from log 8.3-8.6 after production and important reductions occurred in some samples after 28 days of storage (Table 5.2). CFU values of *L. delbrueckii ssp bulgaricus* after fermentation were lower than those of *S. thermophilus* and viability losses during storage also occurred (Table 5.2). In the 4 treatments based on milk, there was a positive correlation (R^2 between 0.76 and 0.78) between the CFU data after fermentation and the buffering capacity of the milk. Thus, in this range of milk solids, the higher the milk solids level, the higher were the populations in the fermented milk. This is in agreement with the literature (Badran & Reichart, 1994). However, when the data of lentil-supplemented yogurts were combined with those of milk, in the regression analyses, the R^2 values dropped to 0.60 (streptococci) and 0.13 (lactobacilli). Therefore, in a given medium, the buffering capacity is a strong regulator of growth, but not when different media are compared. These data show that the nutrients brought by the lentil flour affect growth of the yogurt cultures differently than did skim milk powder. Interestingly, the addition of lentil flour accelerated the rate of acidification, but did not increase the CFU values in the fermented products (Table 5.2) as compared to skim milk powder supplementation. The nature of the stimulatory factors in lentil flour remains unknown. It is hypothesized that complex carbohydrates (e.g., resistant starch, sucrose, raffinose, stachyose, verbascose and oligosaccharides) made this ingredient a very good source of potential prebiotic components (Wang and Daun, 2004). Amino acids, vitamins and minerals

have also been shown to stimulate the growth of starter cultures in milk (Smith, Hillier & Lees, 1975). There were important losses in viability during storage, particularly in the control (Table 5.2). This has been noted for many yogurt products and the phenomenon is strongly strain-related (Dave & Shah, 1997a). Supplementation with lentil flour significantly improved the stability of the lactobacilli (Table 5.2). Antioxidants (Dave & Shah, 1997b) and carbohydrates (Silva, Carvalho, Pereira, Teixeira & Gibbs, 2004) have been shown to improve the stability of yogurt cultures during storage. It remains to be determined which compounds in the lentil flour have this protective benefit towards the lactobacilli.

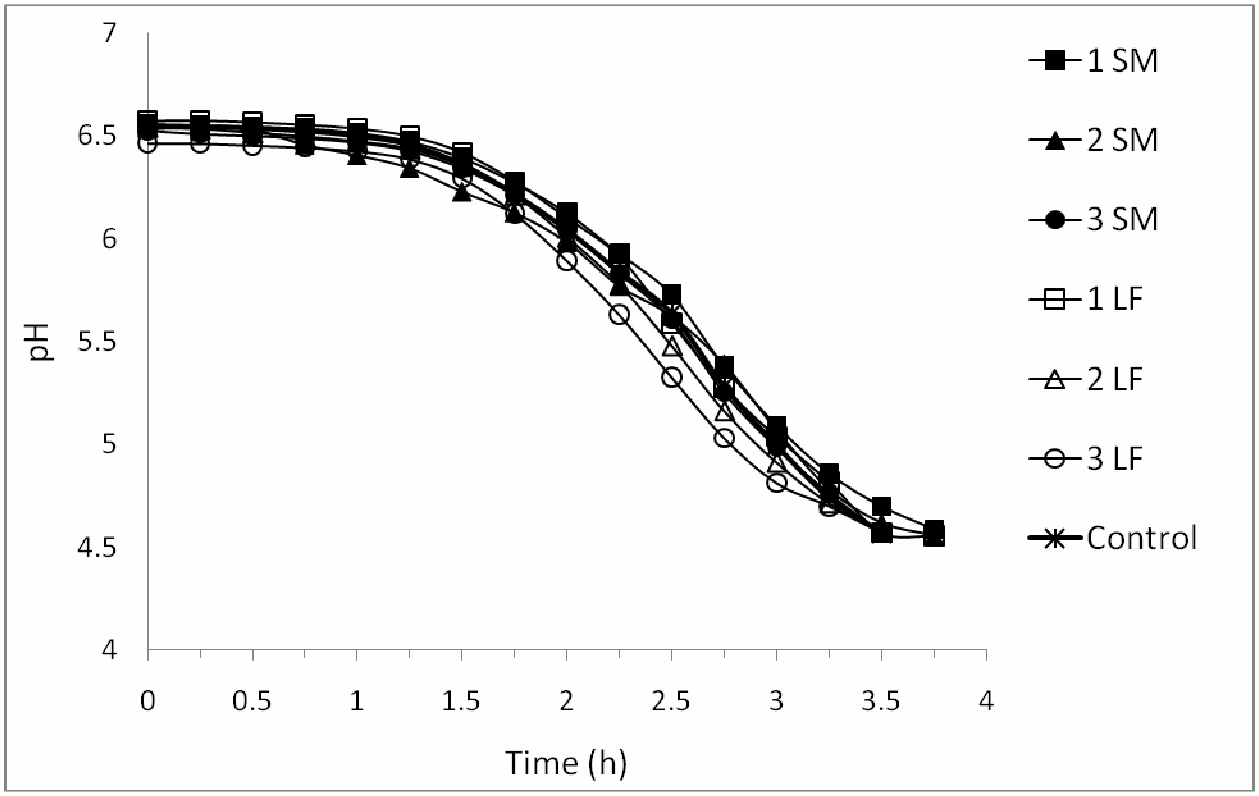


Figure 5.2- Change in pH as a function of incubation time during the acidification of yogurt supplemented with 1-3% lentil flour and 1-3% skim milk powder as well as control yogurt (SM: skim milk, LF: lentil flour)

Table 5.2: Effect of milk supplementation with skim milk powder (SM) or lentil flour (LF), on pH and viable counts after the fermentation as well as after 28 days of storage at 4°C.

Medium	pH		*S. thermophilus* Log CFU/ mL		*L. bulgaricus* Log CFU/ mL	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
1% SM	4.59 ± 0.06 a	4.01 ± 0.01 b	8.44 ab	7.45 a	7.80 ab	6.89 ab
2% SM	4.52 ± 0.03 a	3.95 ± 0.06 c	8.56 a	8.21 a	7.75 ab	6.75 ab
3% SM	4.53 ± 0.04 a	3.99 ± 0.01 c	8.56 a	8.58 a	7.90 a	6.96 ab
1% LF	4.50 ± 0.12 a	4.01 ± 0.01 b	8.33 b	8.01 a	7.91 a	7.76 a
2% LF	4.53 ± 0.03 a	4.03 ± 0.02 b	8.37 ab	8.35 a	7.85 ab	7.55 a
3% LF	4.56 ± 0.04 a	4.19 ± 0.01 a	8.33 b	8.19 a	7.71 b	7.49 a
Control	4.52 ± 0.03 a	4.18 ± 0.01 a	8.39 ab	7.82 a	7.69 c	6.10 b

Means with the same letter are not significantly different, for a given column (P<0.05)

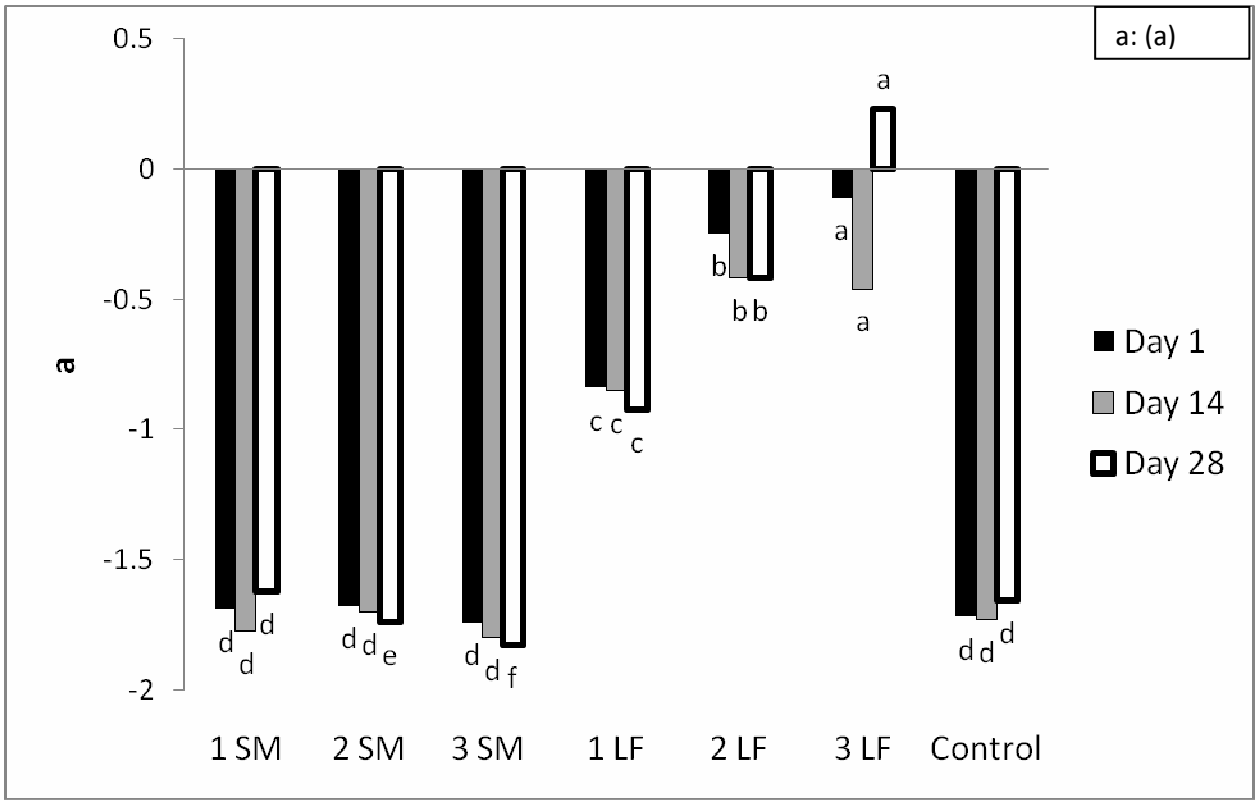
5.3.3 Change in pH during storage

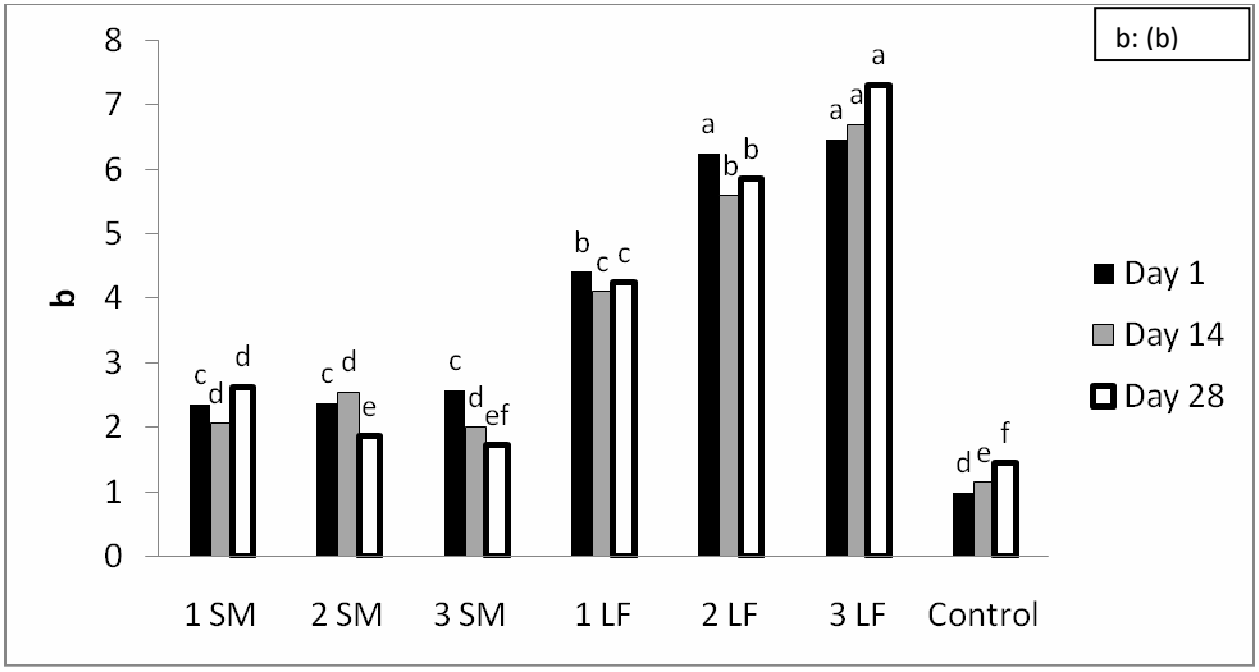
In all samples, the pH decreased from 4.5 to approximately 4.0 over the 28 day storage period (Table 5.2). pH reduction was slightly more in 1% and 2% lentil-supplemented samples in comparison with other samples. According to Kailasapathy, Harmstorf & Phillips (2008), the higher the buffering capacity of yogurt, the lower the pH changes due to the changes in acid content of the food system. Data from this study, however, disagree with these observations since the control had the smallest drop in pH (Table 5.2).

5.3.4 Color

Color parameters are important for marketability of the products and consumer acceptance. Even though a functional food could provide several health benefits to consumers, without

visual attraction to the consumers they cannot be marketable. Thus, the color of the supplemented products should ideally remain unchanged after production and during storage. Figure 5.3 (a, b and c), shows differences in the color (a, b and L values) of the 1-3% lentil flour and 1-3% skim milk supplemented yogurt and control yogurt at days 1, 14 and 28 after production. On the first day of production all lentil flour supplemented samples had significantly less “L” value and higher “a” and “b” values in comparison with other samples; furthermore, the “a” and “b” values were significantly different among the lentil flour supplemented samples (P<0.05). After 28 days, “L” value in lentil supplemented yogurt was significantly higher than skim milk supplemented yogurt and the control sample. The 3% lentil flour supplemented yogurt had the highest “a” and “b” values in comparison with other samples with values of 0.23 and 7.30, respectively (P<0.05). “L”, “a” and “b” represent lightness (100) and blackness (0) , red (+ve)-green (-ve) and yellow (+ve)-blue (-ve) hues (Sanz, Salvador, Jimenez & Fiszman, 2008), and hence the color measurements indicate that immediately after production the lentil flour supplemented yogurt had lower lightness, less greenness and more yellowness hue in comparison with skim milk supplemented samples. After 28 days, the lightness of the lentil supplemented samples increased and the 3% lentil flour supplemented yogurt developed a slightly reddish hue which was significantly different from the other samples.


a: (a)
0.5
0
-0.5
-1
-1.5
-2
a
1 SM
2 SM
3 SM
1 LF
2 LF
3 LF
Control
Day 1
Day 14
Day 28


b: (b)
8
7
6
5
4
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b
1 SM
2 SM
3 SM
1 LF
2 LF
3 LF
Control
Day 1
Day 14
Day 28

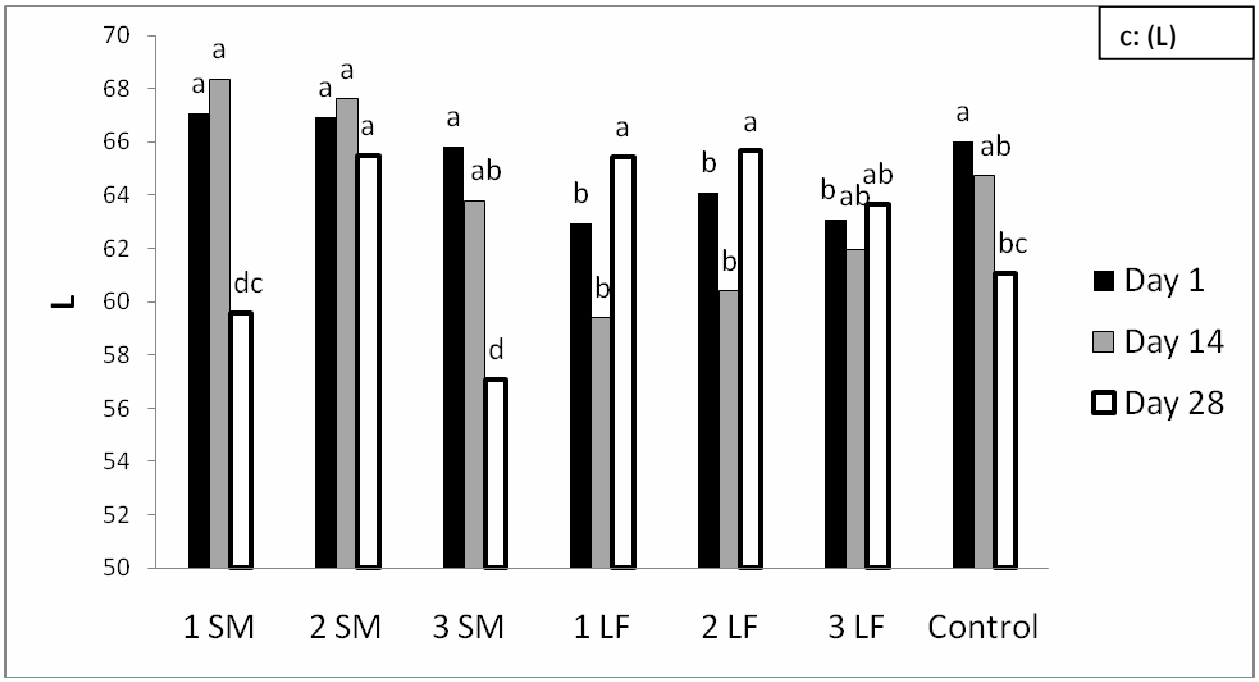


Figure 5.3- Color profile of yogurt supplemented with 1-3% lentil flour and 1-3% skim milk and control sample after production and after 14 and 28 days storage; (SM: skim milk, LF: lentil flour; a (a value) +ve red, -ve green; b (b value) +ve yellow, -ve blue; c (L value): -0 to 100, black to white)

5.3.5 Syneresis

Syneresis provides an indication of the non-homogeneities in the gel system of the yogurt; thus, higher water separation is related to gel instability which is also related to the pH of the yogurt system (Lucey et al., 1998). Figure 5.4 shows the syneresis of lentil flour and skim milk supplemented yogurt and control samples immediately after production and after 14 and 28 days of storage. On day 1, the 3% lentil flour showed the lowest syneresis, which was significantly lower (P<0.05) than for the 1-3% skim milk supplemented samples, whereas the 1-2 % lentil flour supplemented samples had the highest amount of whey separation, which was significantly higher (P<0.05) than all the other samples. After 14 days storage, the 3% lentil flour supplemented yogurt still showed little syneresis. After 28 days, the 1-3% skim milk supplemented yogurt samples had the maximum homogeneity (lowest syneresis), followed by

the 3% lentil flour, control sample, 2% and 1% lentil flour supplemented samples. Yogurt supplementation with an increase in the total solid content, especially protein content, results in stronger texture and less whey separation (Peng et al., 2009; Lucey, 2001). This can explain the lowest syneresis in 3% lentil flour and 3% skim milk supplemented yogurt at day 7 (results not shown) and day 14.

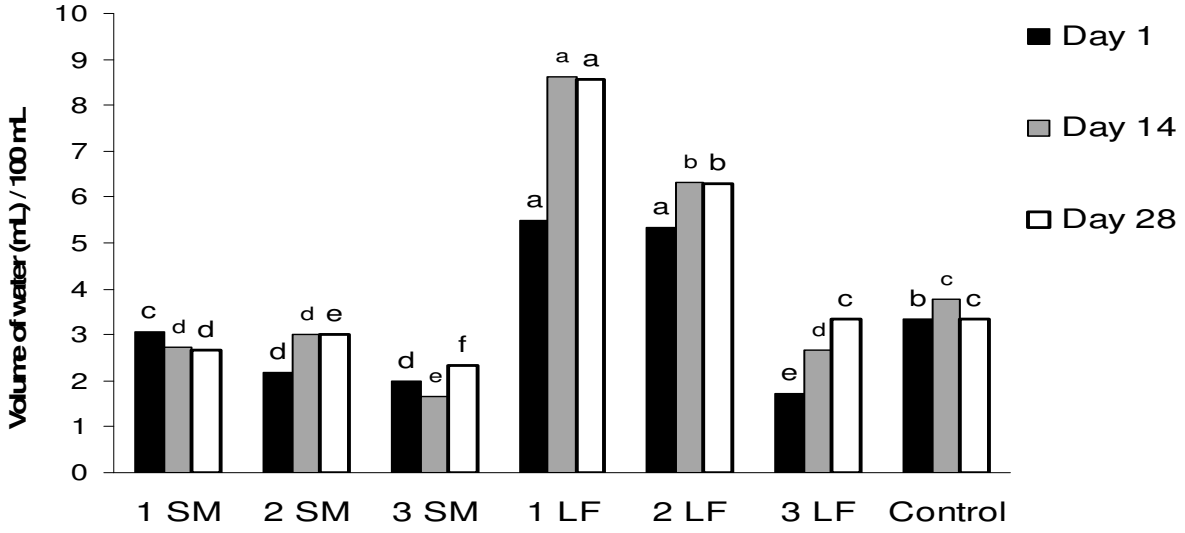


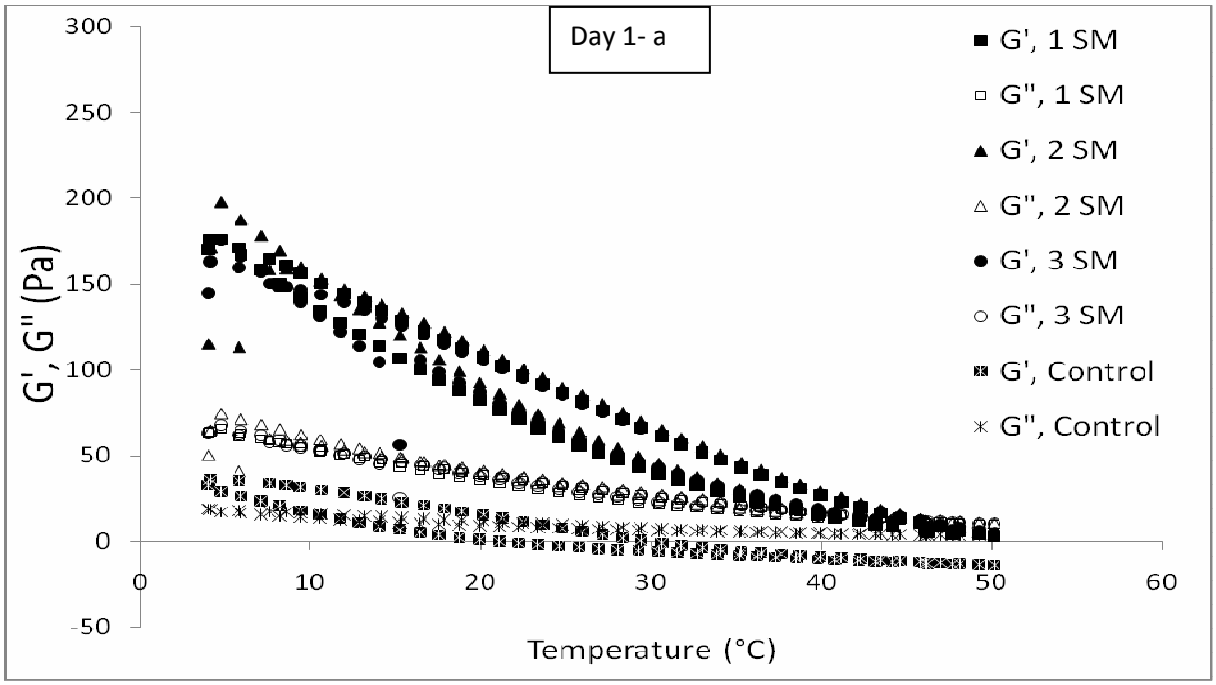
Figure 5.4- Syneresis in 1-3% lentil and 1-3% skim milk supplemented yogurt and control sample during 28 day storage (SM: skim milk, LF: lentil flour)

5.3.6 Rheological properties

Results of storage modulus (G′)(elasticity) and loss modulus (G″)(viscosity) as a function of temperature for the 1-3% lentil flour and 1-3% skim milk supplemented yogurt and control samples at days 1, 14 and 28 of storage are presented in Figures 5.5, 5.6 and 5.7. Dynamic temperature ramp test allows the study of the rheological properties of yogurt during heating and cooling, which occurs in practice when a product is taken out of the refrigerator for consumption and then stored again. All samples demonstrate predominantly an elastic behavior (G'>G") over the range of temperature studied (Figures 5.5, 5.6 and 5.7). G' and G"

parameters follow a hysteresis loop during heating and cooling and they decrease with increasing temperature and increase back with decreasing temperature. Higher percentages of supplementation resulted in higher value of G' and G" which is showing the improvement of viscoelasticity of the samples due to higher solid content. Additionally, when comparing the responses at certain temperatures during heating and cooling, the lentil flour supplemented yogurt behaved differently from the skim milk supplemented yogurt. On day 1, the 3% lentil flour supplemented yogurt had the highest G' and G" value at all temperatures followed by the other supplemented samples; all supplemented samples were significantly higher than the control sample. At day 14, the 2% skim milk supplemented yogurt significantly showed the greatest G' and G" from 4-50 °C and 50-4 °C (P<0.05) followed by 1-3% lentil flour, the 3% and 1% skim milk supplemented yogurt. However over the temperature range studied, the lentil flour supplemented samples generally showed greater viscosity and elasticity which was significantly higher than the control sample, however the difference with skim milk supplemented samples was not significant (P<0.05). At day 28, the 3% lentil flour supplemented yogurt showed the highest G' and G" values which was significantly greater than the control sample. Overall, all supplemented samples had higher viscosity and elasticity than the control sample which means they showed stronger gel and higher stability.

Rheological properties depend on total solid content particularly the amount and type of protein and with higher solids content in yogurt, there is a tendency to increase G' and G" and thus decrease the deformation in yogurt gel. The supplementation level is, therefore, an important factor which can alter gel structure, and affect functional properties such as water absorption (Sendra et al., 2010). Some studies have shown that addition of whey protein isolate improves the physical properties of yogurts such as viscosity (Isleten & Karagul-Yuceer, 2006; Sodini et al., 2005a). Our study shows that supplementation increased the strength of the gel system and lentil flour had either greater or comparable effect to skim milk on the strengthening of the yogurt gel network.



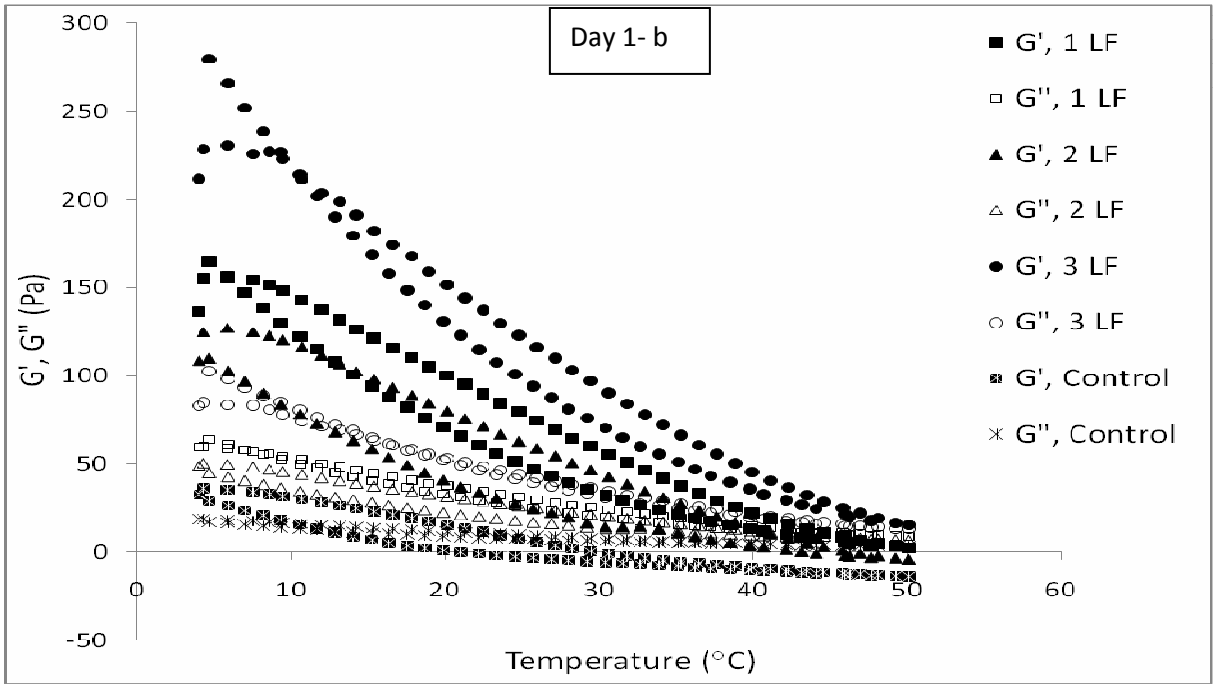
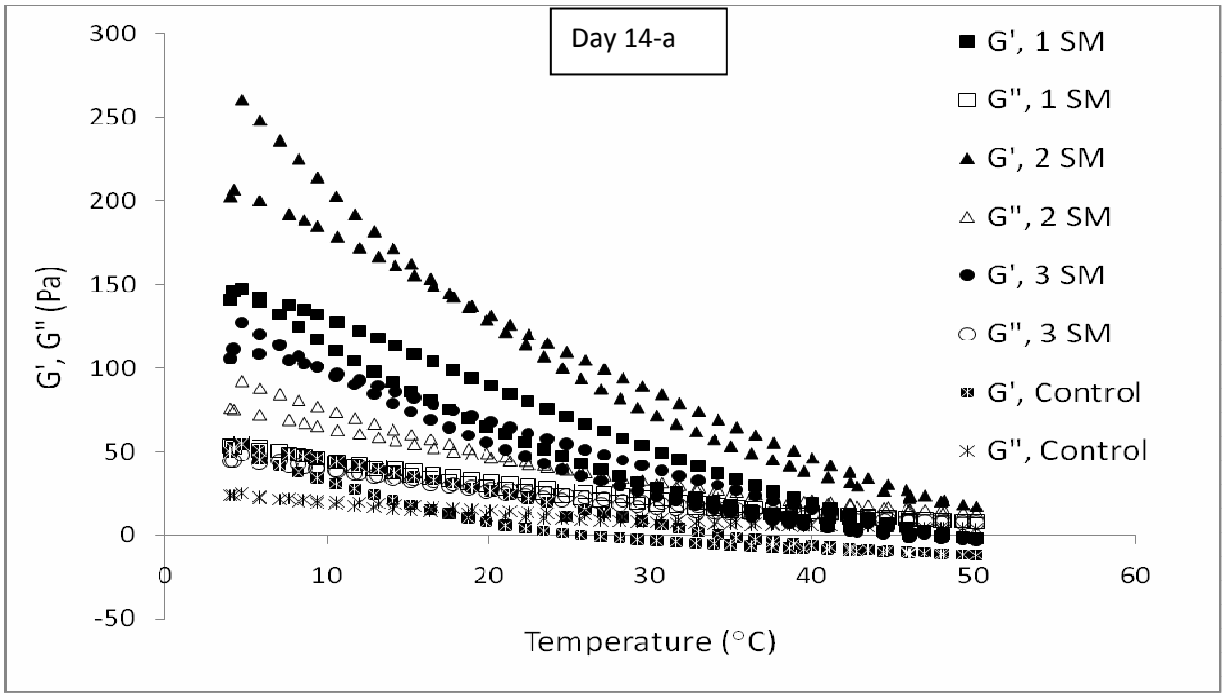


Figure 5.5 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of yogurt supplemented with (a) 1-3% skim milk and (b) 1-3% lentil flour heated from 4-50 ˚C and from 50-4 ^{o}C at Day 1; (SM : skim milk, LF: lentil flour)



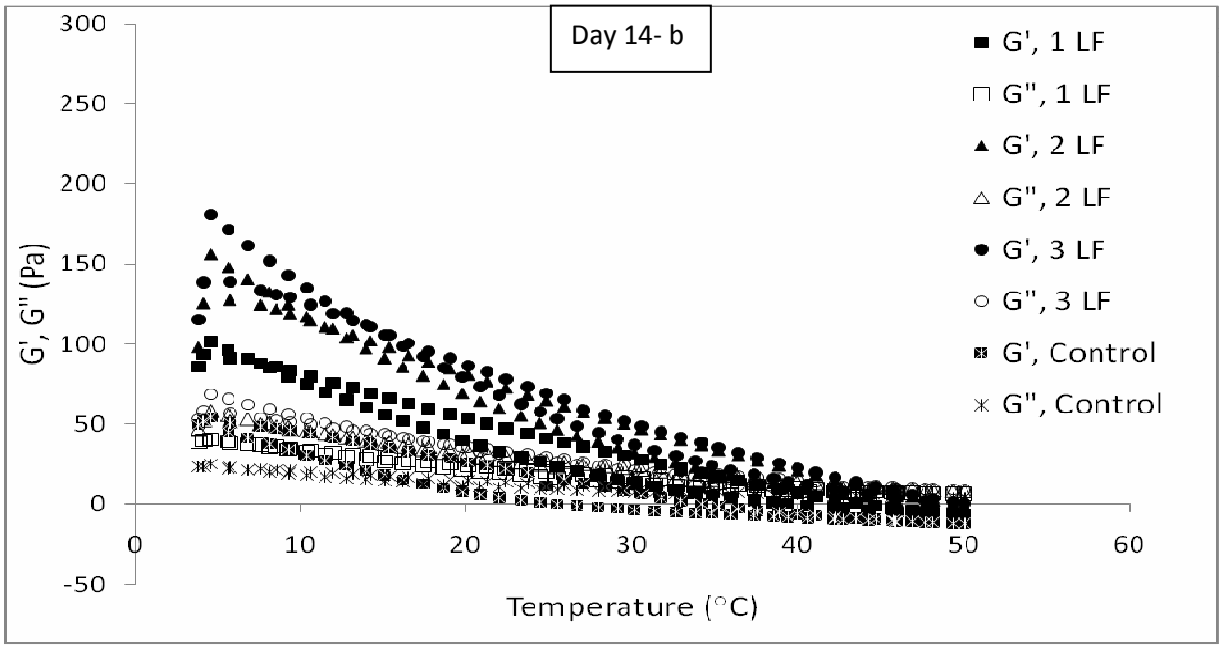


Figure 5.6 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of yogurt supplemented with (a) 1-3% skim milk and (b) 1-3% lentil flour heated from 4-50 ˚C and from 50-4 ^{o}C after 14 days of storage; (SM : skim milk, LF: lentil flour)

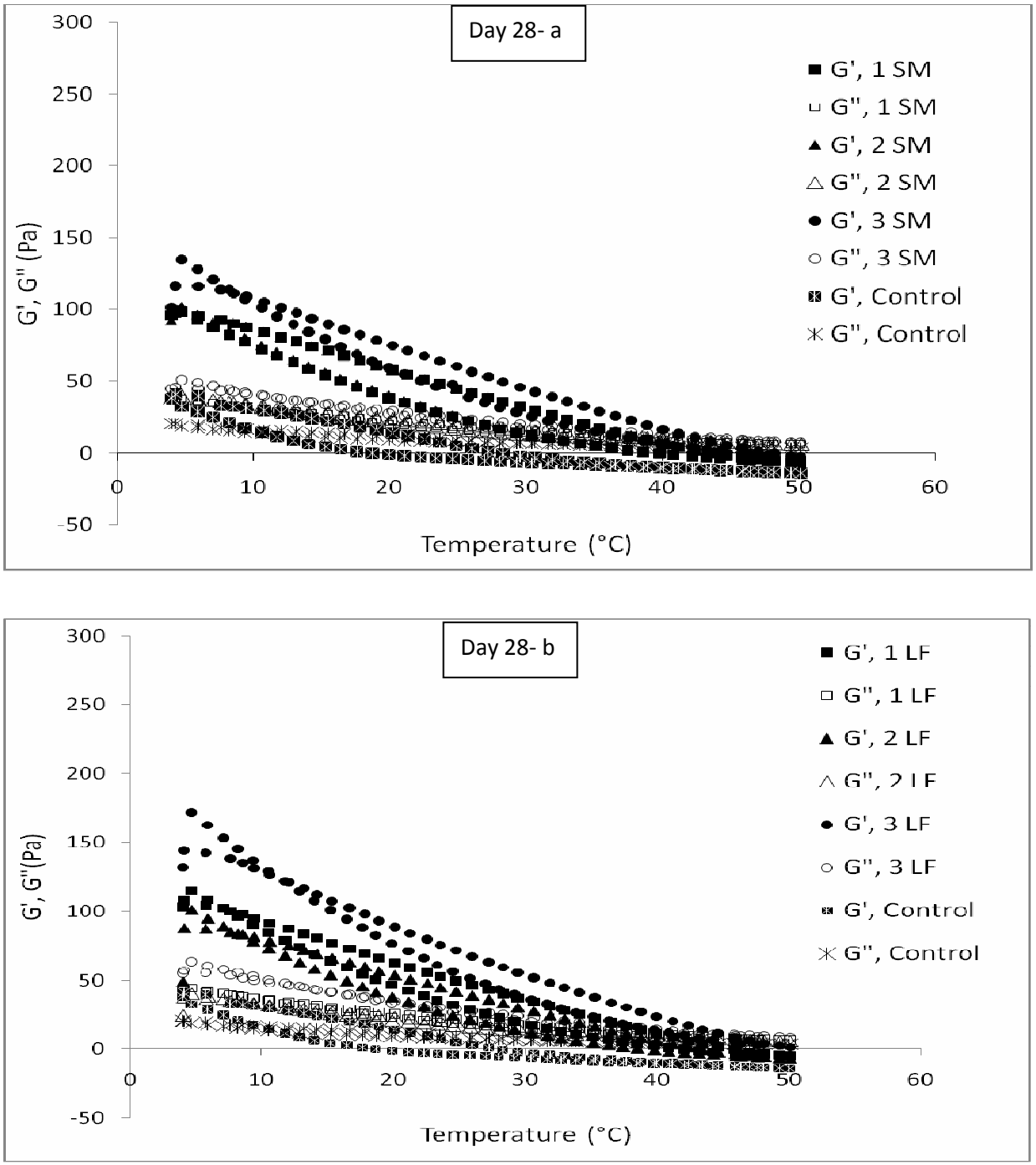


Figure 5.7- Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of yogurt supplemented with (a) 1-3% skim milk and (b) 1-3% lentil flour heated from 4-50 ˚C and from 50-4 ^{o}C after 28 days of storage; (SM : skim milk, LF: lentil flour)

5.3.7 Sensory properties

1-3% lentil flour as well as 1-3% skim milk supplemented yogurt and control samples were ranked in terms of smoothness, graininess, flavor, overall acceptance and color and results are presented in Table 5.3. The lowest numbers represent more desirable, and the highest represent the less desirable traits (i.e., extremely like (1) to extremely dislike (9) with a score of 5 representing neither like or dislike). The 3 % skim milk supplemented yogurt had the lowest scores in all aspects. Although all lentil flour supplemented yogurt had higher scores in comparison with 1-3% skim milk supplemented yogurts, the scores for the 1% and 2% lentil flour supplemented yogurts were not significantly different from those of the 1-2% skim milk supplemented yogurt in terms of smoothness, graininess, flavor and overall acceptance (P<0.05). Additionally, the color of the 1-3% lentil flour supplemented yogurt was not found to be significantly different from the control and 1% skim milk supplemented yogurt (P<0.05). Thus, overall sensory scores for the 1-2% lentil flour supplemented yogurt were found to be comparable to those of the 1-2% skim milk supplemented yogurt and the control sample.

Table 5.3- Sensory properties of control yogurt and yogurt supplemented with 1-3 % lentil flour and 1-3 % skim milk powder (SM: skim milk, LF: lentil flour; 1 - extremely like to 9 - extremely dislike; means with the same letter are not significantly different, for a given column; (P<0.05))

Sample	Smoothness	Graininess	Flavour	Overall acceptance	Color
	Average ± SD	Average ± SD	Average ± SD	Average ± SD	Average ± SD
1 SM	3.00 ± 1.11 a	2.68 ± 1.24 ab	4.04 ± 1.59 ab	3.52 ± 1.38 ab	2.76 ± 1.53 ab
2 SM	2.48 ± 0.65 ab	2.32 ± 0.74 b	3.8 ± 1.77 ab	3.40 ± 1.63 ab	2.08 ± 1.11 b
3 SM	2.08 ± 0.81 b	2.00 ± 0.86 b	2.88 ± 1.53 b	2.72 ± 1.36 b	2.16 ± 0.85 b
1 LF	2.72 ± 1.02 ab	2.76 ± 1.01 ab	3.84 ± 1.34 ab	3.40 ± 0.95 ab	3.60 ± 1.47 a
2 LF	2.88 ± 1.09 ab	3.32 ± 1.28 a	4.44 ± 2.04 a	4.2 ± 1.70 a	3.56 ± 1.36 a
3 LF	3.16 ± 1.74 a	3.44 ± 1.68 a	4.88 ± 1.61 a	4.32 ± 2.09 a	3.44 ± 1.12 a
Control	3.36 ± 1.31 a	2.96 ± 1.09 ab	4.16 ± 1.51 ab	3.92 ± 1.57 ab	3.2 ± 1.19 a

5.4. Conclusion

This study showed that acid production in 1-3% lentil flour supplemented yogurt was significantly higher during fermentation in comparison with 1-3 % skim milk powder supplemented yogurt which may partially be attributed to the lower buffering capacity of lentil supplemented yogurt in comparison with the skim milk supplemented yogurt. Data on the viable counts of the two cultures in the control and supplemented samples however suggest that nutrients brought by the lentil flour may have affected growth of the yogurt cultures differently than did skim milk powder. Our results also showed that yogurt supplementation with 1-3% lentil flour either improved or minimally altered the physical and rheological properties of yogurt in comparison with non-supplemented control yogurt. 1-3% lentil supplemented yogurt demonstrated comparable pH and color with 1-3% skim milk supplemented yogurt and the control sample during 28 days of storage. In terms of syneresis, the 1-2% lentil supplemented yogurt had significantly higher whey separation, while the 3% lentil flour had the least whey separation ranking closely after the 3% skim milk supplemented yogurt at day 14 and was comparable with the control sample at day 28. At any given level of supplementation, the rheological properties of the lentil flour supplemented yogurt were comparable with the skim milk supplemented yogurt and they demonstrated greater viscoelastic properties compared to the control sample. The highest G' and G" was observed for 3% lentil flour during storage which may be due to a stronger gel structure due to its higher fiber content in comparison with the other supplemented samples. 1% and 2% lentil flour were comparable with 1-2% skim milk supplemented yogurt in terms of smoothness, graininess, flavour and overall acceptance.

Overall, on the basis of the microbial, physico-chemical, rheological and sensory properties investigations, the results suggest that lentil flour could be potentially considered as a source of ingredient for yogurt supplementation.

Connecting Statement to Chapter 6

Results from Chapter 4 showed that some pulse ingredients have beneficial effects on yogurt starters and probiotic bacteria in fermented milk. Additionally in Chapter 5 it was shown that yogurt supplementation with 1-3% lentil flour enhanced acid production during fermentation, while the microbial population (CFU) of both *S. thermophilus* and *L.delbrueckii ssp bulgaricus* remained in the same range in all lentil flour and skim milk powder supplemented yogurts. The average pH of samples decreased from 4.5 to 4.1 after 28 days storage. 3% lentil supplementation reduced syneresis during the 28 days storage. With respect to color, lentil supplemented yogurt were comparable with non-supplemented sample. Yogurt with 3% lentil flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the non-supplemented control yogurt. Furthermore, 1-2% lentil flour supplemented yogurt showed comparable sensory properties in comparison with 1-2% skim milk powder supplemented yogurt and the control sample.

Overall, results were very promising for probiotic fermented milk with added lentil flour as lentil addition promoted the growth of *L. rhamnosus* (chapter 4). Further studies were, therefore, needed to investigate the effect of lentil flour on viable counts after production and during storage as well as on the physical properties of probiotics. Therefore, to follow up on our previous studies, skim milk (9.5 % w/v solid content) was supplemented with 1-3% (w/v) lentil flour or skim milk powder, inoculated with a *L. rhamnosus*, fermented and stored at 4˚C. Acid production during the fermentation, microbial growth, physical properties (pH, syneresis, and color) and rheological properties (dynamic oscillation temperature sweep test at 4-50 ˚C), during 28 days of refrigerated storage were studied. For comparison, yogurt (skim milk as the base media for probiotic formulation) was also supplemented with 1-3% skim milk powder and similarly analyzed as well as a non-supplemented control. The results of this research have been presented as follow:

Zare, F., Boye, J.I., Champagne, C.P., Orsat, V., & Simpson, B.K. (2010), Acidification and microbial growth of yogurt and probiotic supplemented with lentil flour, IFT, July 17th-20th, Chicago, IL, USA, (poster presentation).

Zare F., Orsat, V., Champagne, C., Simpson, B.K., Boye, J.I., (2011). Microbial and physical properties of probiotic fermented milk supplemented with lentil flour*, Food Research International,* (Submitted).

Chapter 6: Microbial and physical properties of probiotic fermented milk supplemented with lentil flour

Abstract

In this study, skim milk (9.5 % w/v solid content) was supplemented with 1-3% (w/v) lentil flour or skim milk powder, inoculated with *Lactobacillus rhamnosus* AD200 culture, fermented at 37 ˚C and stored at 4˚C. Acid production during the fermentation, microbial growth, physical properties (pH, syneresis, and color) and rheological properties (dynamic oscillation temperature sweep test at 4-50 ˚C), after production and during 28 days of refrigerated storage were studied. Milk supplementation with 1-3% lentil flour enhanced acid production during fermentation, and the microbial population (CFU) of *L. rhamnosus* were comparable with non-supplemented control sample after production. After 28 days storage, the CFU of 2% and 3% lentil supplemented probiotic were as high as the 1% skim milk supplemented sample. The average pH of samples decreased from 4.5 to 3.9 over 28 days storage. Syneresis in 1-3% lentil flour supplemented probiotic was significantly lower than all other samples. With respect to color, all lentil flour supplemented samples had significantly lower "L" values and higher "b" and "a" values in comparison with other samples after production. Probiotic products with 1-3% lentil flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the non-supplemented control sample. Storage modulus (G′) was higher than loss modulus (G″) in all samples and at all temperatures between 4-50 ˚C and they showed a hysteresis loop over this temperature range when the samples were heated and cooled.

6.1 Introduction

Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit to the host" (Araya et al., 2002). Indeed, humans have been consuming probiotics in the form of fermented foods for many years (Ranadheera et al., 2010). Most common types of probiotics are lactic acid bacteria (LAB) and include species from the

Lactobacillus, Pediococcus and *Bifidobacterium* genera. *Lactobacillus rhamnosus* and *Bifidobacterium* have been the predominant species used as probiotics over the years (Tamime & Robinson, 1999; Ranadheera et al., 2010).

Probiotic health benefits have been known for a long time, with Hippocrates and other scientists in the early ages having reported that fermented milk could heal some digestive disorders (Ranadheera et al., 2010). However, Élie Metchnikoff is considered in recent history to have discovered probiotics (Heller, 2001). He noticed greater longevity in Caucasian populations who frequently consumed fermented milks and proposed that the acid-producing organisms in fermented dairy products could stop the "fouling" in the large intestine which consequently lead to prolongation of a healthy life (Heller, 2001). Probiotics are resistant to bile and survive passage through the gastrointestinal tract without induction of systemic immune or inflammatory reactions. Recent studies have indicated that probiotic bacteria can provide several therapeutic advantages, such as modification of the immune system, blood cholesterol reduction, lessening of lactose intolerance, maintained remission of Crohn's disease, diarrhea healing, and prevention of infections of urogenital organs (Hekmat, Soltani, & Reid, 2009).

Prebiotics, on the other hand, are non-digestible food ingredients that alter the functionality and/or growth of one, or a limited number of probiotic bacteria in the colon (Prado et al., 2008). Oligosaccharides such as lactulose, galactooligosaccharides, inulin, fructooligosaccharides, and other food carbohydrates are some of the well known examples of prebiotics. There is an obvious potential for a symbiotic effect of probiotics and prebiotics, since prebiotics promote the growth and activities of probiotics. Several studies have shown that growth and viability of *L. rhamnosus* could be increased in the presence of resistant starch, inulin, fructooligosaccharides, polydextrose and oligofructose in fermented food products such as yogurt, fermented milk, cheese and ice cream (Ranadheera et al., 2010). Apart from nutritional benefits of prebiotics, addition of these ingredients provides techno-functional benefits that can improve the various quality parameters of the final products. Some plant-based matrices are very rich in prebiotic compounds and inulin-containing chicory is probably the best example of this. Inulin and

oligofructan derived from inulin improve the viscoelastic properties in yogurt and fermented milk products. They increase firmness and viscosity and decrease syneresis (Bozanic, Rogelj, & Tratnik, 2001 and 2002; Dello Staffolo, Bertola, Martino, & Bevilacqua, 2004; Debon, Prudencio, & Petrus, 2010; De Castro, Cunha, Barreto, Amboni & Prudencio, 2008). Several studies have also indicated that the physico-chemical characteristics (pH, acid production, color and water activity) of fermented products such as yogurt, soy yogurt and probiotic fermented milk products improve due to supplementation with prebiotics such as inulin, resistance starch powder (Hi-maize), fiber and calcium, date fiber, β-glucan, glucose and raffinose. This could be due to the nutritional benefits of prebiotics in enhancing the growth of probiotics and promoting acid production during fermentation and storage, as well as their techno-functional properties which could enhance the physical properties of the products (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007; Aportela-Palacois at al., 2005; Vasiljevic et al., 2007; Hashim, Khaul, & Afifi, 2009).

There is great economic interest in finding other prebiotic-rich food matrices. The nutritional composition of lentil which includes complex carbohydrates (e.g., resistant starch, oligosaccharides, sucrose, raffinose, stachyose and verbascose), protein, important vitamins and minerals as well as antioxidants, and only very small amounts of unsaturated fats could make this ingredient a very good source of prebiotic components for human nutrition and probiotics bacteria such as *L. rhamnosus* (Zare, Boye, Orsat, Champagne, & Simpson, 2011a; Wang, & Daun, 2004). Our previous study suggested that yogurt starter culture *(S. thermophilus and L. bulgaricus*) grow better in milk supplemented with lentil flour and acid production during fermentation and storage improved with the addition of lentil flour. Moreover, physical and rheological properties of the final products, either improved or did not change, with 1-3 % of lentil flour supplementation following production and during storage. This study expands on our previous studies and explores lentil supplementation of milk in the presence of a specific probiotic species, *L. rhamnosus.* We investigate the effect of supplementation of fermented milk with 1–3% lentil flour on acid production during fermentation, growth of *L. rhamnosus*, pH, syneresis, color and rheological properties of the final product immediately after production

and during one month of storage. For comparison, skim milk as the base media for fermentation with and without supplementation with 1–3% skim milk powder was similarly analyzed.

6.2. Materials and methods

6.2.1 Cultures and ingredients

Non-fat skim milk powder used was from Agropur (Quebon brand; St. Laurent, QC, Canada); lentil flour was from K2 Milling Company (Tottenham, ON, Canada); *Lactobacillus rhamnosus* AD200 was purchased from ABIASA Inc. (St. Hyacinthe, QC, Canada) (the cultures were obtained in freeze-dried form, packaged in laminated foils and were stored at 4°C until use). Skim milk powder mixed in distilled water (9.5 % w/v) served as the base for supplementation and is referred to as "control". In two series of experimental assays 1-3% (w/v) of lentil flour (K2 Milling Company, Tottenham, ON, Canada) or 1-3% of skim milk powder were added separately to the skim milk base (control).

6.2.2 Fermentation

The culture contained a microbial concentration of 2 x 10^{11} CFU/g. It was re-hydrated at 37 ^{o}C in the sterilized skim milk to obtain 2 X 10^{9} CFU/mL. Subsequently 1 mL of this dilution was added to 100 mL media which represented an inoculation level of approximately 2 x 10^{7} CFU/mL. The experimental protocol used for probiotic supplementation and production are shown in Figure 6.1. Acidification trend in fermented milk by *L. rhamnosus* were measured during fermentation according to the method described by De Brabandere & De Baerdemaeker (1999) using a FACS (Fermentation Acquisition and Control System) installed in a Forma Scientific (OH, US) programmable incubator.

6.2.3 Product characterization

The buffering capacity of the different blends was estimated by acid titration and pH measurements using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada) and a 50 mL

digital burette (Brinkmann Instruments Ltd., ON, Canada). For viable counts culture media; MRS agar from Difco Company (KS, USA) was used for quantifying the *L. rhamnosus.* Viable counts were obtained after 48 hours incubation at 37 °C under aerobic conditions.

pH was measured in the probiotic fermented milk using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada).

Syneresis was determined as the amount of spontaneous whey separation from the fermented product according to the method described by Lucey, Munro, & Singh (1998), with some modifications. The volume of whey drained from 100 mL of undisturbed set yogurt prepared in cylindrical tubes was measured and reported as percentage syneresis.

Color was determined as lightness (*L*), red/greenness (a), and yellow/blueness (*b*), using a colorimeter (Konica Minolta, CM-503 c, NJ, US).

Dynamic oscillation tests were conducted to determine the flow behavior and characterize the viscoelastic properties of yogurt, using a rheometer (TA Instruments, SR-2000, DE, US) fitted with a 40-mm-diameter cone and 0.04 radian degree cone angle and plate geometry with a 4 mm gap. To ascertain the applicable stress and frequency in which storage modulus (G') and loss modulus (G") parameters of yogurt would demonstrate a linear constant rate, dynamic frequency ramp tests (frequency from 1 to 10 Hz and stress set at 3 Pa) and dynamic stress ramp tests (stress from 1 to 10 Pa and frequency set as 2.5 Hz) were conducted at 25 °C. Dynamic temperature ramp tests were done at a stress and frequency of 3.0 Pa and 2.5 Hz, respectively, in a temperature range of 4 to 50 °C (heating) and 50 to 4 °C (cooling), at a rate of 10 °C/min. Aliquots of the samples were carefully removed from the undisturbed yogurt cup and placed on the center of the rheometer plate; the top plate was slowly lowered on the top of the sample prior to analysis.

Viable counts, pH, syneresis, color and rheological parameters were measured after fermentation as well as during 28 days storage at 4 °C at 7 days interval.

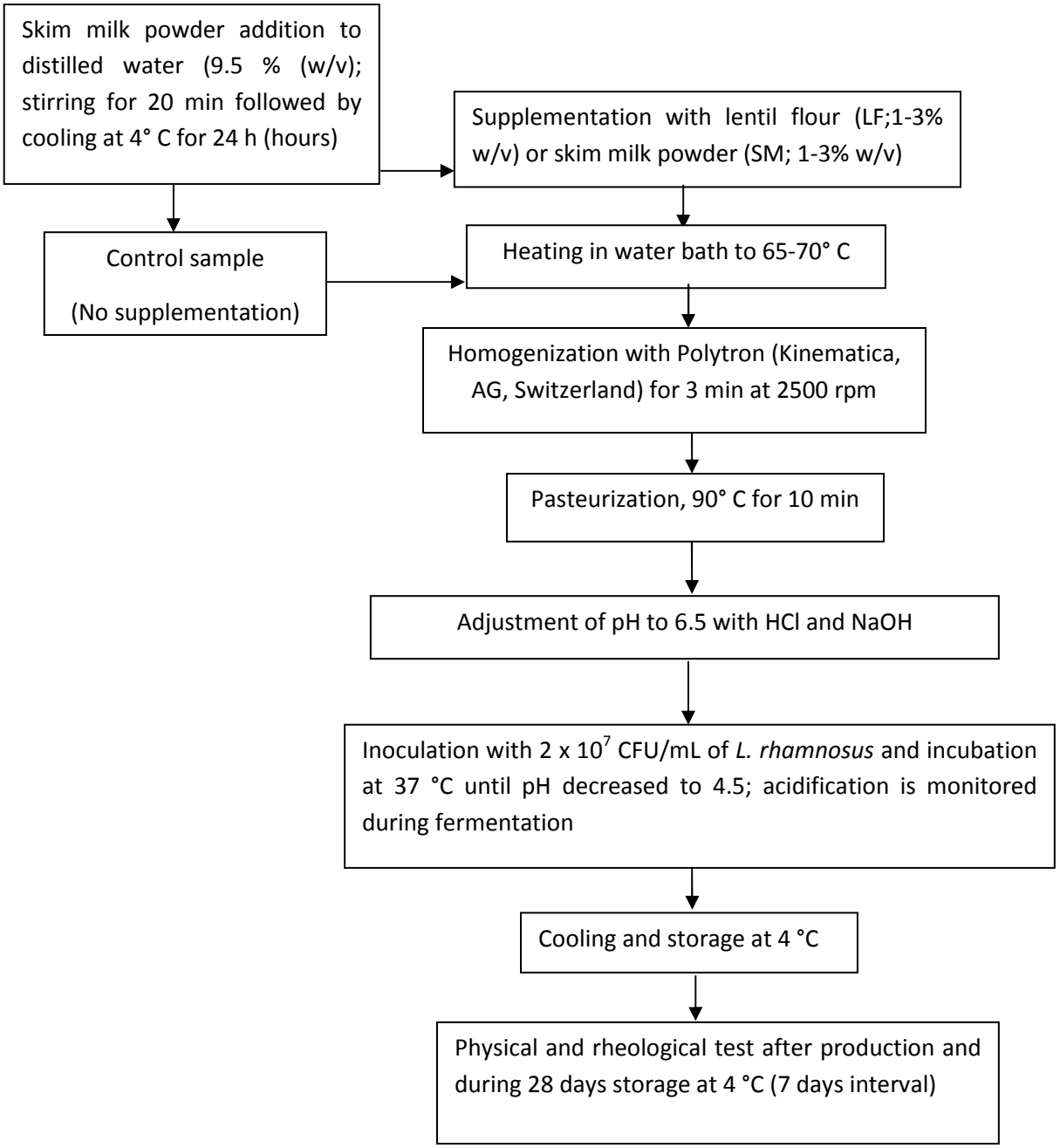


Figure 6.1- Schematic presentation of the process used for the preparation of *L.rhamnosus* probiotic supplemented with skim milk powder (SM) or lentil flour (LF) and the control sample (skim milk base with no supplementation)

6.2.4 Statistical analysis

Statistical test was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two sample t-test for comparison of two means.

6.3. Results and discussion

6.3.1 Acidification by *L. rhamnosus*

Acidification patterns of the various media after inoculation with *L. rhamnosus* is presented in Figure 6.2. For both skim milk powder and lentil powder, supplementation enhanced the acidification rate. The difference in pH between the supplemented media and the control became statistically significant after 8 hours of incubation. After 8 h, the pH in the 2% and 3% skim milk-supplemented products was significantly (P<0.05) lower than the lentil flour-supplemented media. However, after 12 hours of incubation, the 2% and 3% lentil flour-supplemented samples had the lowest pH. As a result, the products with lentil flour reached a pH of 4.5 significantly earlier than skim milk supplemented and control sample (Figure 6.2). This constitutes important time and energy savings in the manufacturing process. These data confirm our preliminary study (Zare et al., 2011b). As milk has greater buffering capacity in comparison with lentil flour (Table 6.1), the greater acidification rates in products supplemented with lentil flour could have been partially due to their lower buffering capacity when compared to the corresponding skim milk supplemented probiotic products. This does not appear to be the case however, when the data with lentil flour are compared to the control. Besides, acid production in the media, which is mainly due to lactic acid, is often linked to the growth of lactic acid bacteria (i.e: *L. rhamnosus)* (Tamime & Robinson, 1999). The acidification data, therefore, suggests that growth of the microorganism may have been stimulated by the lentil flour. Although, viable counts were not followed during the fermentation to assess this hypothesis, some analyses were carried out after production as well as during storage.

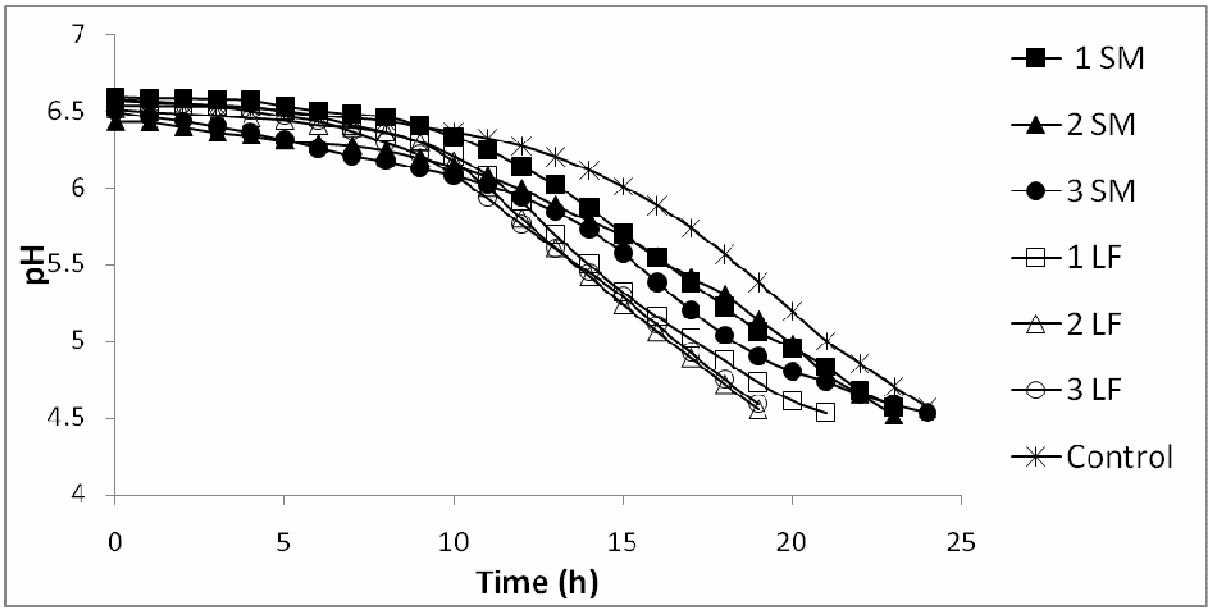


Figure 6.2- Effect of supplementation of skim milk with 1 to 3% lentil flour (1 LF, 2 LF and 3 LF treatments) or 1-3% skim milk (1 SM, 2 SM and 3 SM treatments) on acidification by *L. rhamnosus* AD200 (SM - skim milk (9.5 % solids); LF - lentil flour control (9.5 % solids)

Table 6.1- Amount of HCl (1 M) required to acidify 100 mL of 1-3 % lentil flour and 1-3 % skim milk from pH 6.5 to 4.0 (SM: skim milk, LF: lentil flour; means followed by the same letter are not significantly different ($p < 0.05$))

Sample	Titrable HCl (mL)
	Average ± SD
1 % SM	6.84 ± 0.00 b
2 % SM	7.58 ± 0.07 c
3 % SM	9.14 ± 0.00 d
1 % LF	6.51 ± 0.01 a
2 % LF	6.83 ± 0.21 b
3 % LF	7.06 ± 0.03 b
Control	6.38 ± 0.00 a

6.3.2 Microbial growth and survival in the supplemented products after production and during storage

There is no universally accepted number of viable cells required to obtain a health benefit (Reid, 2008). In yogurt products, in order to provide health benefits targeted towards lactose digestion using probiotics, a minimum of 10^8 CFU per serving is required (EFSA, 2010). Canadian legislation (CFIA, 2009) allows limited non-strain linked claims if the product contains a billion viable cells per portion, and there is a trend, at least in Canada, towards this CFU level in yogurt and fruit juices available on the market. To maintain these numbers, it is important to follow the probiotics viability during manufacture and storage (Damin et al., 2006).

Viable counts of *L. rhamnosus* in fresh (day 0) products supplemented with lentil flour or skim milk powder varied from log 8.11 to log 8.71 (Table 6.2). These counts are lower than those obtained for *L. rhamnosus* in fruit-based media, which are well above 10^9 CFU/mL (Champagne & Gardner, 2008). Growth and acidification of *L. rhamnosus* in milk is rather slow (Gaudreau et al., 2005), and low CFU counts in milk-based products are also encountered with other probiotic cultures (Champagne, Tompkins, Buckley, & Green-Johnson, 2010). Therefore, the data on growth and acidification with *L. rhamnosus* in milk is in line with that of the literature. Although important CFU reductions occurred in some samples after 28 days of storage, all supplied the minimum 1 billion per portion amount of probiotics required for a non-strain related health claim (CFIA, 2009), presuming a portion to represent about 100 mL. Furthermore, many had the 10^8 CFU/mL threshold required to market the high-density probiotic products such as DanActive™ (Danone) or BioBest Maximmunité™ (Parmalat), which contain 10 billion CFUs per portion. Supplementation with 1 to 3% skim milk powder significantly improved *L. rhamnosus* CFU levels in the fresh products (Table 6.2). Addition of 2 and 3 % lentil flour also increased CFU counts in the fresh products (Table 6.2). The high CFUs in the fresh products, supplemented milk and lentil flour powders remained higher than the control treatment during the 28 days of cold storage (Table 6.2). It is noteworthy; however, that the addition of lentil

flour accelerated the rate of acidification, but did not increase the CFU values in the fermented probiotic products (Table 6.2) as compared to skim milk powder supplementation.

The nature of the stimulatory factors in lentil flour, thus, remains unknown and it is hypothesized that lentil flour could serve as a prebiotic source due to its nutrients components such as protein, resistant starch, sucrose, raffinose, stachyose, verbascose and oligosaccharides for *L. rhamnosus* in probiotic products (Wang and Daun, 2004). Also, supplementation with lentil flour significantly maintained the stability of the *L.rhamnosus* during storage (Table 6.2). Antioxidants (Dave & Shah, 1997b) and carbohydrates (Silva et al., 2004) have been shown to improve the stability of lactobacilli during storage. It remains to be determined which compounds in the lentil flour have this protective benefit towards the lactobacilli.

A regression analysis was carried out to ascertain the role of the buffering capacity of the ingredients (Table 6.1) on the viable counts obtained at the end of the fermentation (Table 6.2). There was a positive correlation (R^2 = 0.79) between the CFU data after fermentation and the buffering capacity of the skim milk supplementation samples. Also, there is a positive correlation (R^2 = 0.88) between the CFU data after fermentation and the buffering capacity of the lentil flour supplemented samples. This high correlation data suggest that, for *L. rhamnosus* AD200, while total solid content increases, the buffering capacity is a strong regulator of growth, in both skim milk and lentil flour at this range of milk solids. Thus, the very different nutritive content of milk and lentil flour had a negligible effect on final CFU levels when added to milk in the 1-3% supplementation range. The importance of the buffering capacity and biomass levels is in agreement with the literature (Badran & Reichart, 1994). However, data on acidification rates showed a different picture. One would expect that the higher buffering capacity would slow the rate of pH reduction. A regression analysis between pH values at time = 18 h (hour) and the buffering capacity of the media showed absolutely no correlation (R^2 < 0.01). Therefore the acidification rate itself is completely independent of the buffering capacity of the ingredients, and is presumably directly linked to the nutrient content. It can be concluded,

however, that the nutrients brought by lentil flour accelerated the acidification rate of L. *rhamnosus* AD200 much more than that of skim milk.

6.3.3 Change in pH during storage

The pH reduction is due to the acid produced by *L. rhamnosus* during fermentation which continues slightly during storage. In all samples, the pH decreased by 0.1 to 0.5 units over the 28 day storage period (Table 6.2). This drop in pH was greater in products supplemented with 1-3 % lentil flour in comparison with 1-3% skim milk supplemented and control samples. The level of skim milk supplementation did not affect the pH reduction after 28 days of storage (P<0.05). However, this was not the case in products containing the lentil flour. Indeed, the pH in the 3% lentil-supplemented product was more stable during storage (Table 6.2) than the products with 1-2% lentil flour, and this difference actually became statistically significant (P<0.05) after 14 days of storage (data not presented). Data was examined to determine if there was a link between these pH data and that from buffering capacity or viable counts (Table 6.1). There was no correlation between the pH at the end of storage and the buffering capacity of the medium (R^2 < 0.1) nor with the viable counts at the beginning of storage (R^2 = 0.26). These data are in line with other studies which show that acidification during storage does not necessarily parallel CFU counts (Seo et al., 2009). According to Kailasapathy et al., 2008), the higher the buffering capacity of media, the smaller the pH changes due to the changes in acid content of the food system. This was not the case in our study; the skim milk control had the lowest buffering capacity (Table 6.1) but was the product having the most stable pH during storage (Table 6.2). Supplementation with lentil flour, therefore, seems to increase the acidifying ability of the lactobacilli during storage.

There was a concern with respect to the stability of the probiotics during storage, because a higher buffering capacity of the medium tends to increase the survival of live culture bacteria (Kailasapathy et al., 2008). This was not a problem in this study. Indeed, the viable counts in the lentil flour supplemented products dropped on average by 0.3 log CFU/g while that of the skim milk supplemented milks had viability losses of approximately 0.5 by log CFU/g (Table 6.2).

Although, the increased acidification rates during storage did not negatively affect the losses in viability, pH is known to affect texture. Therefore, analyses of color and texture were carried out on the fresh products as well as on the stored ones.

Table 6.2: Effect of milk supplementation with skim milk powder (SM) or lentil flour (LF), on viable counts of *L. rhamnosus* and pH after fermentation and during 28 days of storage at 4°C

Medium	***L. rhamnosus* (Log CFU/mL)**					**pH**	
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 0	Day 28
1% SM	8.44 c	8.28 b	8.22 b	8.00 bc	7.96 bc	4.57 b	4.44 a
2% SM	8.55 b	8.51 a	8.28 b	8.04 b	8.02 b	4.51 d	4.42 a
3% SM	8.71 a	8.58 a	8.35 a	8.21 a	8.15 a	4.53 cd	4.42 a
1% LF	8.15 de	8.03 d	8.00 cd	7.88 d	7.83 d	4.53 cd	4.00 c
2% LF	8.21 d	8.14 c	8.03 c	7.94 cd	7.92 c	4.55 cb	4.10 bc
3% LF	8.21 d	8.14 c	8.04 c	8.00 bc	7.99 bc	4.59 a	4.15 b
Control	8.11 e	8.10 cd	7.96 d	7.91 d	7.71 e	4.56 b	4.53 a

Means followed by the same letter are not significantly different; for a given column ($p < 0.05$)

6.3.4 Color

Color is one of the most important factors for marketability of food products and consumer acceptance. Although a probiotic product could provide several health benefits to consumers, without visual acceptance by the consumers they will not be marketable. The color of lentil flour supplemented probiotic product should, therefore, be comparable to non-supplemented or skim milk supplemented probiotic or other fermented dairy product. Thus, the color of the supplemented products should ideally remain unchanged after production and during storage. Figure 6.3 (a, b and c), shows differences in the color (a, b and L values) of the 1-3% lentil flour

and 1-3% skim milk supplemented probiotic yogurt type and control samples at days 1 and 28 days after production. On the first day of production all lentil flour supplemented samples had significantly lower "L" value and higher "a" and "b" values in comparison with other samples. Also, the level of supplementation significantly affected the "L" and "b" values in skim milk supplemented samples, but in lentil flour supplemented samples, the level of supplementation only affected the "b" values (P<0.05). After 28 days, "L" values in lentil supplemented probiotics decreased less than "L" values for skim milk supplemented samples; which means that skim milk supplemented probiotics may darken during storage significantly more than lentil flour supplemented samples. However, after 28 days of storage, the 3% lentil flour supplemented sample had the lowest "L" value and still the highest "a" and "b" values in comparison with other samples (P<0.05). "L" represents lightness (100) and blackness (0); "a" represents red (+ve) to green (-ve) hue and "b" represents yellow (+ve) to blue (-ve) hues (Sanz et al., 2008), and so the color measurements indicate that immediately after production the lentil flour supplemented yogurt were darker and had less greenness and more yellowness hues in comparison with skim milk supplemented samples. After 28 days, 1-2% lentil flour supplemented probiotics were comparable with 2% skim milk supplemented and control samples, in terms of lightness; in other words, after 28 days storage 1-2% lentil flour supplemented probiotic was as light as the 2% skim milk supplemented and control samples.

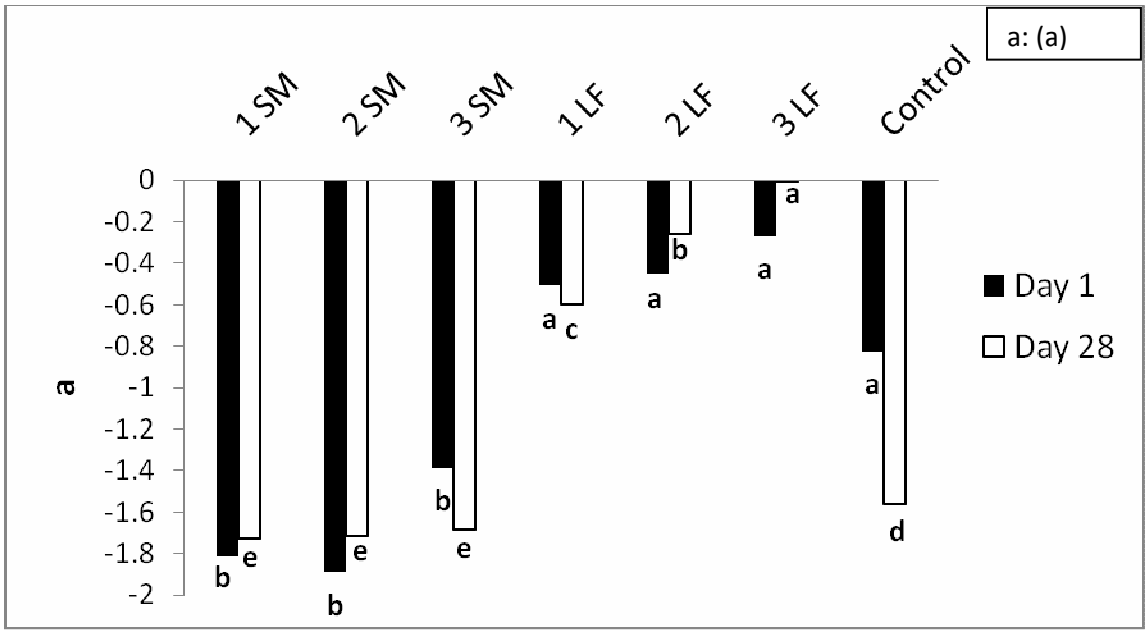
6.3.5 Syneresis

Acid production due to growth of *L. rhamnosus* during fermentation, results in lowering of the pH to the isoelectric point of casein (4.5), which results in protein coagulation and gel formation. Syneresis provides an indication of the non-homogeneities in the gel system; thus, higher water separation (syneresis) is related to gel instability (Lucey et al., 1998). Figure 6.4 shows the syneresis of lentil flour and skim milk supplemented probiotic fermented milk samples and control samples immediately after production and after 14 and 28 days of storage. On day 1, the 1-3% lentil flour showed the lowest syneresis, which was significantly lower than for the 1-3% skim milk supplemented and control samples (P<0.05). The level of supplementation

affected syneresis in lentil flour supplemented samples (i.e., the higher the supplementation level, the lower the syneresis), whereas this factor had a lesser effect on skim milk supplemented samples. After 14 days of storage, although the level of volume separated from the gel dramatically increased in 2-3% lentil flour supplemented samples, the 3% lentil flour supplemented sample still showed the least syneresis compared to all other samples (P<0.05). Most of the increase in syneresis occurred between day 1 and day 14 of storage.

It is hypothesized that the greater changes in syneresis in LF samples were potentially due to the greater decrease in pH due to less solubility of proteins in lower pH. Indeed, acidification during storage was highest in the lentil flour supplemented products (Table 6.2) and this parameter is directly linked to syneresis (Tamime and Robinson, 1999).

Supplementation of probiotic yogurt type products with an increase in the total solid content, especially protein content, results in stronger texture and less whey separation (Peng et al., 2009; Lucey, 2001). This can explain the lowest syneresis in 3% lentil flour and 3% skim milk supplemented samples. Also, considering the starch and fiber contents of lentil flour, which are other hydrocolloid structural compounds, it could be suggested that lentil flour could provide better homogenous texture in comparison with skim milk powder.



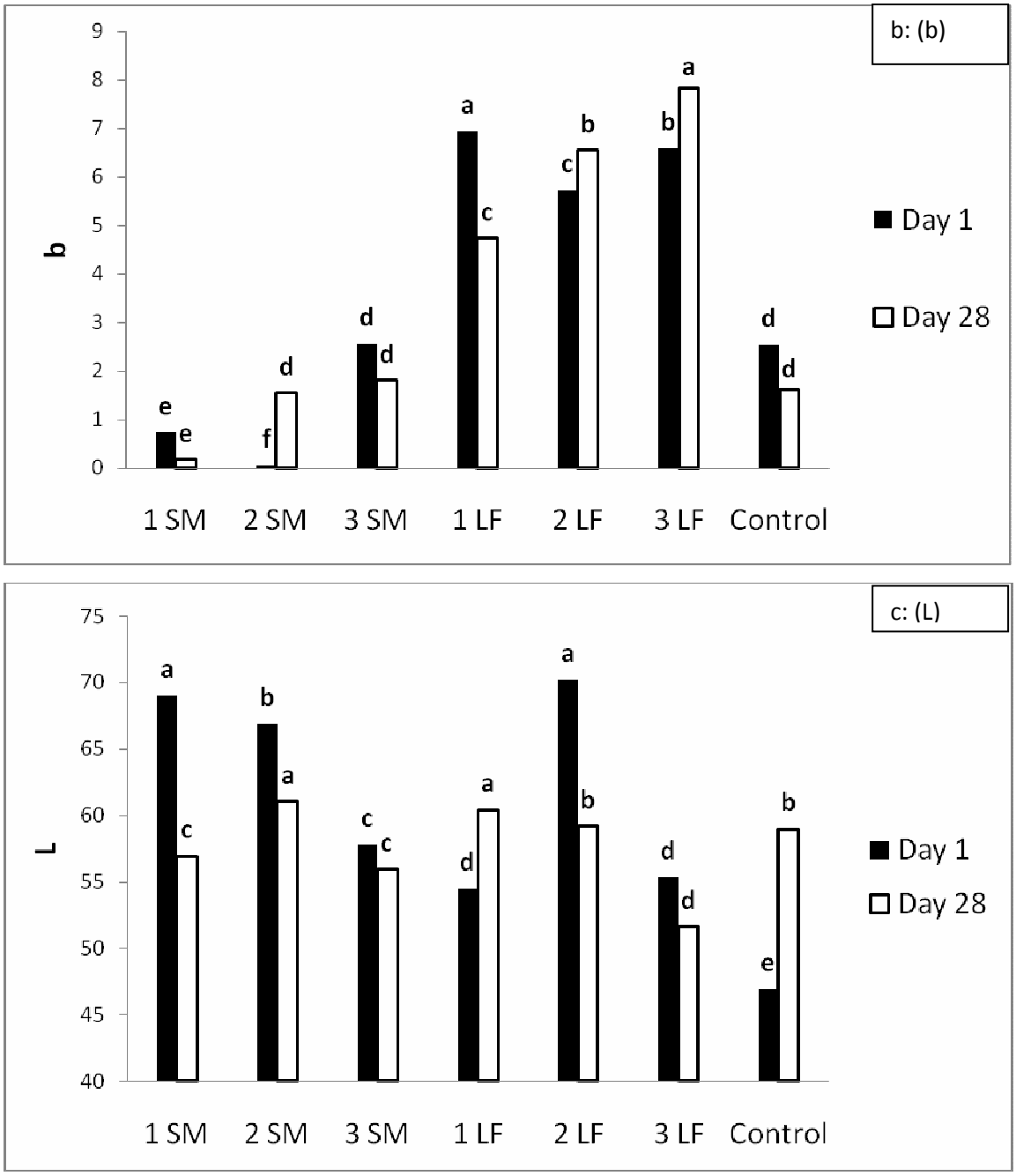


Figure 6.3- Color profile of fermented products supplemented with 1-3% lentil flour or 1-3% skim milk and control sample after production and after 28 days storage; (SM: skim milk, LF: lentil flour; a (a value) +ve red, -ve green; b (b value) +ve yellow, -ve blue; c (L value): -0 to 100, black to white),for a given storage time, means followed by the same letter are not significantly different ($p < 0.05$)

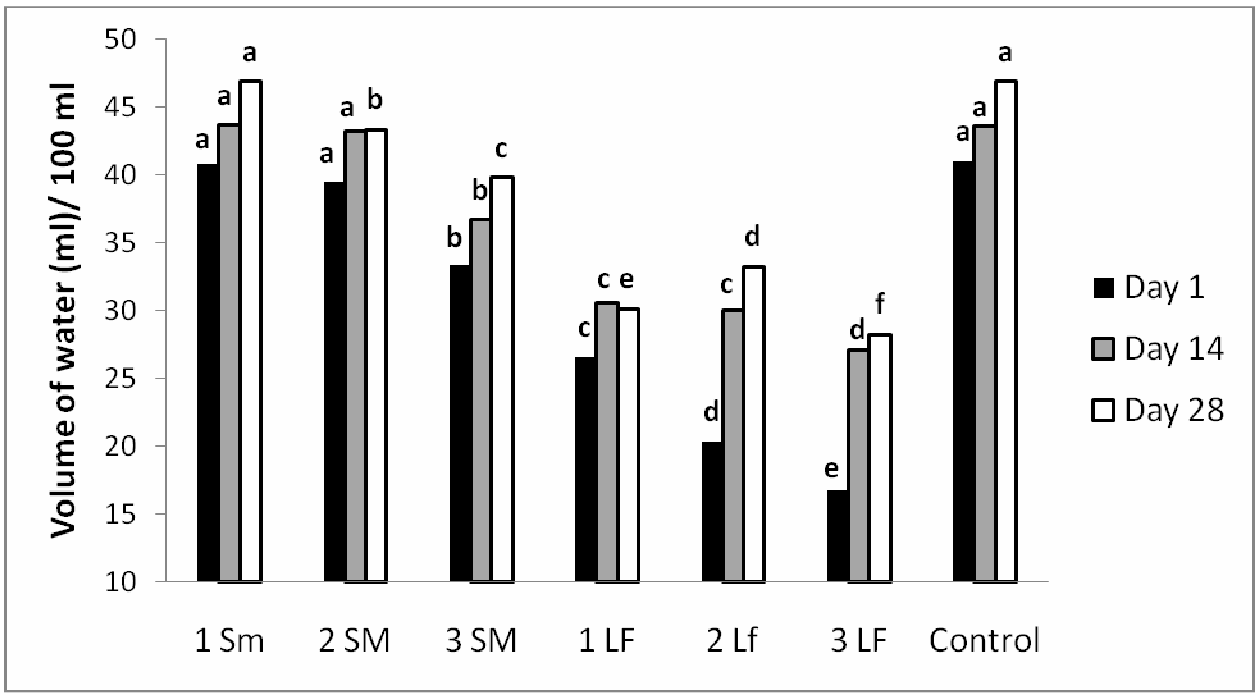


Figure 6.4- Syneresis in products supplemented with 1-3% lentil flour and 1-3% skim as well as control sample during 28 day storage (SM: skim milk, LF: lentil flour), for a given storage time, means followed by the same letter are not significantly different ($p < 0.05$)

6.3.6 Rheological properties

Rheometry is a practical technique for measuring the textural properties of foods. Viscoelastic property measurements give knowledge of the rheological characteristics of foods and give an assessment of the initial experience of a consumer (Kealy, 2006). Oscillatory tests have been used to assess the rheological properties of fermented milk products in several studies (Ozer et al., 1997; Remeuf et al., 2003; Sodini et al., 2005b). When a product is taken out of the refrigerator for consumption and then stored again, the rheological properties could be expected to change. Dynamic temperature ramp test allows the study of the rheological properties during heating and cooling processes.

Results of storage modulus (G′)(elasticity) and loss modulus (G″)(viscosity) as a function of temperature for the 1-3% lentil flour and 1-3% skim milk supplemented probiotic and control

samples at days 1, 14 and 28 of storage are presented in Figures 6.5, 6.6 and 6.7. According to our results, G' and G" parameters follow a hysteresis loop during heating and cooling and G' and G" decrease with increasing temperature and increase back with decreasing temperature in all samples, after production and during storage. Also, over the range of temperatures studied, all supplemented and control samples demonstrated a predominantly elastic behavior (G'>G"). It is also shown that as the level of supplementation increases, either with skim milk powder or lentil flour, the values of G' and G" both after production and during storage increase which means that the higher solid content could improves the viscoelastisity of the products. Thus, the supplementation level and total solids content are important factors which can alter gel structure in probiotic yogurt type products. This finding is in agreement with literature (Sendra, Kuri, Fernandez-Lopez, Sayas-Barbera, Navarro & Perez-Alvarez, 2010).

Additionally, when comparing the responses at certain temperatures during heating and cooling, the lentil flour supplemented probiotic samples behaved differently from the skim milk supplemented yogurt. It is interesting to note that both G' and G" values decreased as a result of heating and subsequently cooling in all samples, but in the lentil flour supplemented samples the reduction was not as big as that in skim milk supplemented and control samples. In other words, the gel structure, specially for 1% and 2% skim milk supplemented probiotics, almost collapsed since G' and G" values were close to zero during the cooling process (from 50-4 ^{o}C). This suggests that the gel structure in lentil flour supplemented probiotic were more stable under temperature stress conditions than the skim milk supplement probiotics. This appears in line with the data on syneresis, where supplementation with LF improved the water-binding property of the gel.

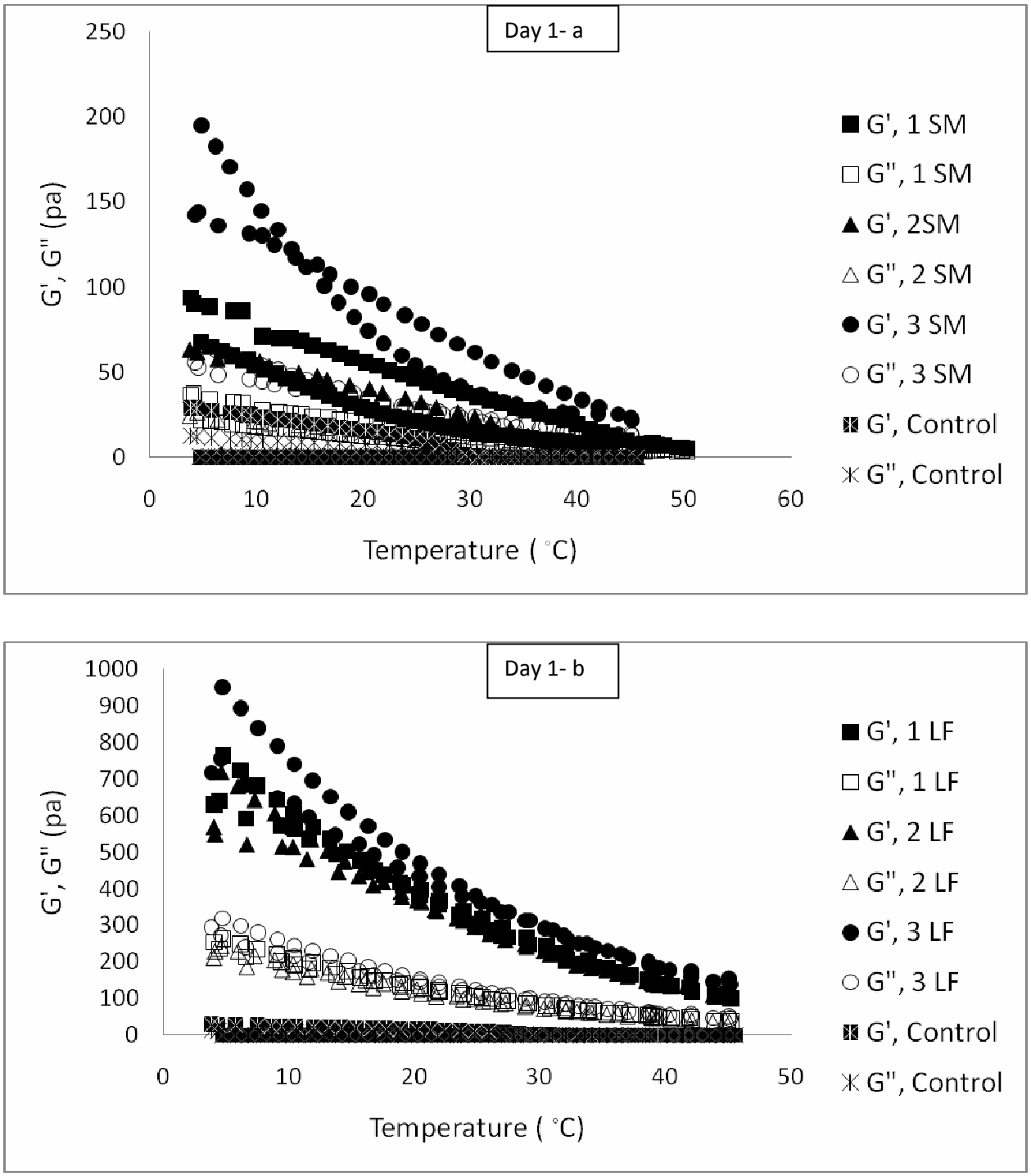
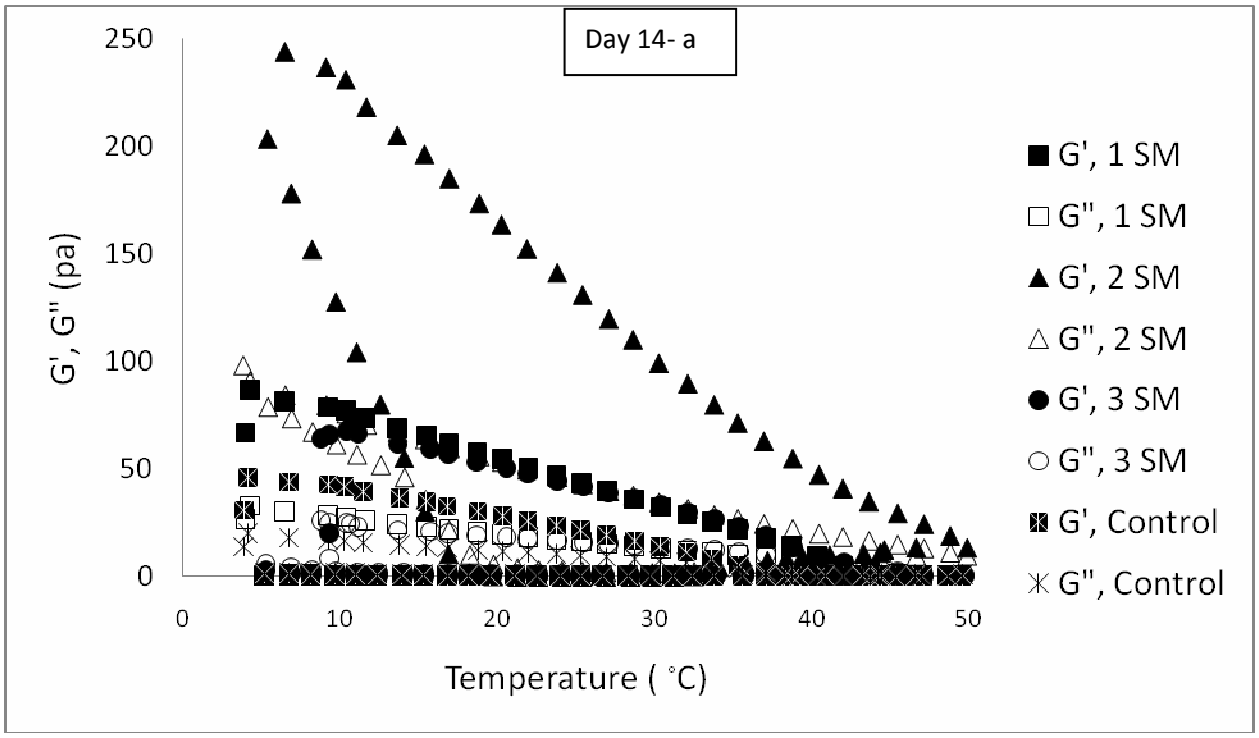


Figure 6.5 - Storage (G′) (elasticity) and loss (G˝) (viscosity) moduli of fermented products supplemented with (a) 1-3% skim milk and (b) 1-3% lentil flour heated from 4-50 ˚C and from 50-4 ^{o}C at Day 1; (SM : skim milk, LF: lentil flour)



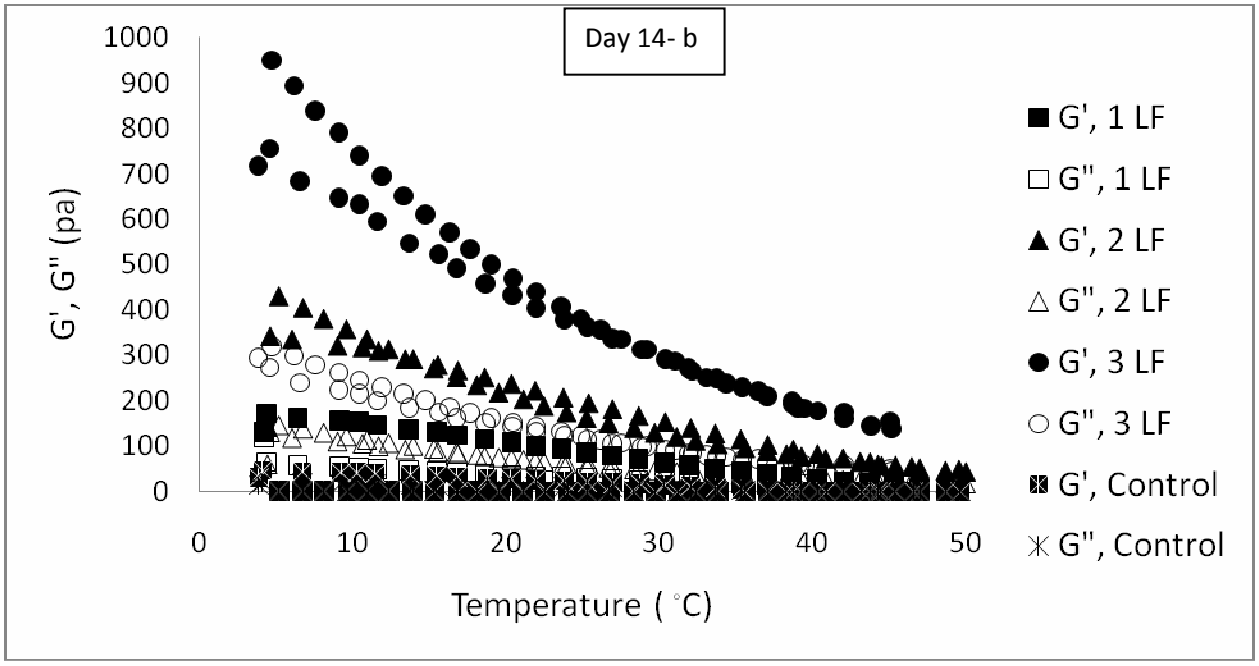
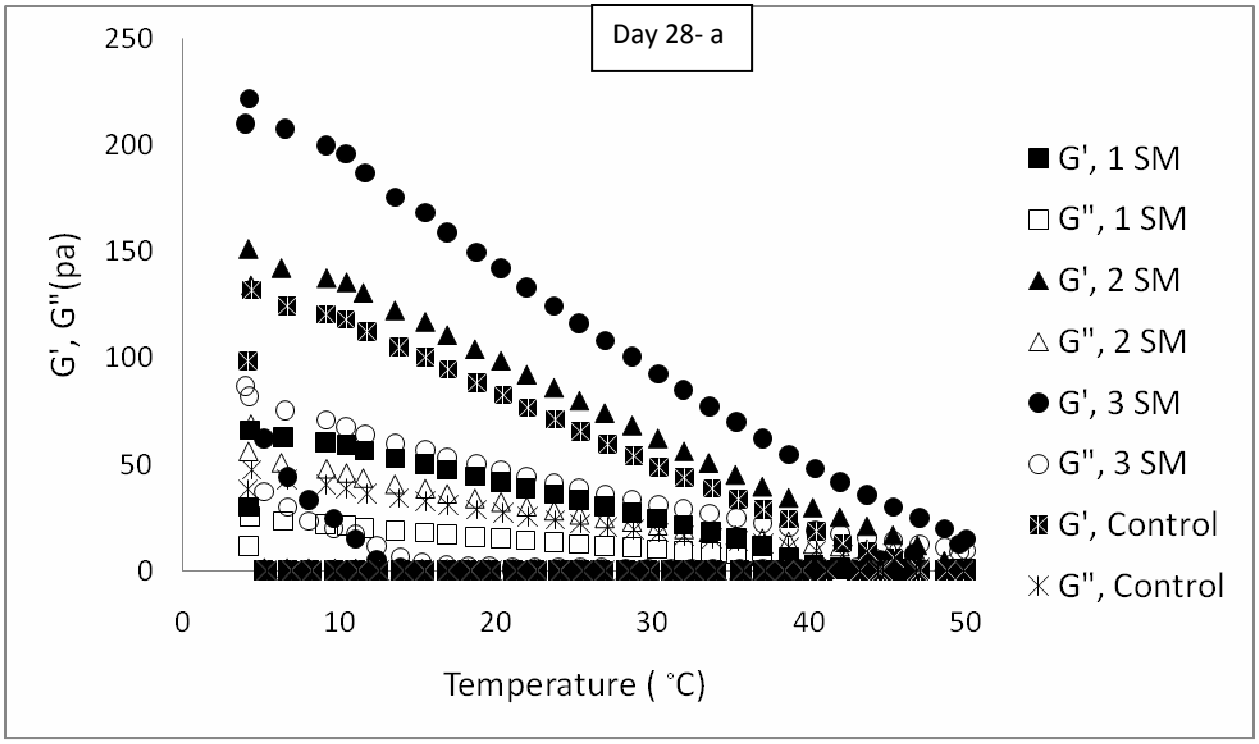


Figure 6.6 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of fermented products supplemented with (a) 1-3% skim milk and (b) 1-3% lentil flour heated from 4-50 ˚C and from 50-4 ^{o}C after 14 days of storage; (SM : skim milk, LF: lentil flour)



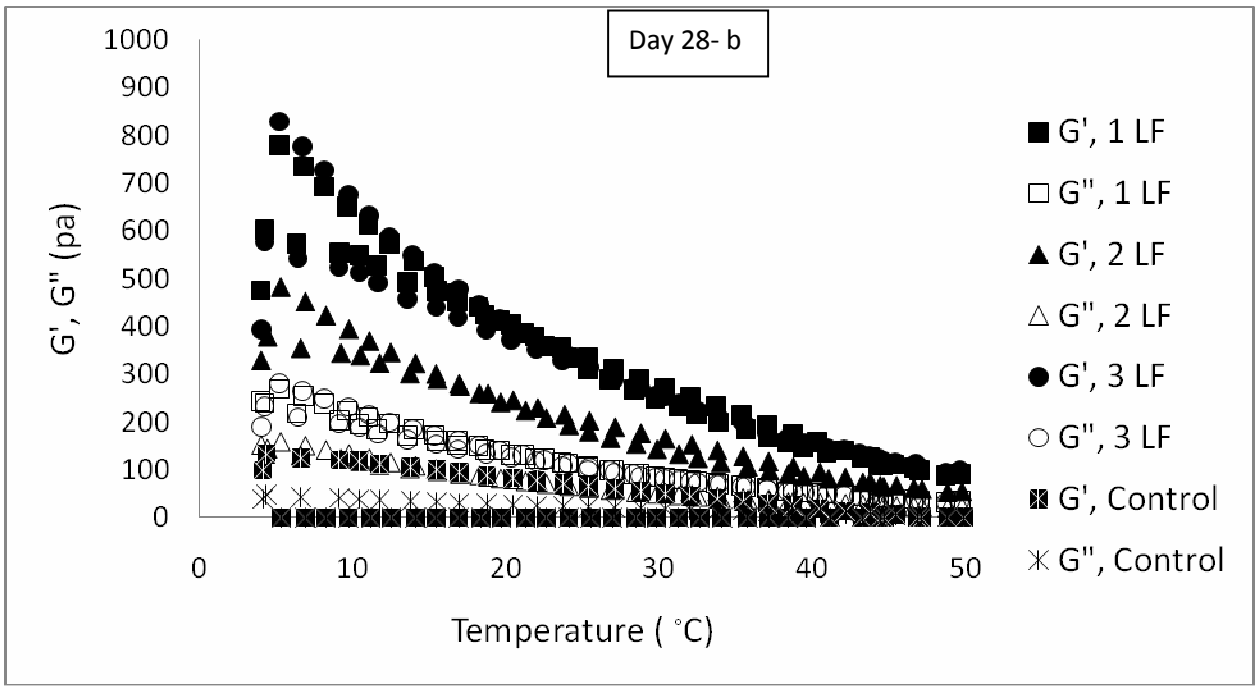


Figure 6.7- Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of fermented products supplemented with (a) 1-3% skim milk and (b) 1-3% lentil flour heated from 4-50 ˚C and from 50-4 ^{o}C after 28 days of storage; (SM : skim milk, LF: lentil flour)

6.4. Conclusion

This study showed that supplementation with 1-3% lentil flour in probiotic products results in a significantly faster lowering of the pH than did the 1-3% skim milk powder enrichment. The fermentation process was therefore significantly faster in 1-3% lentil supplemented probiotic samples. This may partially be attributed to the lower buffering capacity of lentil flour, as compared to skim milk as well as to the nutrient enrichment. Indeed, the viable counts of *L. rhamnosus* in the fermented products enriched by the lentil flour were higher than the non-supplemented control sample, for the same final pH level (pH = 4.5). The stability of *L. rhamnosus* during storage at 4°C for 28 days in lentil supplemented samples was at least as good as in skim milk supplemented probiotic samples. Our results also showed that probiotic supplementation with 1-3% lentil flour improved the physical and rheological properties of products in terms of the viscoelastic properties and syneresis and they minimally altered the color of probiotic sample in comparison with 1-3% skim milk supplemented and control probiotic after production and during storage. pH in 1-3% lentil supplemented probiotic was lower than 1-3% skim milk supplemented samples after 28 days storage, which could not solely be attributed to the lower buffering capacity of lentil supplemented media. In terms of syneresis, following 28 days of storage, lentil flour supplemented probiotic samples had lower syneresis than corresponding products enriched with skim milk powder. At any given level of supplementation, the G' and G" value of the lentil flour supplemented probiotic were higher than the skim milk supplemented probiotic and control sample. The highest G' and G" may be due to a stronger gel structure attributed to the high fiber and complex carbohydrate content of lentil flour.

Overall, on the basis of the microbial, physico-chemical and rheological properties investigations, our results suggest that lentil flour could be potentially considered as a source of prebiotic and texture improvement ingredients for supplementation in *L. rhamnosus* fermented milk products.

Connecting Statement to Chapter 7

In chapters 5 and 6, supplementation of yogurt and probiotic with lentil flour demonstrated the positive effect of lentil flour on growth and stability of the bacteria in fermented products. In addition, lentil flour supplementation improved the viscoelastic and syneresis properties of the supplemented food. Sensory properties of lentil flour supplemented yogurt were comparable for samples supplemented at 1-2%; skim milk supplemented yogurt, however, had more desirable acceptability for the panellist.

Results from the preliminary studies showed that selected pulse ingredients have beneficial effects on yogurt starters and probiotic bacteria in fermented milk which indicated that yogurt starters may grow better in milk supplemented with pea protein and pea fibre (chapter 4). Considering these results, further investigation to ascertain the effects of pea flour on the viable counts of yogurt starters as well as to determine effects on physical and sensory properties of final product was deemed necessary. In chapter 7, we investigated the effect of yogurt supplementation with 1-3% pea flour on acid production during fermentation, growth of yogurt starters, pH, syneresis, color, rheological and sensory properties of the final product immediately after production and during one month of storage. For comparison, yogurt (skim milk as the base media for yogurt formulation), was also supplemented with 1-3% skim milk powder and also analyzed for all aspects as well as a non-supplemented control yogurt. The results of this research have been presented as follow:

Zare F., Simpson, B.K., Champagne, C., Orsat, V., Boye, J.I., (2011). Yogurt supplementation with pea flour: Study of microbiological, physicochemical and sensory impacts*, Innovative Food Science and Emerging Technologies, (Subm*itted).

Chapter 7: Microbial, physical and sensory properties of yogurt supplemented with pea flour

Abstract

In this study, the effect of supplementation of milk with 1-3% pea flour or 1-3% skim milk powder on physical (pH, syneresis, and color), rheological (dynamic oscillation temperature sweep test) and sensory properties (flavor, mouth feel, overall acceptance and color) were studied during yogurt fermentation with starter cultures as well as during 28 days of cold storage (4˚C). Acid production, growth and stability of the yogurt culture strains were monitored, after production and after 28 days of cold storage (4˚C). Milk supplementation with 1-3% pea flour enhanced acid production during fermentation and pea flour supplementation resulted in slightly shorter fermentation times in comparison with other supplements. The populations (CFU) of *S. thermophilus* and *L. delbrueckii ssp bulgaricus* were in the same range in all pea flour and skim milk powder supplemented yogurts, after production and during 28 days of storage, while pea flour supplementation enhanced growth and stability of *L. delbrueckii ssp bulgaricus*. The average pH of yogurt samples decreased from 4.5 to 3.75 after 28 days of storage. pH reduction during storage was less in 1-3% skim milk supplemented yogurt in comparison with pea flour supplemented yogurt (1-3%). Syneresis was less at higher levels of supplementation for all samples, whereas in 2-3% skim milk-supplemented yogurts, the syneresis was significantly lower than all other samples. With respect to color values (“a”, “b” and “L”), after production and 28 days of storage, pea flour supplementation did not alter redness or greenness of yogurts, but the yellowness in pea flour supplemented yogurt was significantly higher than other samples. Pea flour supplemented yogurt had the same lightness as other samples after production and after 28 days storage. In terms of viscoelastic properties, yogurt with 1-3% pea flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the control sample. In all samples and at all temperatures between 4-50 ˚C, the storage modulus (G′) was higher than loss modulus (G″) and they showed a hysteresis loop over the temperature range. 1-2% pea flour

supplemented yogurt showed comparable sensory properties, except for flavour in 2% PF-yogurt; which was less desirable, in comparison with 1-3% skim milk powder supplemented yogurt and control sample.

7.1. Introduction

Yogurt is one of the most popular fermented dairy products in the world. It is produced by fermenting milk with *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*, which is the starter culture responsible for producing lactic acid. Due to pH reduction in the milk system, casein becomes unstable at its isoelectric point (pH = 4.5), and coagulates to produce a firm gel. The yogurt gel is composed of casein micelles, with whey entrapped within this matrix, which is interlocked by hydrogen bonds, forming a firm yogurt texture (Tamime & Robinson, 1999; Yang et al., 2006; Damin, Alcantara et al., 2009). According to Statistic Canada, Canadians consumed 5.4 liters of yogurt in their diet in 2008, more than twice as much as they had a decade ago. Yogurt consumption has been progressively increasing over the years, beginning with 0.03 liters in 1960, to reach 2.4 liters in 1998 and 5.4 liters in 2008 (STATCAN, 2011). Growth in yogurt consumption can be attributed to several factors, including the appreciation of yogurt's health benefits, availability of different options such as reduced fat yogurt and flavored yogurt, convenience packaging, and the influence of diverse cultural cuisines (AAFC, 2011).

Physical properties, especially texture, are amongst the most important attributes for yogurt quality. Texture is mainly affected by milk base solid content, protein content, heating conditions, starter culture and yogurt shearing after fermentation (Damin et al., 2009; Sodini, Remeuf, Haddad & Corrieu, 2004). To improve the physical properties of yogurt due to adequate protein and solids content, there are some mechanisms such as supplementation with protein powders (skim milk, whey protein concentrates, caseinates) or reducing water content by either evaporation of water from milk under vacuum or whey removal using membrane filtration. Protein supplementation, mostly whey protein and skim milk, is the most important parameter that establishes yogurt’s textural properties and several studies report

this method for texture improvement (Tamime, Robinson & Latrille, 2001; Lucey, Munro, & Singh, 1999; Remeuf, Mohammed, Sodini, & Tissier, 2003; Sodini, Montella, & Tong, 2005a; Damin et al., 2009). In addition to protein supplements, carrageenan, starch, pectin and other hydrocolloids have also been added to yogurt in order to enhance firmness and improve the texture. Pea flour, however, has not been examined for this purpose, and data are needed with respect to its potential to contribute to yogurt quality.

As supplementation influences the physical properties of yogurt, a variety of measurements therefore need to be studied. Various methods are available to assess physical attributes of yogurt such as viscoelastic properties, syneresis, color, etc.. Viscoelastic property measurements provide an understanding of the rheological properties of foods and give an estimate of the initial experience of a consumer in terms of mouth feel (Kealy, 2006). Several papers report the use of oscillatory tests to measure the rheological characteristics of yogurt (Ozer et al., 1997; Remeuf et al., 2003; Sodini et al., 2005b, Zare, Boye, Orsat, Champagne, & Simpson, 2011a). Syneresis, defined as the separation of whey from the yogurt without the application of an external force (Peng et al., 2009), is also an indicator of gel stability and so it is important to report for texture evaluation of yogurt, especially during storage. Color is another important physical property as it is a visual factor influencing product marketability and consumer acceptance. Yogurt as affected by supplements may have variable color values which can be represented by “L”, “a” and “b” values (Hashim et al., 2009). Although the physical properties can often be assessed using instruments, this is insufficient in characterizing the product, since they are related to sensory perception of food products (Sodini et al., 2004; Kealy, 2006). Most of the consumers rely on sensory attributes of foods to judge the freshness and quality of a product (Kealy, 2006) while modern food development practices require a clear understanding of the sensory aspects correlating measurements with sensory perception (Cruz, Cadena, Walter, Mortazavian, Granato, Faria, & Bolini, 2010). Sensory properties such as flavor, mouth feel and color can be assessed by trained or untrained panelists and consumer testing can provide the most meaningful and reliable information on the textural quality and

acceptability of yogurt (Jaworska et al., 2005). Therefore, in this study, both instrumental tests to assess physical properties and sensory testing using human panelists were performed.

Apart from the importance of physical properties of yogurt, there are several health benefits owing to the presence of viable microorganisms such as probiotics. Probiotics are live microorganisms which are consumed in large enough quantities in yogurt or supplements to improve the host's intestinal microbial population (Oliveira, Florence, Silva, Perego, Converti, Gioielli & Oliveira, 2009). In yogurt, probiotics have been demonstrated to enhance lactose digestion for individuals with lactose maldigestion. Stability of viable yogurt starters and probiotic cultures is an important factor to achieve desired health benefits with sustained consumption. However there is no universally accepted number of cells required to obtain a health benefit from yogurt microorganisms (Reid, 2008), although, according to the European Food Safety Authority, a minimum of 10^8 CFU per serving of probiotics is required in order to provide health benefits towards lactose digestion (EFSA, 2010). To maintain these numbers, it is important to follow the viability of yogurt starter microorganisms during manufacture and storage (Damin et al., 2009).

Canada is the world's largest producer and exporter of field peas, growing predominantly yellow and green types. Pea flour, pea protein, pea starch and pea fibre are some of the derived ingredients produced by the Canadian pulse industry (Pulse Canada, 2010). Canadian pea contains approximately 23.7% protein, 45.5% starch, 1.3% fat, 2.8% ash and the total mineral content of (mostly potassium, magnesium, phosphosus, calcium, iron, zinc and copper) 1.72%, which is a very good source of nutrients (Wang and Daun, 2004).

Preliminary data suggest that yogurt starters grow better in milk supplemented with pea protein and pea fibre which suggests a prebiotic potential (Zare et al., 2011b). In this study, therefore, we investigated the effect of yogurt supplementation with 1-3% pea flour (as a complex supplement of protein, fibre, carbohydrate, minerals and vitamins) on acid production during fermentation, growth of yogurt starters, pH, syneresis, color, rheological and sensory properties of the final product immediately after production and during 28 days storage. For

comparison, yogurt (skim milk as the base media for yogurt formulation), with and without supplemention with 1-3% skim milk powder was similarly prepared and analyzed.

7.2. Materials and methods

7.2.1 Production of yogurt

The non-fat skim milk powder used in this experiment was sourced from Agropur (Quebon brand; St. Laurent, QC, Canada); yellow pea flour was from Best Cooking Pulses Inc. (Rowatt**,** SK, Canada); Mixed yogurt starter cultures containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus (*Yogotherm M133) was from ABIASA Inc. (St. Hyacinthe, QC, Canada); the cultures were obtained in freeze-dried form, packaged in laminated foils. They were stored at 4°C until use. The experimental protocols used for yogurt supplementation and production are shown in Figure 7.1. Skim milk powder mixed in distilled water (9.5 % w/v) served as the base for supplementation and is referred to as the "control". In two series of experimental assays, 1-3% (w/v) of pea flour or 1-3% of skim milk powder was added separately to the skim milk base (control).

7.2.2 Product characterization

Acidification trends in yogurt were measured during yogurt fermentation according to the method described by De Brabandere & De Baerdemaeker (1999) using a FACS (Fermentation Acquisition and Control System) installed in a Forma Scientific (OH, US) programmable incubator. The buffering capacity of the different blends was estimated by acid titration and pH measurements using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada) and a 50 mL digital burette (Brinkmann Instruments Ltd., ON, Canada).

For viable counts, two culture media - acidified MRS agar (pH 5.2) from Difco Company (KS, USA) and M17 agar from Oxoid Company (ON, Canada) were used for quantifying the S*treptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus,* respectively, after production and during 28 days of storage at 4°C at 7 days interval. pH, syneresis, color, rheological and sensory

parameters were measured on the first day of production and during 28 days storage at 4 °C, at 7 days interval.

Syneresis was determined as the amount of spontaneous whey separation from yogurt according to the method described by Lucey, Munro, & Singh (1998), with some modifications. Volume of whey drained from 100 mL of undisturbed set yogurt prepared in cylindrical tube was measured and reported as percentage.

Color was determined as lightness (*L*))(0-black, 100-white), redness (+ve)/greenness (-ve) (a), and yellowness (+ve)/blueness (-ve) (*b*), using a colorimeter (Konica Minolta, CM-503 c, NJ, US).

Dynamic oscillation tests were conducted to determine the flow behavior and to characterize the viscoelastic properties of yogurt, using a rheometer (TA Instruments, SR-2000, DE, US) fitted with a 40-mm-diameter cone and 0.04 radian degree cone angle and plate geometry with a 4 mm gap. To ascertain the applicable stress and frequency in which storage modulus (G') and loss modulus (G") parameters of yogurt would demonstrate a linear constant rate, dynamic frequency ramp tests (frequency from 1 to 10 Hz and stress set at 3 Pa) and dynamic stress ramp tests (stress from 1 to 10 Pa and frequency set as 2.5 Hz) were conducted at 25 °C. Dynamic temperature ramp tests were done at a stress and frequency of 1.0 Pa and 2.5 Hz, respectively, in a temperature range of 4 to 50 °C (heating) and 50 to 4 °C (cooling), at a rate of 10 °C/min. Aliquots of the samples were carefully removed from the undisturbed yogurt cup and placed on the center of the rheometer plate; the top plate was slowly lowered on the top of the sample prior to analysis.

Sensory analyses (flavor, smoothness, graininess, overall acceptance and color) of the pea flour and skim milk powder supplemented samples as well as control sample were evaluated by 25 untrained panelists using a 9-point hedonic scale. Panelists were asked to score samples from extremely like (1) to extremely dislike (9). The sensory room was equipped with red light to blind the panelists to the color for the first 4 questions (flavor, smoothness, graininess and overall acceptance) and also white light for the question of color evaluation.

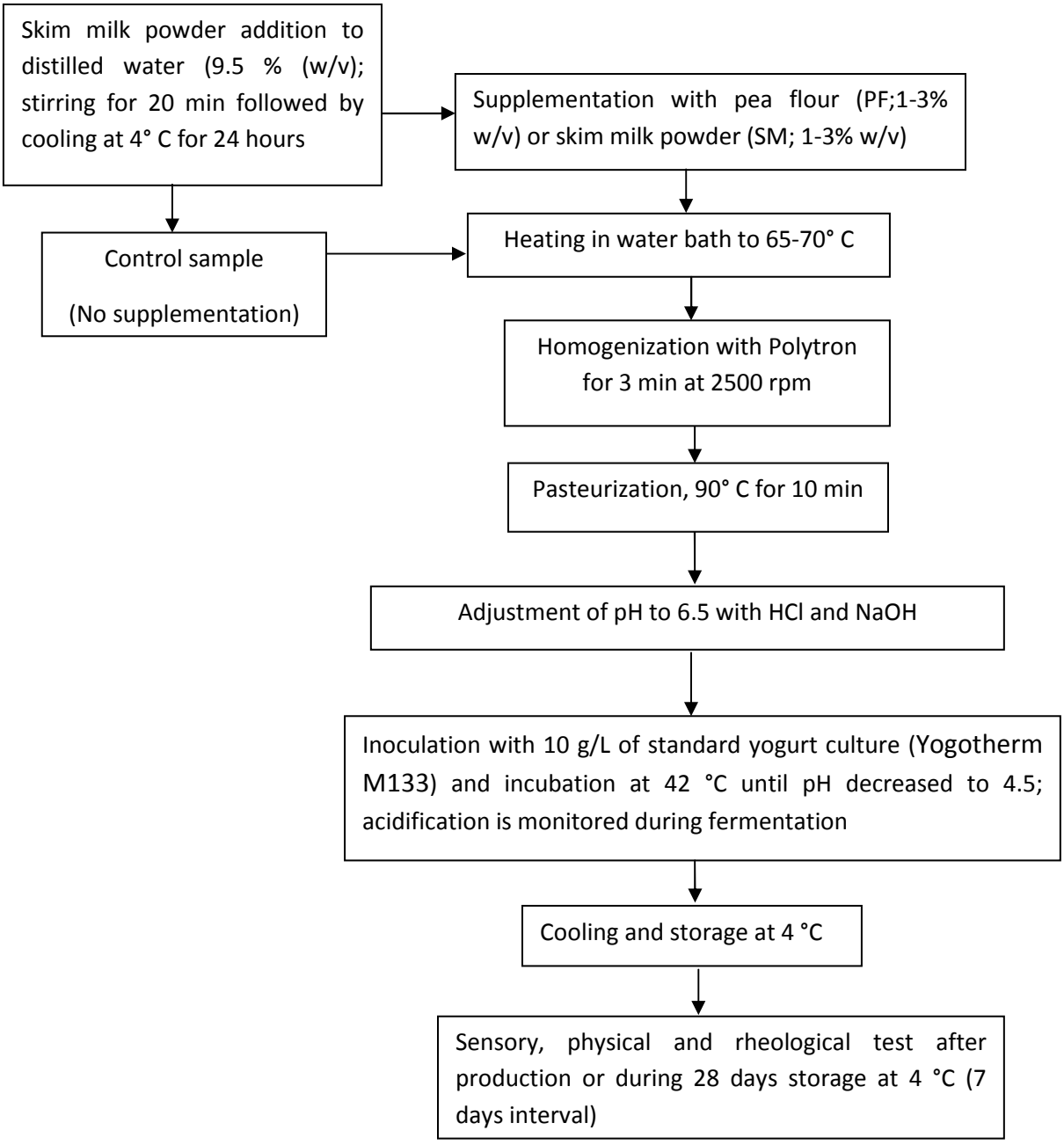


Figure 7.1- Schematic presentation of the process used for the preparation of the yogurt supplemented with skim milk powder (SM) and pea flour (PF) and the control yogurt (skim milk base with no supplementation)

7.2.3 Statistical analysis

Three independent repetitions were carried out. Statistical test was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two-ample t-test for comparison of two means.

7.3. Results and discussion

7.3.1 Acidification trend during yogurt fermentation

Figure 7.2 presents pH profiles as a function of incubation time during the acidification of yogurt supplemented with 1-3% pea flour and 1-3% skim milk powder as well as control yogurt. After 1 hour of incubation, the level of acidification was found to be statistically significant in 2% skim milk supplemented yogurt, followed by 3% pea flour supplemented yogurt ($p < 0.05$). The addition of 1 and 2% pea flour also resulted in faster acidification but this only became significant after 2.5 hours of incubation. After 3.5 hours incubation, the lowest pH was observed for the 1% and 2% pea flour supplemented yogurt. Milk has greater buffering capacity in comparison with pea flour (Table 7.1), therefore, the greater acidification rates in products supplemented with pea flour could simply be due to their lower buffering capacity when compared to corresponding skim milk supplemented yogurts. As was also observed for lentil, this was not the case when the pea flour data is compared with the control. Fermentation stopped after pH dropped to 4.5 in all supplemented yogurt and control samples, while the 1% and 2% pea flour supplementation lowered the pH about 15-45 min earlier compared with other supplements and control samples. This indicates that the addition of pea flour increased the acidification rate and the growth of the yogurt strains to a greater extent than was observed for the skim milk supplemented and control yogurt.

7.3.2 Microbial growth in yogurt after production and during storage

Nutritional benefits of yogurt and fermented dairy products depend on the viable counts of probiotics (10^8 CFU/serving (EFSA, 2010)), hence the viable counts of microorganisms were

monitored after fermentation and during storage (Table 7.2). Viable counts of *S. thermophilus* in pea flour and skim milk powder supplemented yogurt, varied from log 8.46 to 8.66, after production and between log 7.95 to 8.45 after 28 days of cold storage. These losses in viability over 28 days are rather small, and they were not statistically different (P = 0.78) between corresponding pea flour and skim milk supplemented samples. As a result, there was no significant difference between the viable count of *S. thermophilus* in pea flour supplemented yogurt and other samples, after production and 28 days storage (Table 7.2).

CFU values of *L. delbrueckii ssp bulgaricus* after fermentation were slightly lower than those of *S. thermophilus* and varied between log 7.95 to 8.66 (Table 7.2). All supplemented yogurt (1-3% skim milk and 1-3% pea flour) contained higher CFU values in comparison with control sample. The growth of *L. bulgaricus* was on the average 0.45 log CFU higher in pea flour supplemented yogurt as compared to the corresponding skim milk yogurt products, and this difference was statistically significant (P = 0.02). There were important losses in viability losses of *L. delbrueckii ssp. bulgaricus* during storage, particularly in the control samples (Table 7.2). This has been noted for many yogurt products and the phenomenon is strongly strain-related (Dave & Shah (1997a). Viability losses of the lactobacilli were generally greater than those noted for the *streptococci* (P = 0.01). The drop in *L. delbrueckii ssp. bulgaricus* CFUs during storage was greater in the skim milk supplemented yogurts than those in the pea flour supplemented products (P = 0.04). These finding suggest that the stability of *L. bulgaricus* in yogurt was significantly enhanced by pea flour supplementation. Thus, the higher *L. bulgaricus* CFU levels obtained in stored pea flour yogurt products were linked to greater growth during the fermentation as well as greater stability during storage.

In the 4 treatments based on milk, there was some correlation between the CFU data after fermentation and the buffering capacity of the milk but it was not high (R^2 of 0.57 and 0.80 for *streptococci* and *lactobacilli* respectively). Thus, in this range of milk solids, increasing solids level tended to generate higher populations in the fermented milk and this was partially related to an increased buffering capacity. Such a link is in agreement with the literature (Badran & Reichart, 1994). However, when the data on pea flour supplemented yogurts were combined with those of milk in the regression analyses, the R^2 values dropped to 0.03 (*streptococci*) and

0.01 (*lactobacilli*). Thus, when enriched milk with supplements of various sources is examined, the growth levels of the cultures are not directly associated with the buffering capacity of the media. These data show that the nutrients brought by the pea flour affect growth of the yogurt cultures differently than did skim milk powder. Generally it could be concluded that the addition of pea flour accelerated the rate of acidification, and also increased the CFU values in the fermented products (Table 7.2) when compared to skim milk powder supplementation. The nature of the stimulatory factors in pea flour has not yet been identified. As was the case for lentil, it is hypothesized that the presence of complex carbohydrates (e.g., resistant starch, sucrose, raffinose, stachyose, verbascose and oligosaccharides) made this ingredient a very good source of potential prebiotic components (Wang and Daun, 2004). Amino acids, vitamins and minerals have also stimulated the growth of starter cultures in milk (Smith et al., 1975).

Table 7.1- Amount of HCl (1 M) required to acidify 100 mL of 1-3 % pea flour or 1-3 % skim milk supplemented yogurt and non-supplemented control samples from pH 6.5 to 4.0 (SM: skim milk, PF: pea flour)

Sample	Titrable HCl (mL)
	Average ± SD
1 % SM	6.84 ± 0.00 d
2 % SM	7.58 ± 0.07 b
3 % SM	9.14 ± 0.00 a
1 % PF	6.42 ± 0.08 e
2 % PF	6.77 ± 0.11 d
3 % PF	7.15 ± 0.07 c
Control	6.38 ± 0.00 e

Means with the same letter are not significantly different ($P<0.05$)

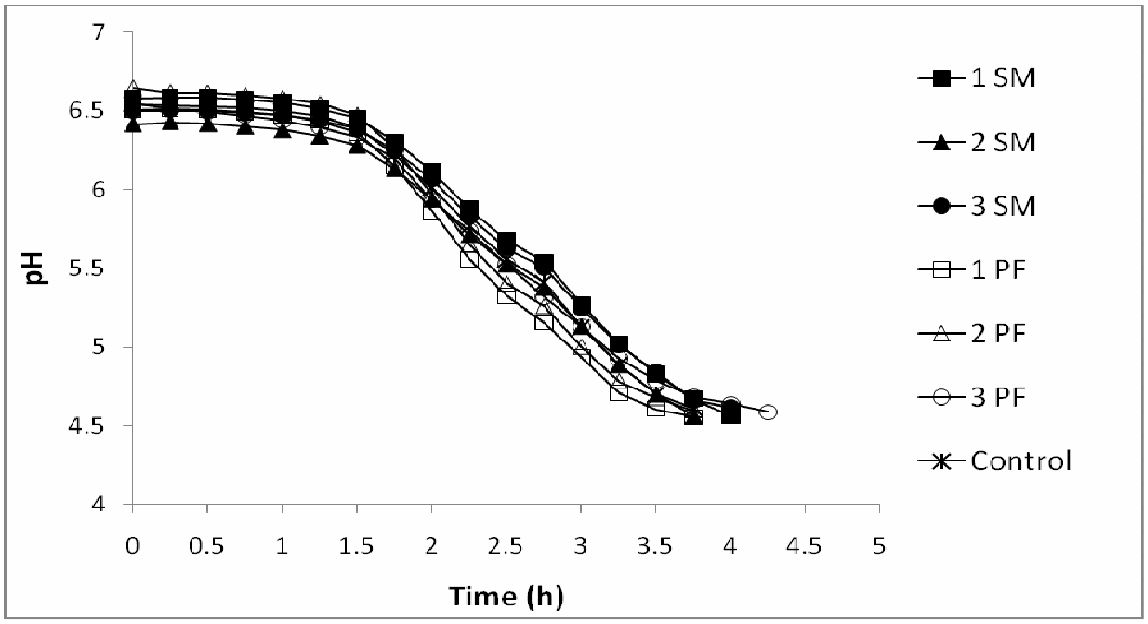


Figure 7.2- Change in pH as a function of incubation time during the acidification of yogurt supplemented with 1-3% pea flour and 1-3% skim milk powder as well as control yogurt (SM: skim milk, PF: pea flour)

7.3.3 Change in pH during storage

Table 7.2 shows the pH changes in pea flour and skim milk supplemented and control yogurt after 28 days storage. According to Kailasapathy et al., 2008, when the buffering capacity of yogurt system is higher, as the acid content increases, due to the starters' growth, the pH would not decrease or only decrease slightly. Our results indicate that after 28 days, pH dropped in all samples and the lowest pH was observed in 1-3% pea flour supplemented yogurt (P<0.05). Considering the buffering capacity of the samples (Table 7.1), relatively to the solid content; the pH reduction was slightly more in 1-3 % pea flour supplemented yogurt in comparison with 1-3% skim milk supplemented samples (P<0.05), This finding is in agreement with reports from Kailasapathy et al. (2008).

Table 7.2: Effect of milk supplementation with skim milk powder (SM) or pea flour (PF), on pH and viable counts of supplemented yogurt after the fermentation as well as after 28 days of storage at 4°C

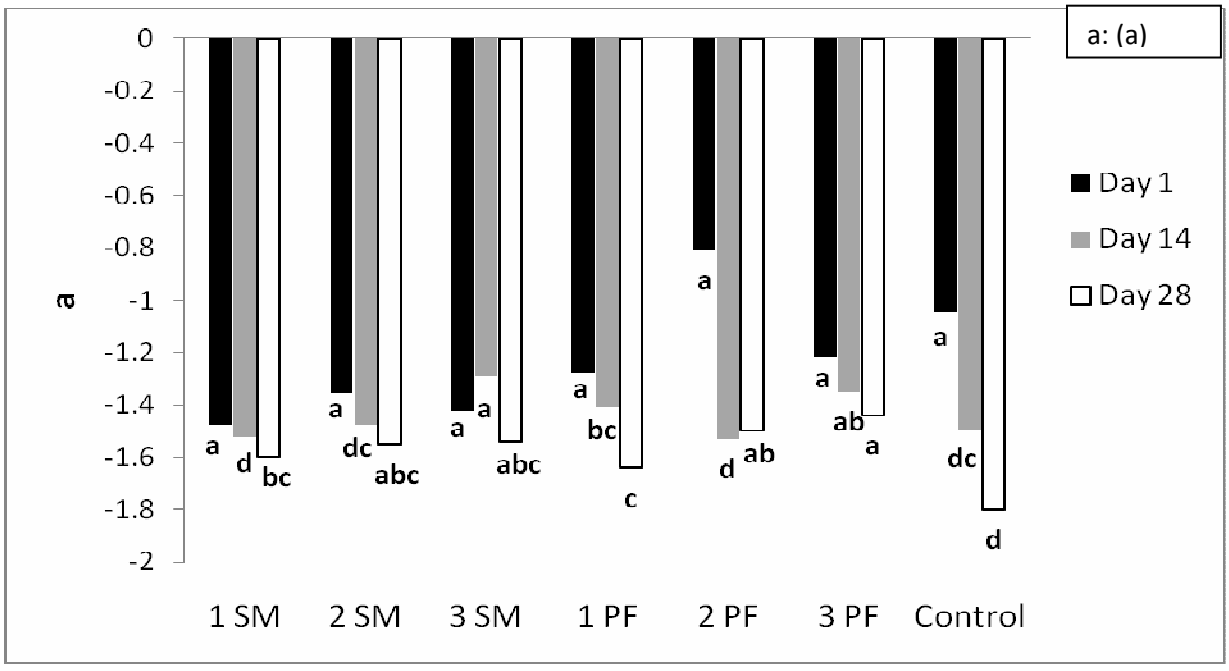
Medium	pH		*S. thermophilus* Log CFU/ mL		*L. bulgaricus* Log CFU/ mL	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
1% SM	4.55 ± 0.00 a	4.00 ± 0.01 a	8.52 ab	7.95 a	8.02 d	7.21 d
2% SM	4.56 ± 0.00 a	3.94 ± 0.01 b	8.60 a	8.36 a	7.95 d	7.08 de
3% SM	4.56 ± 0.01 a	4.00 ± 0.00 a	8.58 a	8.45 a	8.23 bc	7.21 d
1% PF	4.55 ± 0.00 a	3.81 ± 0.01 c	8.61 a	8.35 a	8.38 bc	7.66 bc
2% PF	4.57 ± 0.02 a	3.80 ± 0.01 c	8.65 a	8.41 a	8.51 ab	8.03 ab
3% PF	4.56 ± 0.01 a	3.75 ± 0.00 e	8.66 a	8.33 a	8.66 a	8.44 a
Control	4.55 ± 0.00 a	3.97 ± 0.01 d	8.46 b	8.07 a	7.79 e	6.60 de

Means with the same letter are not significantly different; for a given column ($P < 0.05$).

7.3.4 Color

To enhance marketability, the color of supplemented yogurt/probiotic products should not differ very much from regular products. Figure 7.3 (a, b and c), presents the color profile of the 1-3% pea flour and 1-3% skim milk supplemented yogurt and control yogurt at days 1, 14 and 28 after production. After production, there was no significant difference between “a” values in all pea flour supplemented yogurt and other samples ($P<0.05$), while “b” value showed highly significant differences, which varied between 1.48 to 2.9 in skim milk supplemented yogurt and 2.48 to 4.7 in pea flour supplemented yogurt ($P<0.05$). “a” and “b” values decreased slightly during storage in all samples. After 28 days storage, 1-3% pea flour supplemented yogurt had higher “a” and “b” values in comparison with control sample ($P<0.05$). “a” and “b” values

represent “red (+ve)-green (-ve)” and “yellow (+ve)-blue (-ve) hues” (Sanz et al., 2008). So, our results indicated that after production and 28 days storage, pea flour supplementation did not alter redness or greenness of the yogurt, but the yellowness in pea flour supplemented yogurt was significantly higher than for other samples. This result is expected since the yellow pea flour adds its original color to the yogurt. “L” values were in the same range; between 57.69 to 65.40, after production in all supplemented and control samples, except in 2% pea flour supplemented yogurt (P<0.05) where it increased slightly after 28 days storage. The “L” values were not significantly different (P<0.05) for skim milk and pea flour supplemented samples, except for the 3% skim milk. “L” value represents lightness (100) to darckness (0) (Sanz et al., 2008), and hence our results show that the pea flour supplemented yogurt had the similar lightness to other samples after production and after 28 days storage.



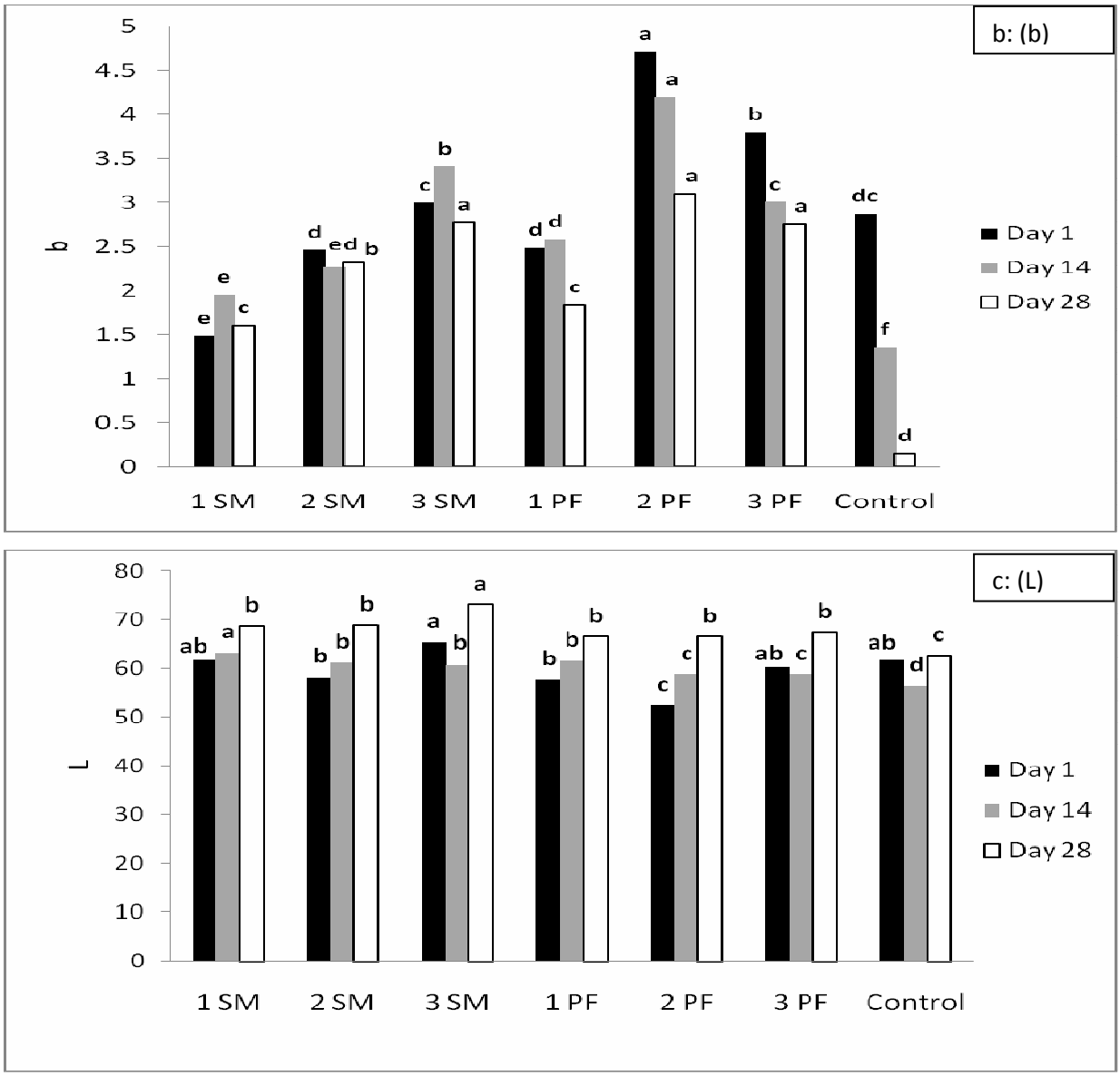


Figure 7.3- Color profile of yogurt supplemented with 1-3% pea flour and 1-3% skim milk and control sample after production and after 28 days storage; (SM: skim milk, PF: pea flour; a (a value) +ve red, -ve green; b (b value) +ve yellow, -ve blue; c (L value): -0 to 100, black to white)

7.3.5 Syneresis

Figure 7.4 presents the volume of water, separated from the yogurt gel in pea flour and skim milk supplemented yogurt and control samples, after production and after 14 and 28 days of

storage. The volume of water separating from the yogurt, without the application of an external force is known as syneresis. Our results show that, syneresis after production varies from 2.2 %- 3 % in skim milk supplemented samples, while they vary from 3.4 %- 6.5 % in pea flour supplemented samples. The higher level of supplementation resulted in significantly less syneresis in both skim milk and pea flour supplemented samples. After production, the best results were observed in yogurt supplemented with 3% and 2% skim milk supplemented yogurt (P<0.05). After 14 days and 28 days storage syneresis increased in all supplemented yogurt; however, skim milk supplemented yogurt still had the lowest syneresis. Syneresis in 1- 3% pea flour supplemented yogurt also, decreased over the storage period. After 28 days the resulrs vary from 2.5 %-3.1% for skim milk vs 3.2 %-8 % for pea flour supplemented samples. These results indicate more stability of the gel in skim milk supplemented yogurt in comparison with pea flour supplemented yogurt, after production and after storage.

Syneresis is related to the water holding capacity of the solid material in yogurt system. The water holding capacity attributed to the hydrophilic sites of protein molecules and fiber which could be affected by pH and processing treatments (Heller & Hackler, 1977; Lin et al., 1974). Several studies have reported that, the higher the solid content especially protein content or milk solids concentration, the higher the water holding capacity in yogurt systems with the gels being stronger and having lower syneresis (Sodini et al., 2004; Peng et al., 2009; Lucey, 2001). This confirms the decreasing rate of syneresis due to increasing the supplementation level in both group of skim milk and pea flour with supplemented yogurt. Fiber components could also strengthen the yogurt gel (Bozanic et al., 2001; Bozanic et al., 2002; Aportela-Palacois et al., 2005). In our study though, pea flour supplemented yogurt containing higher fiber, in comparison with skim milk supplemented yogurt; showed higher water separation. This finding could be attributed to the lower pH in supplemented pea flour or the processing treatment of the pea flour.

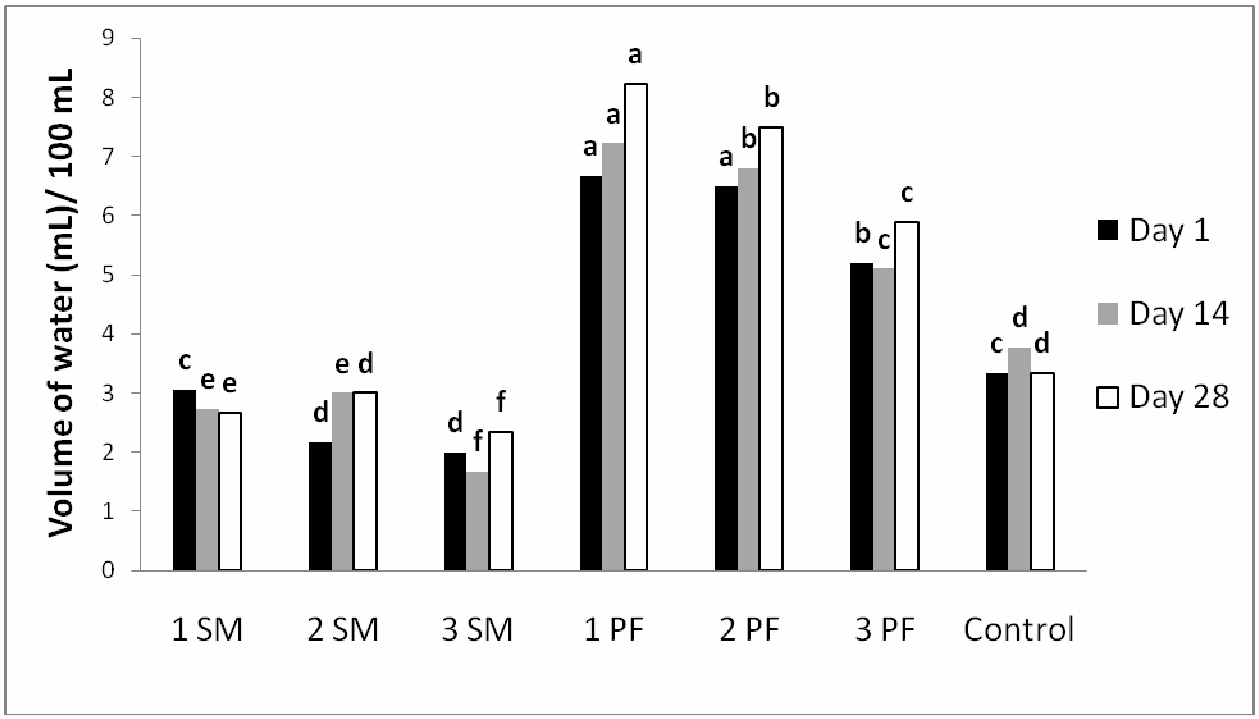


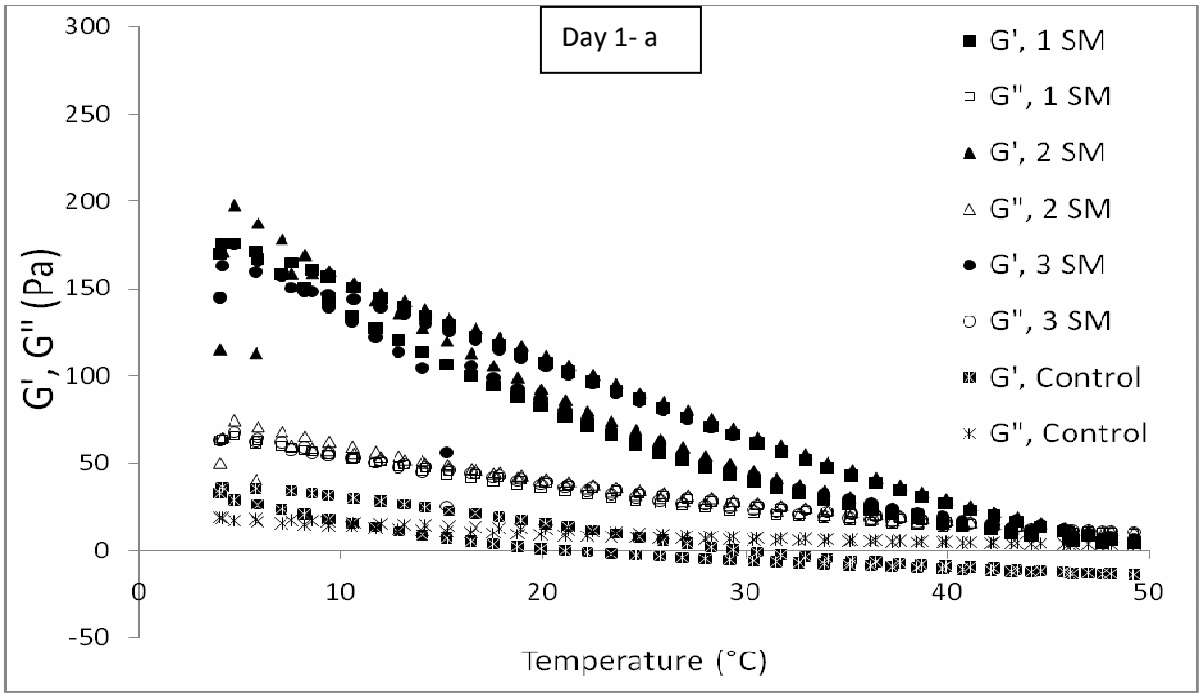
Figure 7.4- Syneresis in 1-3% pea flour and 1-3% skim milk supplemented yogurt and control sample during 28 day storage (SM: skim milk, PF: pea flour)

7.3.6 Rheological properties

Figures 7.5, 7.6 and 7.7 present the results of storage modulus (G′)(elasticity) and loss modulus (G″)(viscosity) as a function of temperature for the 1-3% pea flour and 1-3% skim milk supplemented yogurt and control samples at days 1, 14 and 28 of storage. In order to study the rheological behavior of yogurt when it is taken out of the refrigerator for consumption and then stored again, the dynamic temperature ramp test under conditions of changing temperature can be a useful test. Our results showed that all samples have predominantly an elastic behavior (G'>G") over the range of temperature studied (4-50 °C). A hysteresis loop was observed for G' and G" parameters, during heating and cooling and these parameters decreased with increasing temperature (4 to 50 °C) and increased back with decreasing temperature (50 to 4 °C). Total solid content, especially the amount and type of protein content, affect the rheological properties of yogurt. The higher solid content in yogurt, results in the

higher G’ and G” values and thus a lower deformation in yogurt gel. The supplementation level is, therefore, an important factor which can alter gel structure, and affect functional properties such as water absorption (Sendra et al., 2010, Sodini, at al., 2004). Our results also show that the higher percentages of supplementation resulted in higher values of G’ and G” in both groups of pea flour (1-3%) and skim milk powder (1-3%) supplemented samples. 2% and 3% pea flour supplementation in comparison with 1-3% skim milk powder and control sample, had significantly greater effects in increasing G’ and G” (P<0.05), after production and during storage. Storage period (after 14 days, and 28 days) was also an important factor with increasing G’ and G” for both skim milk and pea flour supplemented yogurts.

In regards to the effect of heating and cooling on the yogurt texture, our results showed that heating decreased G’ and G” as the temperature increased, until the lowest values was reached at 50 ^{o}C. After cooling back the samples (i.e; cooling from 50 to 4 ^{o}C) the pea flour supplemented yogurt, recovered its G’ and G” (G’ and G” increased again) to values which were very close to those observed during the heating process. However, skim milk supplemented yogurt behaved differently, suggesting that the texture of pea flour supplemented yogurt was less subject to change due to the heating process or temperature abuse in comparison with skim milk supplemented yogurt.



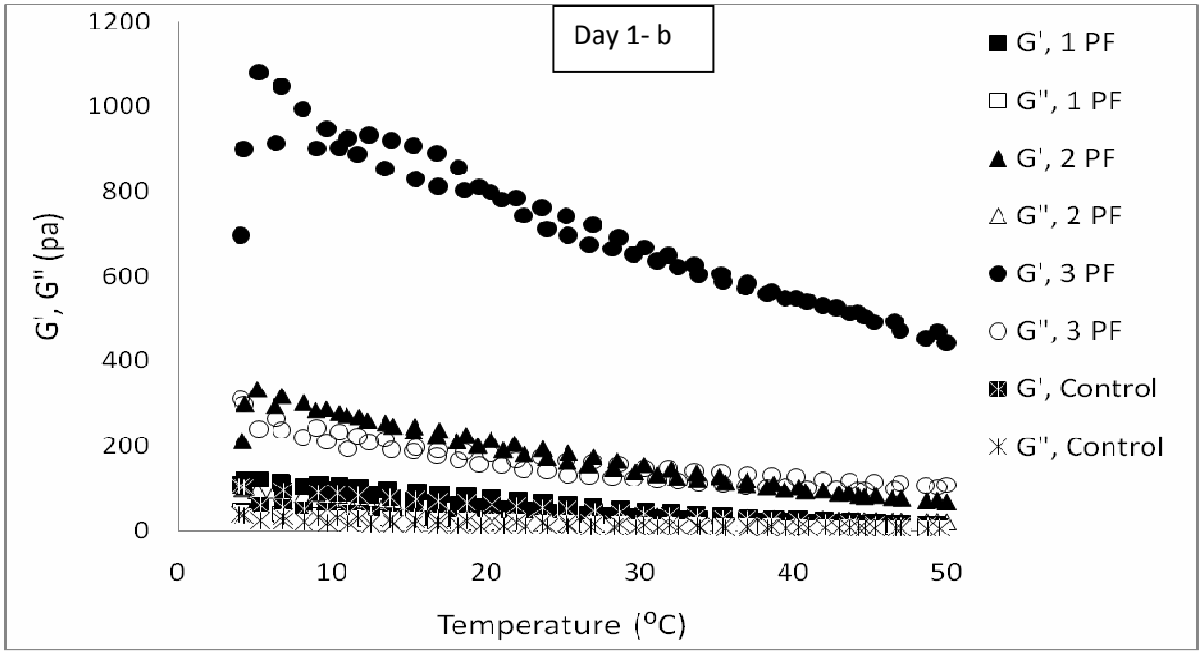
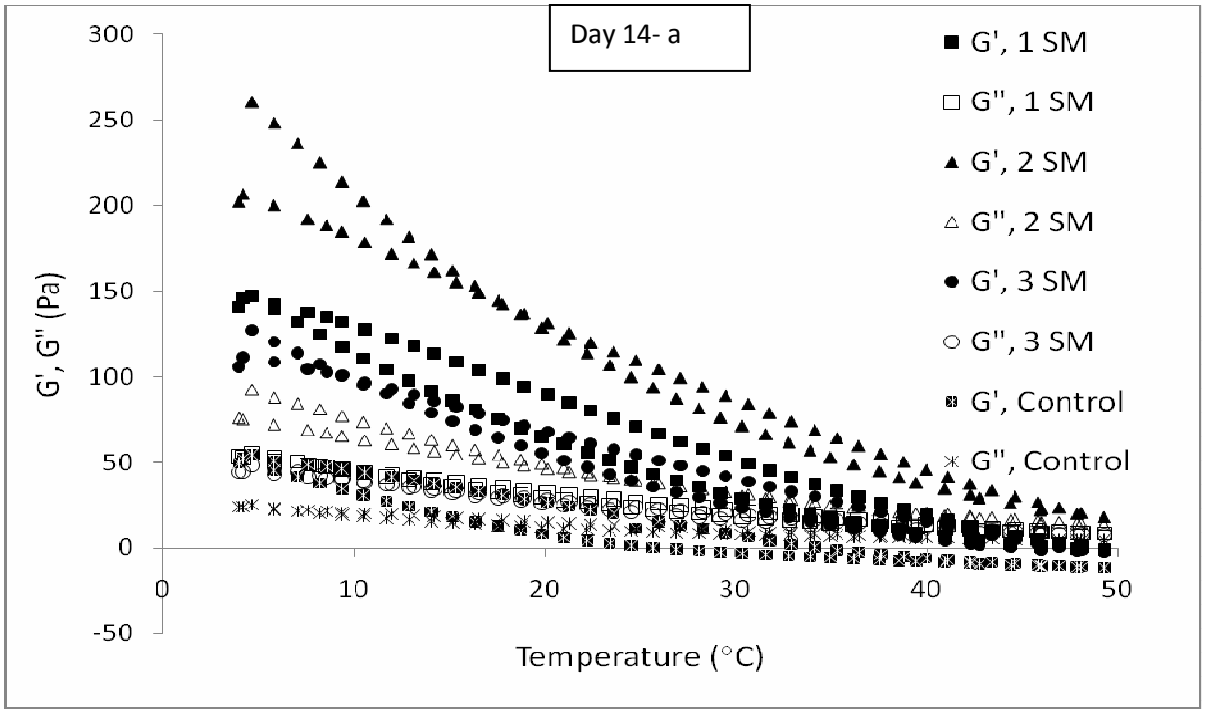


Figure 7.5 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of yogurt supplemented with (a) 1-3% skim milk and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 °C at Day 1; (SM: skim milk, PF: pea flour)



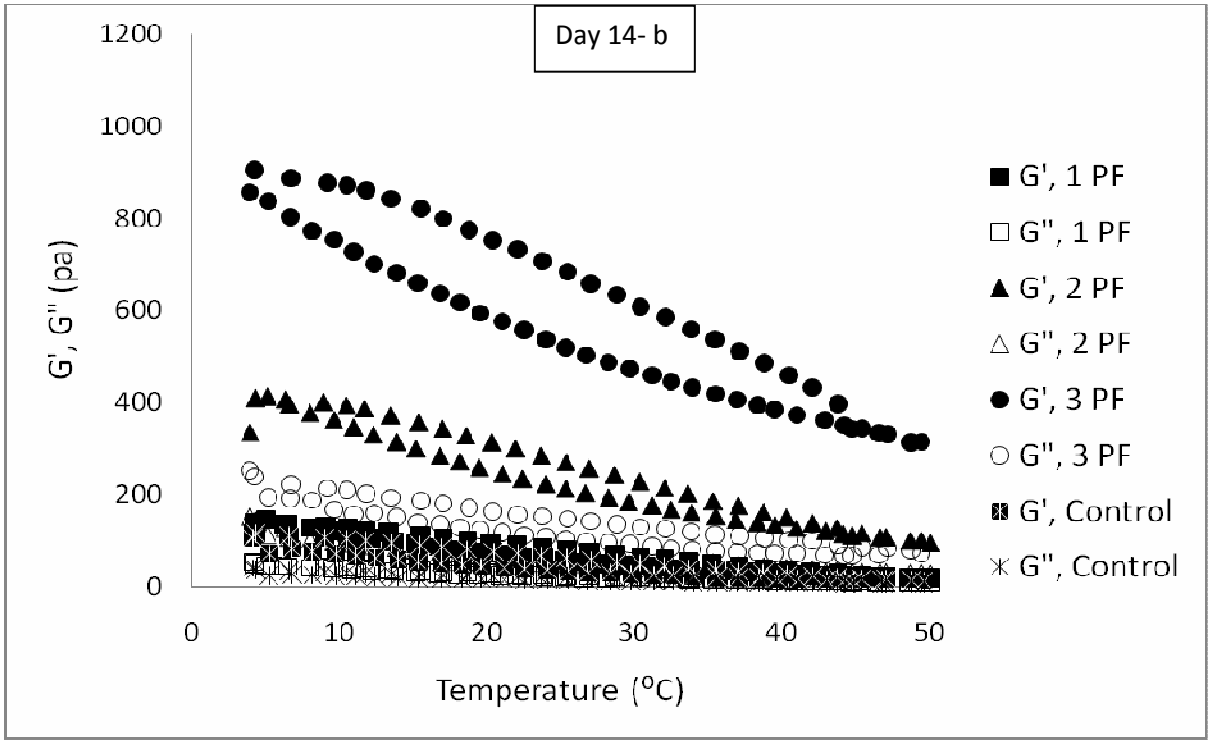


Figure 7.6 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of yogurt supplemented with (a) 1-3% skim milk and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 °C after 14 days of storage; (SM : skim milk, PF: pea flour)

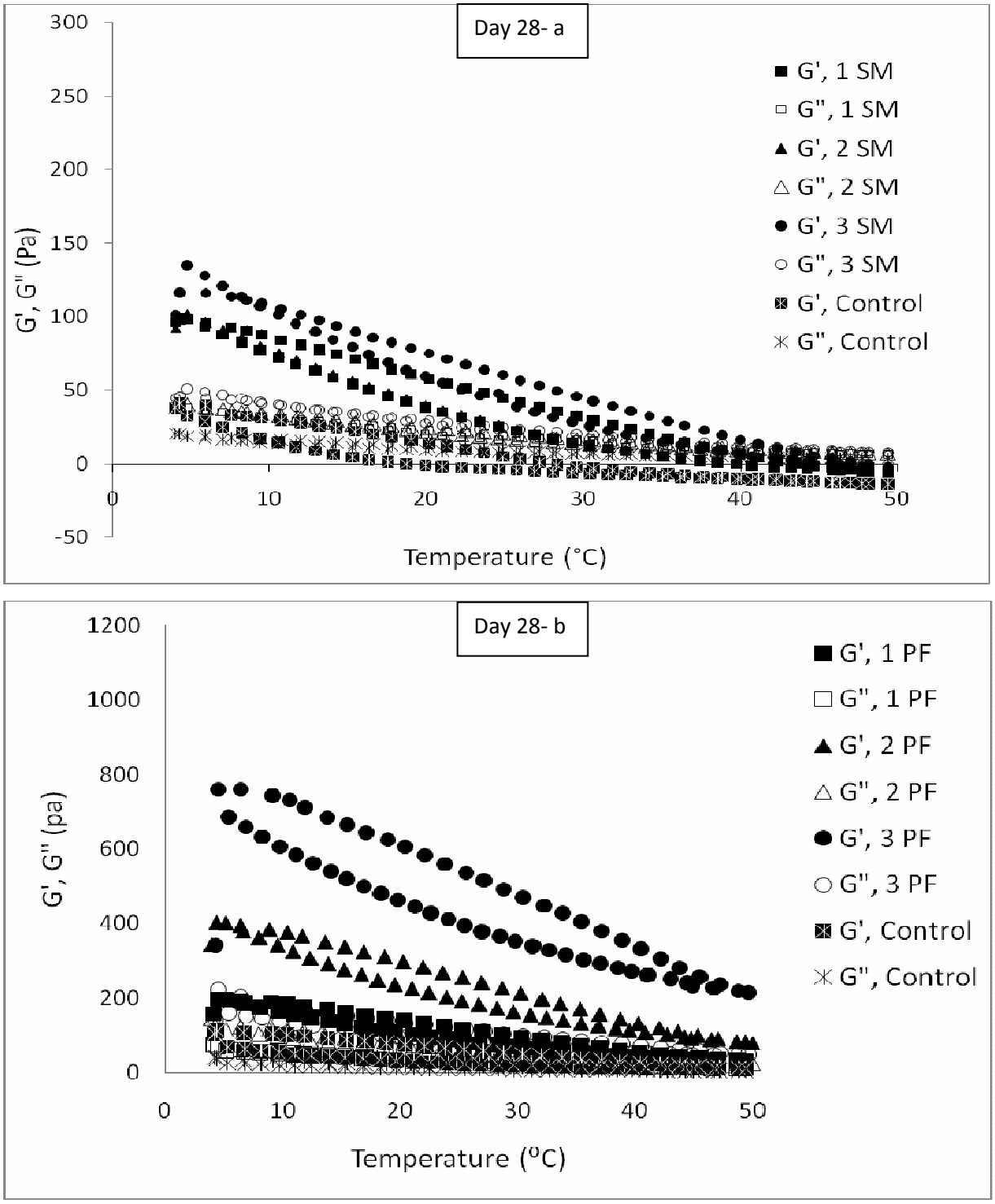


Figure 7.7- Storage (G′) (elasticity) and loss (G˝) (viscosity) moduli of yogurt supplemented with (a) 1-3% skim milk and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 ^{o}C after 28 days of storage; (SM : skim milk, PF: pea flour)

7.3.7 Sensory properties

The 1-3% pea flour, as well as 1-3% skim milk supplemented yogurt and control samples was evaluated in terms of smoothness, graininess, flavor, overall acceptance and color (Table 7.3). The lowest numbered scores represent the more desirable and the highest represent the least desirable samples (extremely like (1) to extremely dislike (9)). In terms of the acceptability of yogurt supplemented with different food ingredients, it was mostly expected that the supplementation would not change dramatically the sensorial aspects of the product. Published studies showed that the sensory properties of yogurt containing 1.5-4.5% of fiber such as inulin, wheat or bamboo fibers were not significantly different from the control yogurt (Aryana et al., 2007). Some other studies however, did not support the above report and showed that organoleptic quality of yogurt decreased with increasing inulin concentration (2-3%) in yogurt (Guven et al., 2005). Our results showed that pea flour supplementation changed the sensory properties of yogurt, but the differences were not significant ($P<0.05$, Table 7.3). In 1-3% skim milk supplemented yogurt, when the level of supplementation increased, the samples were ranked lower, meaning more desirable, but this was not the case for pea flour supplementation. 1% pea flour supplementation did not alter any sensory aspects of yogurt and was rated similar to 1-3% skim milk supplemented yogurt and control samples ($P<0.05$). When the level of supplementation increased to 2% pea flour, the supplemented yogurt was still ranked as desirable (comparable to the 1-3% skim milk supplemented and control sample) for all aspects, except flavor ($P<0.05$). Our results also showed that 3% pea flour supplemented yogurt was comparable to other samples in terms of smoothness and color, but not for graininess, flavor and overall acceptance ($P<0.05$). We can therefore conclude that 1-2% pea flour supplemented yogurts are comparable with 1-3% skim milk supplemented yogurt and control samples in terms of sensory properties, however some modification in the yogurt formula could help to improve all sensorial aspects and marketability especially at the 3% supplementation level.

Table7. 3- Sensory properties of control yogurt and yogurt supplemented with 1-3 % pea flour and 1-3 % milk powder (SM: skim milk, PF: pea flour)

Sample	Smoothness	Graininess	Flavor	Overall acceptance	Color
	Average ± SD	Average ± SD	Average ± SD	Average ± SD	Average ± SD
1 SM	2.72 ± 1.4 a	2.84 ± 1.40 b	3.40 ± 1.73 b	3.32 ± 1.37 b	3.00 ± 1.22 a
2 SM	2.64 ± 1.07 a	2.96 ± 1.51 b	3.00 ± 0.95 b	2.92 ± 0.75 b	2.80 ± 1.19 a
3 SM	2.52 ± 1.35 a	2.64 ± 1.52 b	3.32 ± 1.84 b	3.12 ± 1.64 b	2.52 ± 1.15 a
1 PF	2.68 ± 1.14 a	2.88 ± 1.16 b	3.76 ± 1.58 b	3.44 ± 1.32 b	2.72 ± 1.10 a
2 PF	3.12 ± 1.16 a	3.76 ± 1.47 ab	4.84 ± 1.92 a	4.08 ± 1.55 ab	2.96 ± 1.05 a
3 PF	3.12 ± 1.58 a	4.28 ± 2.07 a	5.40 ± 2.14 a	4.76 ± 2.02 a	3.04 ± 1.27 a
Control	3.00 ± 1.35 a	3.08 ± 1.15 b	3.40 ± 1.52 b	3.40 ± 1.25 b	3.12 ± 1.26 a

Means with the same letter are not significantly different; for a given column (P<0.05)

7.4. Conclusion

This study showed that acid production in 1% and 2% pea flour supplemented yogurt was significantly higher after 3.5 h fermentation in comparison with 1-3 % skim milk supplemented yogurt and control samples. Since milk has greater buffering capacity in comparison with pea flour, the greater acidification rates in products supplemented with pea flour could be due to their lower buffering capacity. The CFU/ml of *S. thermophilus* of all samples were not significantly different at the end of the fermentation process (log CFU/ml: 8.53-8.66), whereas the 2% and 3% pea flour increased significantly the CFU values of *L. bulgaricus* in comparison with other samples, after fermentation. pH decreased slightly in all samples after 28 days storage and color changed slightly in 1-3% pea flour supplemented yogurt. In terms of syneresis, the higher level of supplementation decreased the whey separation in 1-3% skim milk supplemented samples and 2-3 % pea flour supplemented yogurt had significantly less whey separation than all other samples. At any given level of supplementation, for both skim milk

and pea flour supplementation, all samples demonstrated greater viscoelastic properties compared to the control sample and the G' and G" moduli in pea flour supplemented yogurt were higher than skim milk supplemented yogurt and control samples. 1% pea flour supplemented yogurt was comparable with 1-3% skim milk supplemented yogurt and control samples in terms of smoothness, graininess, flavor and overall acceptance while for the 2% pea flour supplemented yogurt there was a significant change in flavor. Overall, on the basis of the microbial, physico-chemical, rheological and sensory properties investigations, the results suggest that pea flour could be potentially considered as a source of prebiotic and texture improvement ingredient for yogurt supplementation.

Connecting Statement to Chapter 8

Results from chapter 7, showed that yogurt supplementation with 1-3% pea flour enhanced acid production during fermentation, as well as the microbial populations (CFU) of both starter cultures *S. thermophilus* and *L.delbrueckii ssp bulgaricus*. Furthermore, in chapter 4 we demonstrated that pea protein and pea fiber have beneficial affects on probiotic cultures in particular *L. rhamnosus*, however the effect of pea flour on this probiotic microorganism has not been investigated. Since pea flour contains protein, carbohydrate and fiber, it could be a complex nutrient food for probiotic growth and so complementary studies were deemed necessary to investigate the effect of pea flour on the viable counts after production and during storage as well as on the physical properties of probiotic yogurt.

Therefore to follow up on our previous studies, skim milk (9.5 % w/v solid content) was supplemented with 1-3% (w/v) pea flour or skim milk powder, inoculated with *L. rhamnosus*, fermented and then stored at 4˚C. Acid production during the fermentation, microbial growth, physical properties (pH, syneresis, and color) and rheological properties (dynamic oscillation temperature sweep test at 4-50 ˚C), during 28 days of refrigerated storage were studied. For comparison, yogurt (skim milk as the base media for probiotic formulation), was also supplemented with 1-3% skim milk powder and analyzed for all aspects as well as a non-supplemented control. The results of this research have been presented as follow:

Zare F., Boye, J.I., Champagne, C., Orsat, V., Simpson, B.K., (2011), Supplementation of *L. rhamnosus* probiotic fermented milk with pea flour: Effect on microbial and physical properties, *Innovative Food Science and Emerging Technologies, (Subm*itted).

Chapter 8: Microbial and physical properties of probiotic fermented milk supplemented with pea flour

Abstract

In this study, skim milk (9.5 % w/v solid content) was supplemented with 1-3% (w/v) pea flour (PF) or skim milk (SM) powder, inoculated with *Lactobacillus rhamnosus* AD200 probiotic culture, fermented at 37 ˚C and stored at 4 ˚C. Acid production during the fermentation, microbial growth, physical properties (pH, syneresis, and color) and rheological properties (dynamic oscillation temperature sweep test at 4-50 ˚C), after production and during 28 days of refrigerated storage were studied. Milk supplementation with 1-3% pea flour enhanced acid production during fermentation, and the populations of *L. rhamnosus* were comparable with skim milk supplemented and non-supplemented control sample after production and after 28 days storage. At day 28, the CFU of 3% pea flour supplemented probiotic fermented milk was the highest followed by 2-3% SM and 1-2% PF supplemented samples. The average pH in all samples decreased from 4.5 to 4.04 over 28 days of storage. Syneresis in 1-3% pea flour supplemented probiotic was significantly lower than all other samples. With respect to color, pea flour supplementation slightly changed the color which was not as light as skim milk supplemented samples and there was more yellowness in the final product after production and storage. Probiotic fermented milk with 1-3% pea flour showed higher storage (G′) and loss (G″) moduli in comparison with samples supplemented with 1-3% skim milk powder and the non-supplemented control samples. Storage modulus (G′) was higher than loss modulus (G″) in all samples and at all temperatures between 4-50 ˚C and a hysteresis loop over this temperature range was observed when the samples were heated and cooled.

8.1 Introduction

Foods that contain some health-promoting component are known as functional foods. These foods, beyond their traditional nutrients, can supply additional health benefits and are sometimes also defined as medicinal foods, nutraceuticals, therapeutic foods, super foods,

foodiceuticals, medifoods and designed foods. Generally, a functional food is modified in some manner to confer 'health-functionality' (e.g., supplementation with nutrients or probiotic microorganisms) (Shah, 2007). Probiotics which originates from the meaning "for life" is defined as a food containing live microorganisms that would improve the health status of the host by balancing the gut's microflora (Araya et al., 2002; Fuller, 1992). Probiotics bacteria (and yeast) include *Lactobacillus, Leuconostoc, Pediococcus, Bifidobacterium,* and *Enterococcus*, but the main species believed to have probiotic distinctiveness are *L. acidophilus, Bifidobacterium spp.,* and *L. casei*. *Lactobacillus* and *Bifidobacterium* are the probiotics known as safe in the manufacture of dairy products and are also encountered as part of the normal gastrointestinal microflora. There are several human or animal clinical evidences on the health promoting effects of probiotics, which are either well established or promising; these include antimicrobial activity and reduced gastrointestinal infections, improvement in lactose metabolism, antimutagenic properties, anticarcinogenic properties, reduction in serum cholesterol, anti-diarrhoeal properties, immune system stimulation, improvement in inflammatory bowel disease and suppression of helicobacter pylori infection (Shah, 2007). The health benefits related to probiotics are strain specific, for instance it is reported that *L. rhamnosus GG (Valio), S. cerevisiae Boulardii (Biocodex), L. casei Shirota (Yakult),* and *B. animalis Bb—12 (Chr. Hansen)* can bring health improvements to human with respect to lactose malabsorption, rotaviral diarrhoea, antibiotic-associated diarrhoea, and *Clostridium difficile* diarrhea (Playne, Bennet, & Smithers, 2003; Shah, 2007).

Interests in consumption of probiotic products has increased in recent years, especially for probiotics incorporated in dairy foods, due to their reported potential health benefits. The level of consumer knowledge of different types of probiotics has risen in the last 5 years as a result (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). The global market for probiotic ingredients, supplements and foods was worth US$14.9 billion in 2007 and US $16 billion in 2008 (Granato et al., 2010). It is estimated that total sales of probiotic products would reach US$19.6 billion in 2013. Additionally, a survey of 2000 consumers in North-America conducted by the same authors found that 19% of adults in 2008 had purchased a pre/probiotic yogurt in the previous

3 months compared to 11% in 2006. Probiotic bacteria have the ability to survive passage through the gut and they seek to consume prebiotic foods in the colon to sustain their growth. Prebiotics are non-digestible food compounds (such as complex carbohydrates) that influence the host colon microflora beneficially by stimulating the growth and/or activity of one or a limited number of bacteria. There are some oligosaccharides which are not digestible by humans due to their chemical structure; these are either partly degraded or not degraded and, therefore, become available for fermentation by gut bacteria. These compounds (e.g., fructo-oligosaccharides, lactulose, lactitol, galacto-oligosaccharides, and soyabean oligosaccharides) play a role as energy source for bacteria. Some other components such as resistant starch and non-starch oligosaccharides are classified as colonic foods, but not as prebiotics, because they are not metabolized by certain beneficial bacteria in the colon (Ranadheera et al., 2007). "Synbiotic" products combine prebiotics and probiotics and it is expected that this association would enhance the health benefits of the related functional food. In terms of the use of *L. rhamnosus*, as a probiotic, several studies have showed that the growth and viability of the probiotics could be increased in yogurt or fermented milk products in the presence of some prebiotics such as resistant starch, inulin, fructooligosaccharides, polydextrose and oligofructose (Ranadheera et al., 2010). Regarding the marketability of a probiotic product, not only the nutritional benefits are important, but also the physical property of the probiotic food and the prebiotic ingredients may help to improve the physical property of the product as well as the nutritional properties. Various quality parameters of the final food products such as color, pH, visco-elasticity and texture could be improved by some plant-based matrices, such as the now widely used inulin from chicory. According to the literature, inulin and oligofructan derived from inulin improve viscoelastic properties, increase firmness and decrease syneresis of fermented milk products such as yogurt (Bozanic et al., 2001 and 2002; Debon et al., 2010; De Castro et al., 2008). Physico-chemical characteristics (pH, acid production, color and water activity) of fermented products such as yogurt, soy yogurt and probiotic fermented milk products, also have been reported to be improved by supplementation with inulin, resistance starch powder (Hi-maize), fiber and calcium, date fiber, β-glucan, glucose and raffinose. The improved sensory qualities noted with many prebiotics could be due to enhanced growth of

probiotics microorganisms as well as to their techno-functional properties (Donkor et al., 2007; Vasiljevic et al., 2007; Hashim et al., 2009).

Canadian peas contain approximately 23.7% protein, 45.5% starch, 1.3% fat, 2.8% ash and a total mineral content of 1.72% (mostly potassium, magnesium, phosphosus, calcium, iron, zinc and copper) (Wang and Daun, 2004). The carbohydrate content of pea flour is comprised of sucrose (2.8%), raffinose (0.7%), stachyose (2.7%), vebrascose (1.0%) and oligosaccharides (4.4%)(Wang and Daun, 2004). Our preliminary study showed that pea protein and pea fiber individually, improved the acidification rate of *L. rhamnosus* (Zare et al., 2011b), but no data were available on neither viable counts nor sensory propreties. In this study, therefore; the effect of supplementation of fermented milk with 1–3% (w/v) pea flour on acid production during fermentation, on growth of *L. rhamnosus*, pH, syneresis and color of the final product immediately after production and during one month of storage has been investigated. For comparison, skim milk as the base media for fermentation was also supplemented with 1–3% (w/v) skim milk powder and analyzed for all quality aspects as well as a non-supplemented control probiotic sample.

8.2. Materials and methods

8.2.1 Cultures and ingredients

Non-fat skim milk powder was purchased from Agropur (Quebon brand; St. Laurent, QC, Canada); pea flour was obtained from Best Cooking Pulses Inc. (Rowatt**,** SK, Canada); *Lactobacillus rhamnosus* AD200 was purchased from ABIASA Inc. (St. Hyacinthe, QC, Canada); the cultures were obtained in freeze-dried form, packaged in laminated foils and stored at 4°C until use. Skim milk powder rehydrated at 9.5% solids (w/v) served as the base for supplementation and will be referred to as the "control". In two series of experimental assays, 1-3% (w/v) of pea flour or 1-3% of skim milk powder was added separately to the 9.5% skim milk base (control). The heat treatments and processing parameters of the various milk-based blends are presented in Figure 8.1.

8.2.2 Fermentation

The culture contained a microbial concentration of 2 x 10^{11} CFU/g. It was re-hydrated at 37 ^{o}C in the sterilized skim milk to obtain 2 X 10^{9} CFU/mL. Subsequently 1 mL of this dilution was added to 100 mL media which represented an inoculation level of approximately 2 x 10^{7} CFU/mL. The experimental protocols used for probiotic supplementation and production are shown in Figure 8.1. Acidification trends in fermented milk by *L. rhamnosus* were measured during fermentation according to the method described by De Brabandere & De Baerdemaeker (1999) using a FACS (Fermentation Acquisition and Control System) installed in a Forma Scientific (OH, US) programmable incubator.

8.2.3 Product characterization

The buffering capacity of the different blends was estimated by acid titration and pH measurements using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada) and a 50 mL digital burette (Brinkmann Instruments Ltd., ON, Canada). For viable counts culture media; MRS agar from Difco Company (KS, USA) was used for quantifying the *L. rhamnosus.* Viable counts were obtained after 48 hours incubation at 37°C in aerobic conditions.

pH was measured in the probiotic fermented milk using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada).

Syneresis was determined as the amount of spontaneous whey separation from the fermented product according to the method described by Lucey et al., 1998, with some modifications. The volume of whey drained from 100 mL of undisturbed set fermented milk prepared in cylindrical tubes was measured and reported as percentage of syneresis.

Color was determined as lightness (*L*), red/greenness (a), and yellow/blueness (*b*), using a colorimeter (Konica Minolta, CM-503 c, NJ, US).

Dynamic oscillation tests were conducted to determine the flow behavior and characterize the viscoelastic properties of yogurt, using a rheometer (TA Instruments, SR-2000, DE, US) fitted with a 40-mm-diameter cone and 0.04 radian degree cone angle and plate geometry with a 4 mm gap. To ascertain the applicable stress and frequency in which storage modulus (G') and loss modulus (G") parameters of yogurt would demonstrate a linear constant rate, dynamic frequency ramp tests (frequency from 0.0 to 10 Hz and stress set at 3.0 Pa) and dynamic stress ramp tests (stress from 1 to 10 Pa and frequency set as 0.3 Hz) were conducted at 25 °C. Dynamic temperature ramp tests were done at a stress and frequency of 3.0 Pa and 0.3 Hz, respectively, in a temperature range of 4 to 50 °C (heating) and 50 to 4 °C (cooling), at a rate of 10°C/min. Aliquots of the samples were carefully removed from the undisturbed yogurt cup and placed on the center of the rheometer plate; the top plate was slowly lowered on the top of the sample prior to analysis. Viable counts, pH, syneresis, color and rheological parameters were measured after fermentation as well as during 28 days storage at 4°C at 7 days intervals.

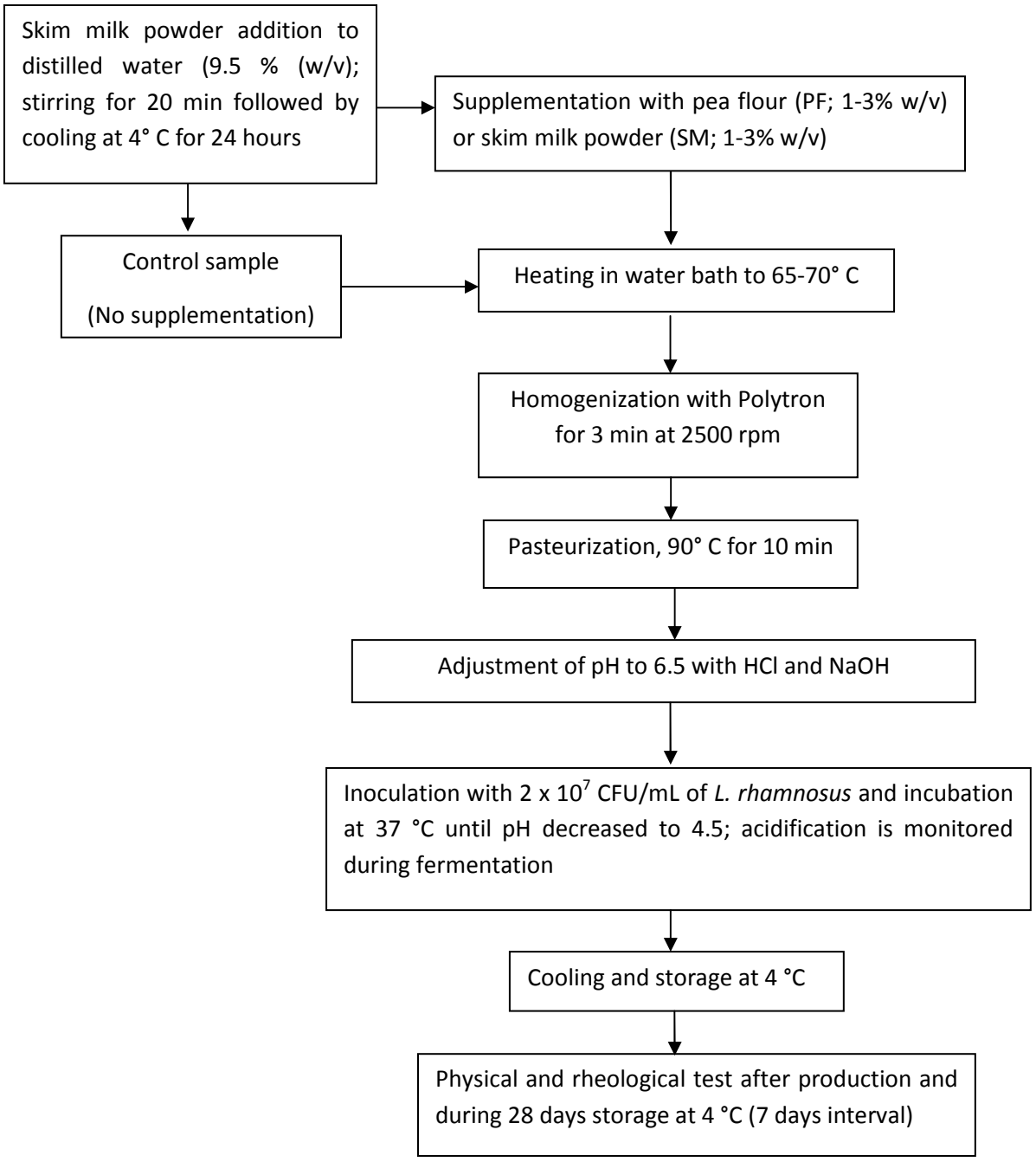


Figure 8.1- Schematic presentation of the process used for the preparation of probiotic supplemented milk (*L. rhamnosus)* with skim milk powder (SM) or pea flour (PF) and the control sample (skim milk base with no supplementation)

8.2.4 Statistical analysis

Statistical test was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two sample t-test for comparison of two means.

8.3 Results and discussion

8.3.1 Acidification by *L. rhamnosus*

According to our findings, presented in Figure 8.2; the acidification rate of the fermented milk was enhanced by supplementation with both skim milk powder and pea flour. The results show that pea flour supplementation made the pH reduction process faster in the probiotic fermented milk in comparison with control sample and skim milk supplemented samples. There was a significant difference in pH between the supplemented media and the control sample after 5 hours of incubation, especially for 2-3% skim milk and 2-3% pea flour supplemented probiotics (P<0.05). At this point (h=5), the pH in the 2% and 3% pea flour supplemented products was significantly lower than 1% pea flour, 1% skim milk supplemented and control sample. After 12 hours of incubation, all supplemented samples (both groups of skim milk supplemented and pea flour supplemented) showed a pH significantly lower than control sample. At this point (h=12), 3% pea flour-supplemented probiotic had the lowest pH followed by 2% and 1% PF- supplemented samples which were significantly lower than skim milk supplemented samples (P<0.05). After 18 hours of fermentation, the rate of acid production was still higher in 1-3% pea flour supplemented samples in comparison with control sample. Since the fermentation is terminated at pH= 4.5, our data showed that 3% pea flour supplemented probiotic reached the pH 4.5 significantly earlier than 1-3% skim milk supplemented and control samples (Figure 8.2). This finding constitutes important time and energy savings in the manufacturing process. These data are in line with those noted previously (Zare et al., 2011b).

Buffering capacity can influence the pH reduction in milk supplemented media and its impact, therefore, needs to be considered (Ranadheera et al., 2010). According to Table 8.1, since milk has greater buffering capacity in comparison with pea flour, the greater acidification rates in products supplemented with pea flour could have been partially due to their lower buffering capacity when compared to the corresponding skim milk supplemented probiotic products. Indeed, the pea lour supplemented products have a higher buffering capacity than the control (Table 8.1), but their drop in pH is nevertheless more rapid (Figure 8.1). However, it was not the case when comparing pea flour supplemented milk and non-supplemented control sample. Acid production in the media, which is mainly lactic acid, is often linked to the growth of lactic acid bacteria (i.e: *L. rhamnosus)* (Tamime & Robinson, 1999). The significant acidification data for pea flour supplementation thus indicates that the growth of *L .rhamnosus* appears to have been stimulated by pea flour. To better understand the microbial growth during acidification, the evolution of the viable counts (CFU) during the fermentation process may be studied. In this study, the CFU analyses were carried out after production and during storage.

Table 8.1- Amount of HCl (1 M) required to acidify 100 mL of 1-3 % pea flour and 1-3 % skim milk from pH 6.5 to 4.0 (SM: skim milk, PF: pea flour)

Sample	Titrable HCl (mL)
	Average ± SD
1 % SM	6.84 ± 0.00 d
2 % SM	7.58 ± 0.07 b
3 % SM	9.14 ± 0.00 a
1 % PF	6.42 ± 0.08 e
2 % PF	6.77 ± 0.11 d
3 % PF	7.15 ± 0.07 c
Control	6.38 ± 0.00 e

Means followed by the same letter are not significantly different ($P < 0.05$)

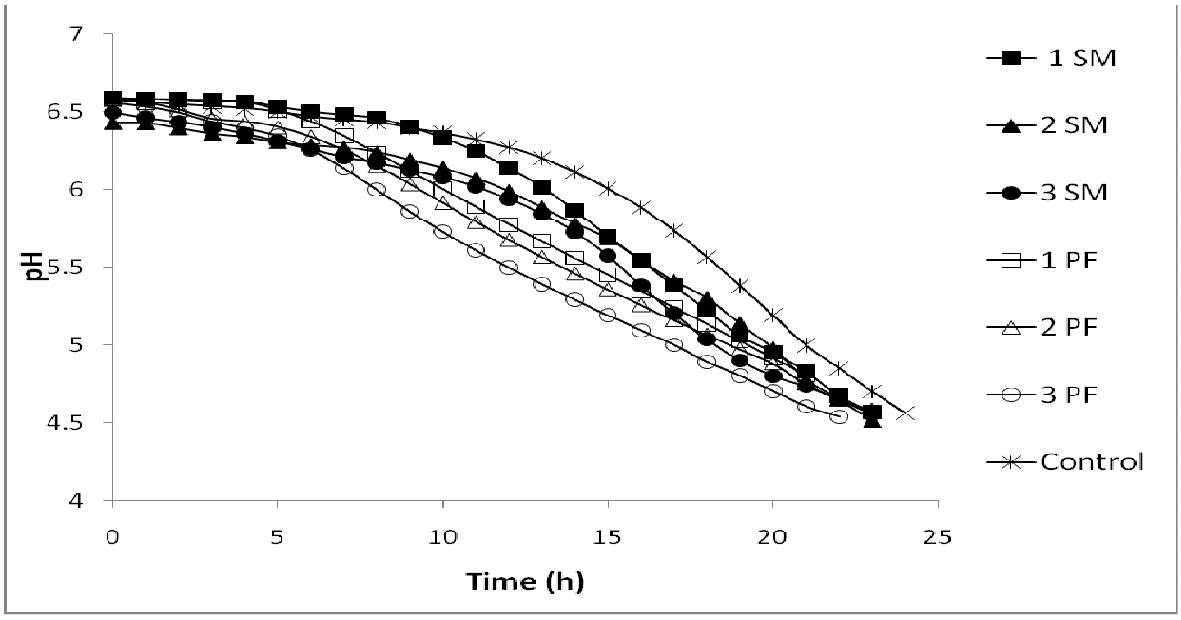


Figure 8.2- Effect of supplementation of skim milk (9.5 % solids), with 1 to 3% pea flour (1 PF, 2 PF and 3 PF treatments) or 1-3% skim milk (1 SM, 2 SM and 3 SM treatments) on acidification by *L. rhamnosus* AD200

8.3.2 Microbial growth and survival in supplemented product after production and during storage

There is no universally recognized number of probiotic bacteria in foods which guarantee a health effect (Reid, 2008). This is why, to provide the health benefits from the consumption of probiotic food products, several proposals of bacterial amounts are found. Thus, as a function of the food or the strains it is suggested that 10^6 CFU/g (Granato et al., 2010), 10^8 CFU per serving (EFSA, 2010) or 10^9 CFU per portion CFIA, 2009) of a probiotic culture be the minimum concentration in the fermented product. To maintain these numbers, it is important to follow the probiotic viability during manufacture and storage (Damin et al., 2006). Viable counts of *L. rhamnosus* in fresh (day 0) products supplemented with pea flour or skim milk powder varied from log 8.11 to log 9.25, while it decreased over 28 days storage between 0.4 and 0.76 log

CFU/mL (Table 8.2). According to our results, supplementation with 1 to 3% pea flour and 1 to 3% skim milk powder significantly improved *L. rhamnosus* CFU levels in the fresh products and after 28 days cold storage, in comparison with non-supplemented control probiotic. It is noticeable that 1-3% pea flour had even greater effect to increase viability since there was on average 0.39 log CFU/mL higher viable counts of *L. rhamnosus* at day 0 in comparison to the equivalent 1-3% skim milk powder-supplemented products; this difference was statistically different (P = 0.04) in a paired t test analysis. However, the highest decreases in CFU's of *L. rhamnosus* during the 28 day storage period tended to occur in the pea flour supplemented products (P = 0.052). Overall, pea flour supplemented probiotic products still showed higher CFU's in comparison with skim milk supplemented and control samples during storage (Table 8.2). For both supplements, there was a good correlation between the CFU values at Day 0 and the milk supplementation level (R^2 = 0.91 for PF and 0.94 for SM). It is noteworthy that pea flour supplementation accelerated both the rate of acidification and CFU values in the fermented probiotic products as compared to skim milk powder supplementation and control sample (Figure 8.2 and Table 8.2).

The growth level of *L. rhamnosus* in fresh pea flour supplemented media gave comparable values to those obtained for in fruit-based media, which were above 10^9 CFU/mL (Champagne & Gardner, 2008). Since milk is not the optimal media for growth and acidification of *L. rhamnosus* (Gaudreau et al., 2005), the growth of *L. rhamnosus* or other probiotic in milk-based media is slow, and may result in rather low CFU counts (Champagne et al., 2010). Therefore, our data on growth and acidification with *L. rhamnosus* in non-supplemented milk are in agreement with that of the literature. Results of this study showed that, although there were CFU reductions in pea flour supplemented samples after 28 days of storage, all of the samples still could supply the minimum 1 billion per portion (100 mL) amount of probiotic required for a non-strain related health claim (CFIA, 2009). Furthermore, all PF-enriched samples had values above the 10^8 CFU/ mL threshold, even after 28 d of storage. These results show that many products had comparable viable counts as those found in commercial high-density probiotic

products such as DanActive™ (Danone) or BioBest Maximmunité™ (Parmalat), which contain 10 billion CFUs per portion.

It could be suggested that the growth improvement of *L. rhamnosus* by pea flour is due to the stimulatory factors in pea flour. However, more research is required to understand what exact component could affect L*. rhamnosus*, and it is hypothesized that pea flour could serve as a prebiotic source due to its nutrients' content such as resistant starch, raffinose, stachyose, verbascose and oligosaccharides favouring the growth of *L. rhamnosus* in probiotic yogurt type product (Wang and Daun, 2004). Other non-prebiotic components such as minerals, sucrose, or protein could also be involved. Therefore, it could be suggested that pea flour supplemented probiotic product could be considered as a very good nutrient source providing both prebiotics and probiotics for human diet.

A regression analysis was carried out to ascertain the role of the buffering capacity of the ingredients (Table 8.1) on the viable counts obtained at the end of the fermentation (Table 8.2). In separate groups of milk supplemented and pea flour supplemented samples there was a positive correlation (R^2 = 0.79 and 0.71, respectively) between the CFU data after fermentation and the buffering capacity of the supplemented samples. This high correlation data suggests that, for *L. rhamnosus* AD200, while total solid content increases, the buffering capacity is a strong regulator of growth in this range of milk solids and this correlation is stronger for skim milk supplemented samples. The importance of the buffering capacity and biomass levels is in agreement with the literature (Badran & Reichart, 1994). However, data on acidification rates showed a different picture. One would expect that the higher buffering capacity would slow the rate of pH reduction. A regression analysis between pH values at time = 18 h (hours) and the buffering capacity of the media showed absolutely no correlation (R^2 < 0.01). Therefore the acidification rate itself is completely independent of the buffering capacity of the ingredients, and is presumably directly linked to nutrient content.

8.3.3 Change in pH during storage

Acid production by *L. rhamnosus* causes the pH reduction during fermentation at 37℃, and continues during storage at 4℃, which is known as post acidification (Shah, 2000). So there was an expected pH difference after 28 days storage in the probiotic samples as shown in Table 8.2. According to our results, in all samples, the pH decreased slightly by 0.03 to 0.47 unit over the 28 day storage period. pH reduction was greater in 1-3% pea flour supplemented products than 1-3% skim milk supplemented and control samples. Regarding the level of supplementation, it is shown that in each group of supplementation, either with skim milk or pea flour; there is no significant difference between pHs after 28 days (P<0.05), while there is a significant difference between pea flour supplemented and skim milk supplemented samples as well as control sample.

The data was examined to determine if there was a link between the pH data and the buffering capacity or viable counts (Table 8.1). There was no correlation between the pH at the end of storage and the buffering capacity of the medium or with the viable counts at the beginning of storage (R^2 < 0.1). These data are in line with other studies which show that acidification during storage is not necessarily parallel to CFU counts (Seo et al., 2009). According to Kailasapathy et al., 2008, the higher the buffering capacity of media, the smaller the pH changes due to the changes in acid content of the food system. This was not the case in our study; the skim milk control had the lowest buffering capacity (Table 8.1) but was the product having the most stable pH during storage (Table 8.2). Supplementation with pea flour therefore seems to increase the acidifying ability of the lactobacilli during storage, in addition to its stimulation of the fermentation rate at 37℃ (Figure 8.2).

There was a concern with respect to the stability and viability of the probiotics during storage, because a higher buffering capacity of the medium tends to increase the survival of live culture bacteria (Kailasapathy et al., 2008). The viable counts in the pea floursupplemented products dropped on the average by 0.67 log CFU/g while that of the skim milksupplemented milks had viability losses of approximately 0.52 by log CFU/g (Table 8.2). This difference was not quite statistically significant (P = 0.052) but it does suggest a trend. It can be hypothesized that the

greater drop in pH during storage observed in the pea flour supplemented products could have generated this higher viability loss. Indeed, Kailasapathy et al. (2008) reported a correlation between the post-storage pH in yogurts and the survival of probiotic bacteria.

The pH of milk-based products does affect their texture. Therefore, analyses on color and texture were carried out on the fresh products as well as on the stored ones.

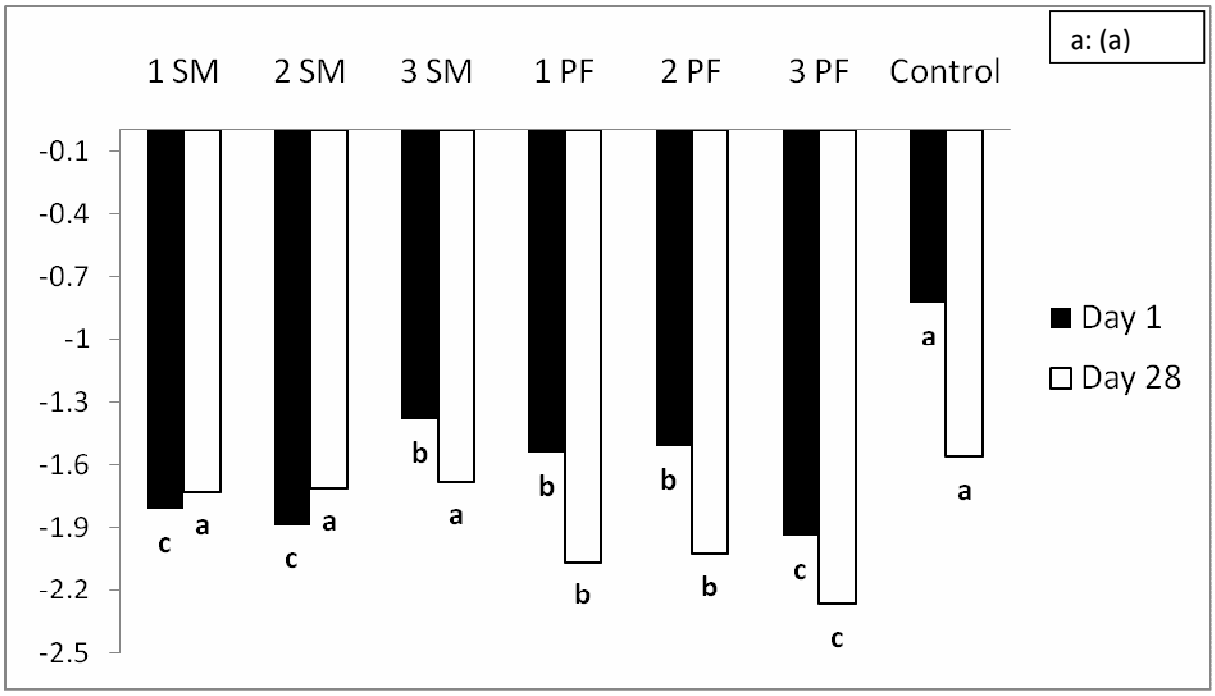
Table 8.2: Effect of milk supplementation (9.5 % solids), with skim milk powder (SM) or pea flour (PF), on viable counts and pH of *L. rhamnosus* after the fermentation and during 28 days of storage at 4°C

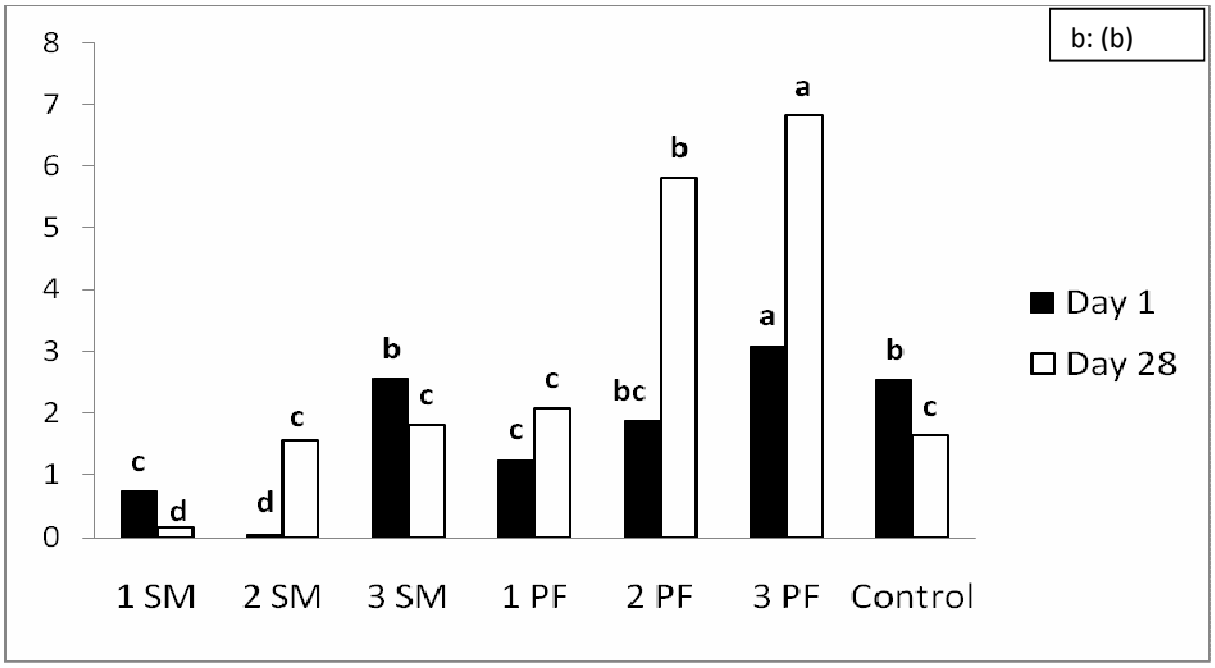
Medium	***L. rhamnosus* (Log CFU/mL)**				**pH**	
	Day 0	Day 7	Day 14	Day 28	Day 0	Day 28
1% SM	8.44 e	8.28 d	8.22 d	7.96 d	4.57 a	4.44 a
2% SM	8.55 d	8.51 bc	8.28 d	8.02 b	4.51 a	4.42 a
3% SM	8.71 c	8.58 a	8.35 c	8.15 b	4.53 ab	4.42 a
1% PF	8.77 c	8.47 c	8.17 f	8.11 bc	4.51 b	4.04 b
2% PF	8.85 b	8.57 a	8.60 b	8.09 c	4.54 ab	4.11 b
3% PF	9.25 a	8.53 ab	8.70 a	8.66 a	4.56 a	4.12 b
Control	8.11 f	8.10 e	7.96 e	7.71 e	4.56 a	4.53 a

Means followed by the same letter are not significantly different; for a given column ($p < 0.05$)

8.3.4 Color

In this study, color was measured by colorimetery. Ideally, the color of the supplemented products should not vary much from the control and should remain unchanged after production and during storage. Figure 8.3 (a, b and c), presents differences in the color (a, b and L values) of the 1-3% pea flour and 1-3% skim milk supplemented probiotic and control samples at days 1 and 28 after production. On the first day of production 1% and 2% pea flour supplemented samples had significantly lower "L" values in comparison with 1-3% skim milk supplemented samples, and 1-2 % skim milk supplemented sample showed the highest "L" values followed by 3% pea flour supplemented sample (P<0.05). This could be due to the presence of pea hulls in the pea flour which made the product darker than the skim milk supplemented samples. The lowest "a" value was observed in 1-2% SM and 3% PF supplemented samples, followed by 1-2% PF and the 3% SM supplemented sample and then control sample (P<0.05). The highest "b" value was observed in 3% pea flour supplemented samples, followed by 2% PF and 3% SM supplemented and control sample (P<0.05). Surprisingly, after 28 days, "L" values were highest in the 1-3% pea flour supplemented samples in comparison with all other samples (P<0.05) and the level of supplementation appeared to increase this value in PF supplemented samples. "a" value for samples stored for 28 days was the lowest in 3% PF supplemented sample followed by 1-2% PF supplemented sample and 1-3% SM supplemented and the control sample (P<0.05). The "b" value, on the other hand, was the highest in 3% PF supplemented sample, followed by 2% and then 1% PF and 1-3% SM supplemented and control sample (P<0.05). "L" value represents lightness (100) and blackness (0); "a" value represent red (+ve) to green (-ve) hue and "b" value represent yellow (+ve) to blue (-ve) hues (Sanz et al., 2008). So the color measurements indicated that after production, pea flour supplemented samples were not as light as skim milk supplemented samples, however, after 28 says storage the lightness of the PF supplemented samples improved and was closer to all other samples. Also after production and following 28 days of storage, 1-3% pea flour supplemented probiotic had more yellowness and more greenness in comparison with other samples, which was expected since the pea flour has a yellow hue.


a: (a)
1 SM
2 SM
3 SM
1 PF
2 PF
3 PF
Control
-0.1
-0.4
-0.7
-1
-1.3
-1.6
-1.9
-2.2
-2.5
Day 1
Day 28


b: (b)
8
7
6
5
4
3
2
1
0
1 SM
2 SM
3 SM
1 PF
2 PF
3 PF
Control
Day 1
Day 28

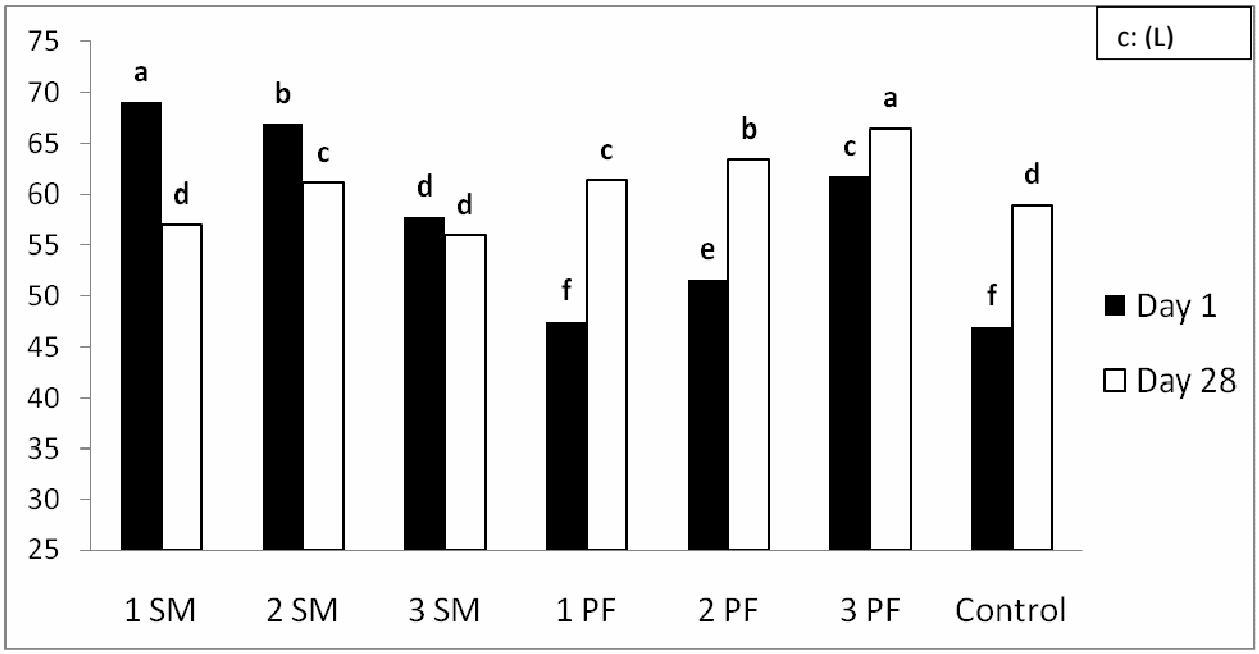


Figure 8.3- Color profile of fermented products supplemented with 1-3% pea flour or 1-3% skim milk and control sample after production and 28 days storage; (SM: skim milk, PF: pea flour; a (a value) +ve red, -ve green; b (b value) +ve yellow, -ve blue; c (L value): -0 to 100, black to white), a,b,c,d,e,f: for a given storage time, means followed by the same letter are not significantly different ($p < 0.05$)

8.3.5 Syneresis

Syneresis provides an indication of the non-homogeneities in the gel formed during the acidification and coagulation of proteins in the fermented milk system. A higher water separation (syneresis) is related to higher gel instability (Lucey et al., 1998). The syneresis of pea flour and skim milk supplemented probiotic and control samples are presented in Figure 8.4, immediately after production and after 14 and 28 days of cold storage. According to our results, on day 1, the highest syneresis was observed in 1% skim milk supplemented and control samples, followed by 2% and 3 % skim milk, 2% and 3% pea flour supplemented samples ($P<0.05$). It could be suggested that the level of supplementation in both skim milk and pea

flour supplemented samples, significantly affects the syneresis (P<0.05). After 14 and 28 days of storage, the level of volume separated from the gel had increased in all samples in comparison with the first day of production. At day 14, the least syneresis was measured in 1-3% pea flour supplemented probiotic followed by 2% and 3% skim milk supplemented samples and then 1% SM and control sample (P<0.05). After 28 days of cold storage, syneresis was the least in 3% pea flour supplemented samples, followed by 1% and 2% pea flour and 3% SM, 2% SM and 1% SM supplemented and control sample (P<0.05).

Overall, our results showed that 1-3% pea flour supplementation could significantly improve the gel stability compared to 1-3% skim milk supplemented and control samples. The greater changes in syneresis in PF samples after 14 and 28 days, were potentially due to decreases in pH, since the acid production was highest in the pea flour supplemented products during storage (Table 8.2) and according to Tamime and Robinson (1999), greater acidification causes more initial water separation from the gel, thus having less water to lose during storage. It could be suggested that syneresis is directly linked to acidification during storage. Also, it is noticeable that an increase in the total solid content, especially protein content, starch and fiber as hydrocolloid structural compounds, results in stronger and more homogenous texture and less water separation (Peng et al., 2009; Lucey, 2001). Therefore, the ability of pea flour to decrease the syneresis could be due to its higher hydrocolloid content which favours both probiotic activity (acidification) and water holding capacity.

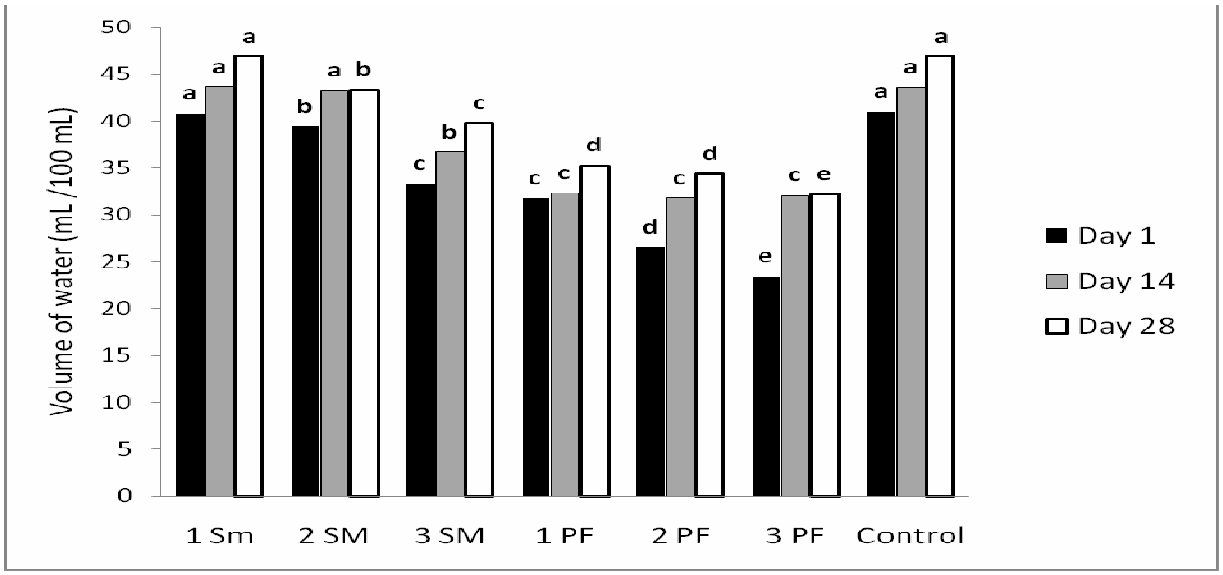
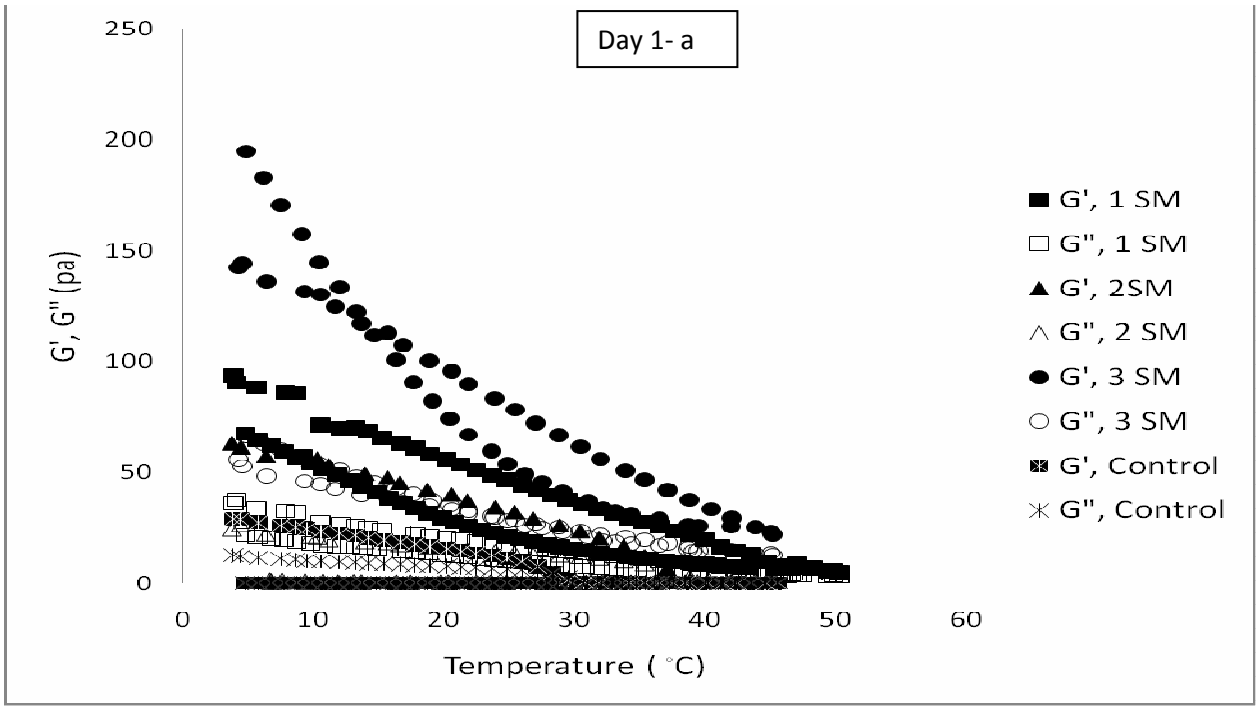


Figure 8.4- Syneresis in products supplemented with 1-3% pea flour and 1-3% skim as well as control sample during 28 day storage (SM: skim milk, PF: pea flour), a, b, c, d, e, f: for a given storage time, means followed by the same letter are not significantly different ($p < 0.05$)

8.3.6 Rheological properties

Although the texture and mouth feel of a food product can be judged by a panel of consumers, rheometry is still considered a practical technique which gives an understanding of the physical properties of the product especially before it is finally formulated for sensory testing. Viscoelastic property measurements give knowledge of the rheological characteristics of foods and give an assessment of the initial experience of a consumer (Kealy, 2006). There are different tests which are designed for this special purpose, such as the oscillatory test, that has been used to evaluate the rheological properties of fermented milk products in several studies (Ozer et al., 1997; Remeuf et al., 2003; Sodini et al., 2005b). Since temperature is an important factor affecting the physical functionality of food components, it is expected that variations in temperature would affect the physical properties of a product. So, when a product is taken out of the refrigerator for consumption and then stored again, the rheological properties could be changed. To understand how temperature would affect the rheological properties of fermented milk products, the dynamic temperature ramp test is practical and it allows studying the

rheological behavior of the product over heating and cooling processes (Duggan, and Waghorne, 2001). Results of storage modulus (G′) (elasticity) and loss modulus (G″) (viscosity) as a function of temperature for the 1-3% pea flour and 1-3% skim milk supplemented probiotic and control samples at days 1, 14 and 28 of storage are presented in Figures 8.5, 8.6 and 8.7. The results of G' and G" parameters follow a hysteresis loop during heating and cooling, especially for skim milk supplemented probiotics. All pea flour and skim milk supplemented and control samples demonstrated a predominantly elastic behavior (G'>G"), in the temperature range studied. It is also shown that for the higher percentages of supplementation; either with skim milk powder or pea flour, the higher the values of G' and G" were obtained, after production and during 14 and 28 days of storage, however, pea flour supplementation resulted in significantly higher G' and G" value in comparison with skim milk supplemented and control samples, at all levels of supplementation, after production and during storage. In pea flour supplemented samples, 2% and 3% pea flour supplementation resulted in significantly higher G' and G", in comparison with 1% pea flour supplementation. Therefore, it could be suggested that the supplementation level and total solids content could alter the gel structure in probiotic products. This finding is in agreement with literature (Sendra et al., 2010). Our results also showed that, 2% and 3% pea flour supplemented probiotic and 2% and 3% skim milk supplemented samples behaved differently from 1% skim milk supplemented, 1% pea flour supplemented and control samples, as affected by temperature ramp. When a sample is affected by heating process, over the 4-50 °C temperature range; G' and G" values decrease, but when cooling the sample from 50-4 °C the sample does not recover its original visco-elastic behavior and the G' and G" get close to zero, suggesting that the gel has almost collapsed. In our study, it is shown that the structure of the gel in 1% pea flour supplemented, 1% skim milk supplemented and control sample is negatively affected by heating and subsequent cooling, while this was not experienced by the other supplemented samples. These results are in line with the obtained data on syneresis, where supplementation with PF improved the water-binding property of the gel in fermented milk systems.



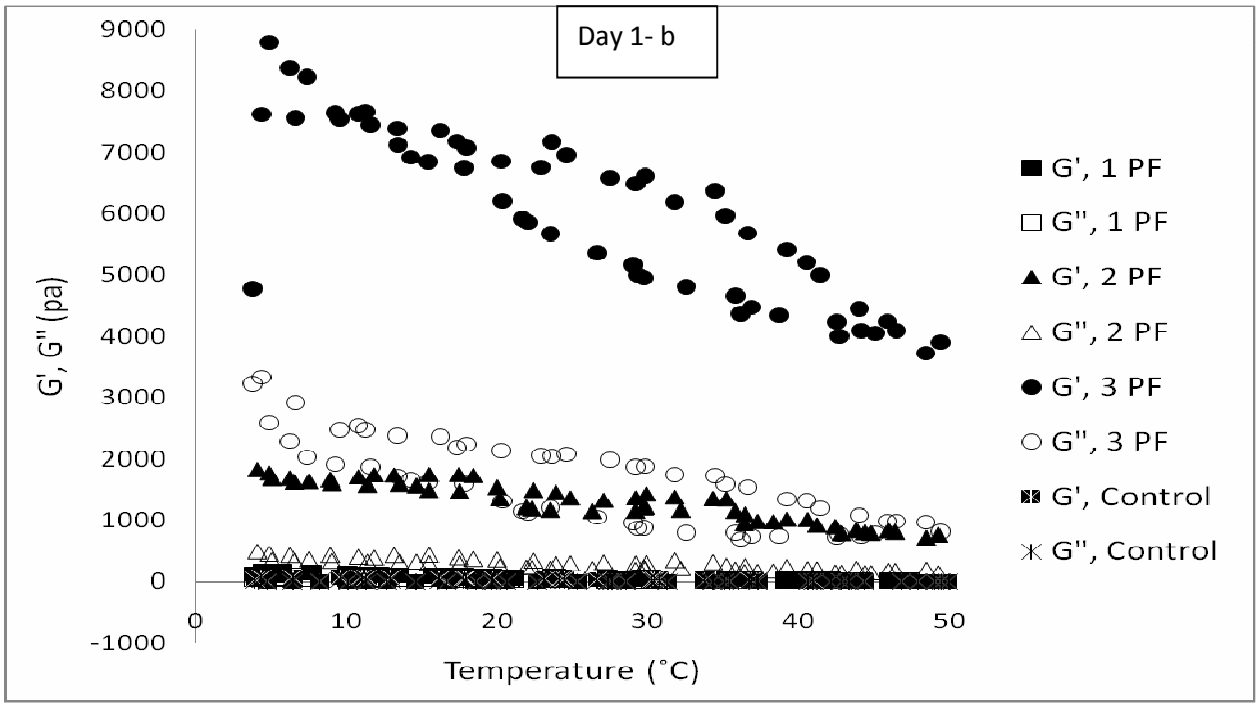


Figure 8.5 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of fermented products supplemented with (a) 1-3% skim milk and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 ^{o}C at Day 1; (SM : skim milk, PF: pea flour)

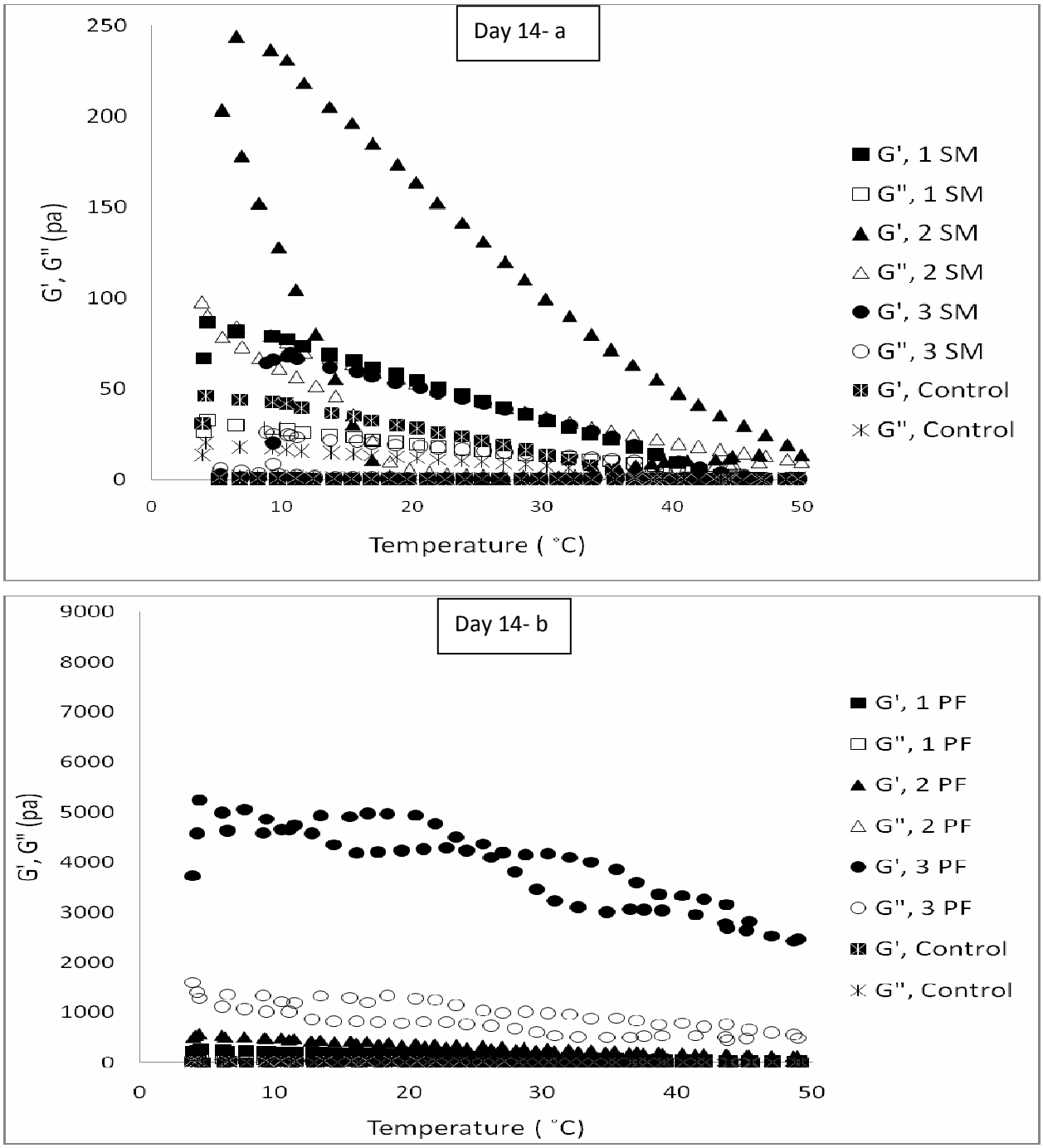
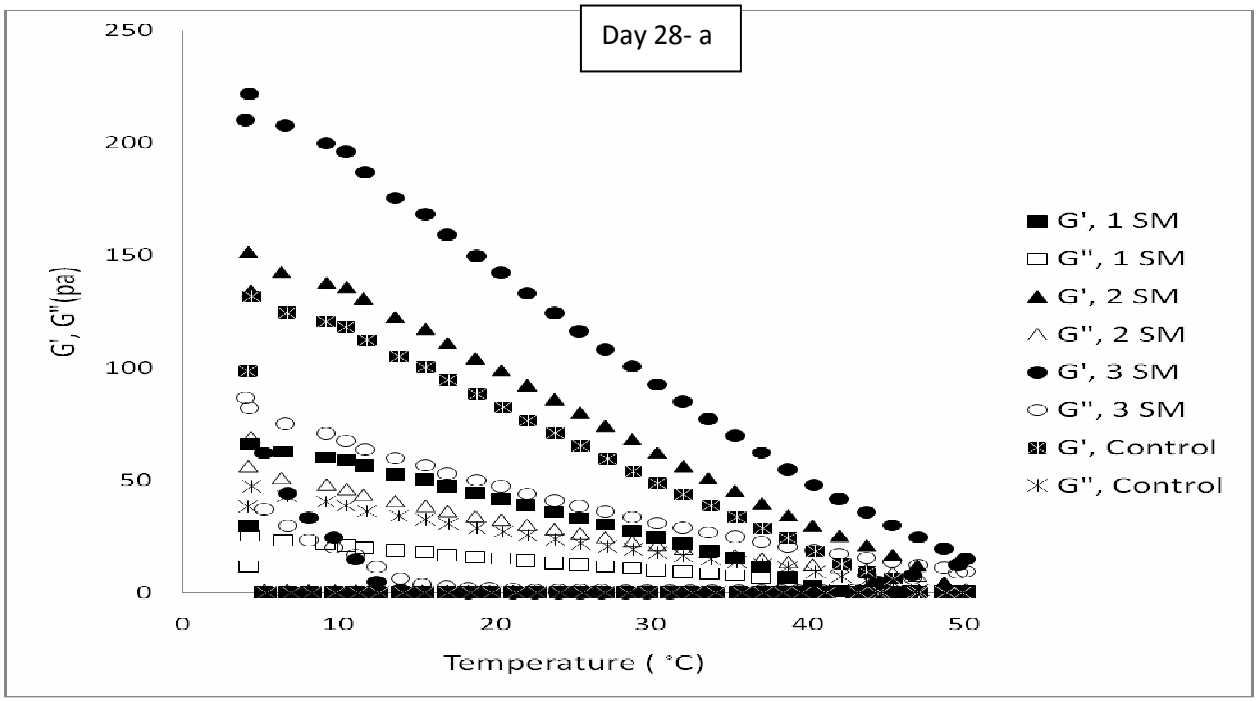


Figure 8.6 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of fermented products supplemented with (a) 1-3% skim milk and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 ^{o}C after 14 days of storage; (SM : skim milk, PF: pea flour)



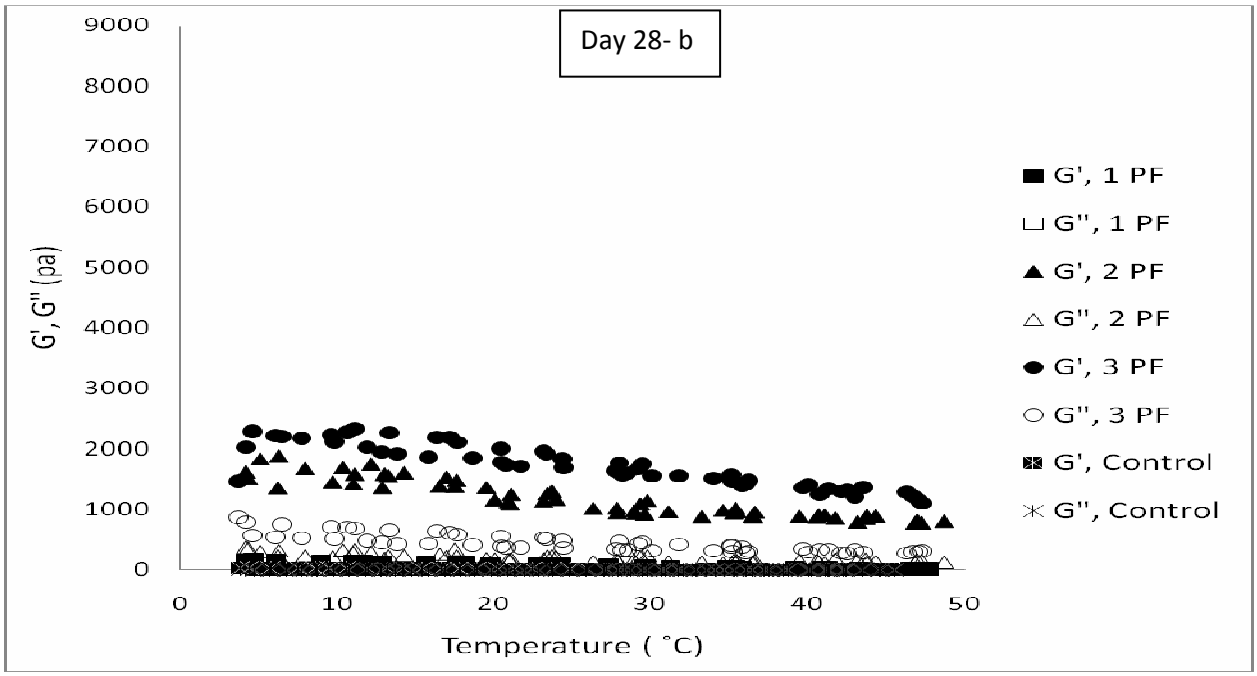


Figure 8.7- Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of fermented products supplemented with (a) 1-3% skim milk and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 ^{o}C after 28 days of storage; (SM: skim milk, PF: pea flour)

8.4 Conclusion

This study showed that supplementation with 1-3% pea flour in probiotic fermented milk products results in a significantly faster lowering of the pH than those with 1-3% skim milk powder supplementation and control samples. However, this may partially be due to the lower buffering capacity of pea flour, as compared to skim milk, while this is not the case when PF samples are compared only with non-supplemented control samples. Nutrient enrichment by pea flour resulted in higher viable counts of *L. rhamnosus* in comparison with skim milk supplemented and non-supplemented control sample, for a same final pH level (pH = 4.5). The stability of *L. rhamnosus* during storage at 4°C for 28 days in 1-3% pea supplemented samples tended to be lower than that of skim milk supplemented probiotic samples, but this might be due to greater post acidification in the pes flour containing products.

Regarding the physical and rheological properties of probiotic fermented products, 1-3% pea flour supplementation improved the stability of the gel structure with lower syneresis and improved viscoelastic properties. The color of pea flour supplemented samples was slightly altered in comparison with 1-3% skim milk supplemented and control probiotic after production and during storage. pH in 1-3% pea supplemented probiotic was lower than 1-3% skim milk supplemented samples after 28 days storage, which could not solely be attributed to the lower buffering capacity of pea supplemented media. The higher level of supplementation resulted in higher G' and G" value in both pea flour and skim milk supplemented probiotic, while the pea flour significantly affected the strength of the gel. Overall, on the basis of the microbial and rheological properties investigations, our results suggest that pea flour could be potentially considered as a source of prebiotic and texture improvement ingredient for supplementation in *L. rhamnosus* fermented milk products.

Connecting Statement to Chapter 9

In this document, the functional properties of pulse ingredients were studied and based on those techno-functional properties, supplemented beverage, yogurt and probiotic fermented milk were formulated. The physico-chemical, microbial and sensory properties of the supplemented food have been studied as well. Previous chapters (3-8) focussed on the effect of supplementation on acid production, microbial growth and physical properties of each microorganisms (yogurt starters and probiotic separately) by each of pulse ingredients (lentil flour and pea flour) individually. However, the comparative effect of supplementation of yogurt or probiotic microorganisms, by lentil flour or pea flour has not been analyzed yet. In chapter 9; a general conclusion from chapters 3-8 is formulated along with an analysis of the acid production, microbial growth, physical and sensory properties of yogurt and probiotic supplemented with lentil flour or pea flour, compared to understand the effect of two different pulse ingredients on the yogurt and probiotic microorganisms and the physical and sensory properties of final products, after production and during storage.

Chapter 9: General Conclusion

9.1 Summary of findings from chapters 3-8

9.1.1 Our results showed clear differences in the functional properties of lentil flour, chickpea flour, pea protein and pea fiber, which may be attributed to differences in composition (e.g., protein, carbohydrate content, carbohydrate type, etc). Additionally, chickpea flour and lentil flour, in spite of their similar protein contents, also exhibited different functionalities. This could be attributed to the different type of proteins, processing treatments and also fiber content.

9.1.2 Beverage supplementation with 1% and 2% of pulse fractions could give satisfactory results in terms of sensory attributes based on results obtained for both orange and apple juice, but not for the turbidity cloud and visual stability and color.

9.1.3 Acid production in 1-3% lentil flour supplemented yogurt was significantly higher during fermentation in comparison with skim milk supplemented yogurt which may partially be attributed to the lower buffering capacity of lentil supplemented yogurt in comparison with the skim milk supplemented yogurt. Data on the viable counts of the two starter cultures (*S. thermophilus* and *L. bulgaricus)* suggest that nutrients brought by the lentil flour improve the growth and stability of the yogurt cultures after production and during 28 days storage.

9.1.4 Yogurt supplementation with 1-3% lentil flour either improved or minimally altered the physical and rheological properties of yogurt which could be due to higher fiber content of lentil flour in comparison with skim milk supplemented and control samples. pH, syneresis and color in 1-3% lentil supplemented yogurt was comparable with 1-3% skim milk supplemented and control yogurt after production and during 28 days storage. 1% and 2% lentil flour supplemented yogurt ranked as acceptable as 1% and 2% skim milk supplemented yogurt in terms of smoothness, graininess, flavour and overall acceptance.

9.1.5 Fermentation process was faster in 1-3% lentil supplemented probiotic in comparison with 1-3% skim milk supplemented samples which is attributed to the lower buffering capacity as well as the nutrient content of lentil flour as compared to skim milk supplemented samples. The viable counts of *L. rhamnosus* in the fermented products supplemented by the lentil flour were higher than the control sample after production and the stability of *L. rhamnosus* during 28 days storage in lentil supplemented samples was at least as good as in skim milk supplemented probiotic samples.

9.1.6 Probiotic supplementation with 1-3% lentil flour either improved or minimally altered the physical and rheological properties of fermented milk in comparison with non-supplemented control yogurt which could be due to higher fiber content of lentil flour in comparison with skim milk supplemented and control samples. pH changes in lentil flour supplemented probiotic were comparable to skim milk supplemented and control sample after production and 28 days storage. Syneresis decreased and color was minimally altered in lentil flour supplemented probiotic in comparison with skim milk supplemented and control samples after production and 28 days storage.

9.1.7 Acid production in 1% and 2 % pea flour supplemented yogurt was significantly higher after 3.5 hours fermentation in comparison with 1-3 % skim milk supplemented yogurt and control samples which could be due to greater buffering capacity of milk in comparison with pea flour. Viable count of *S. thermophilus* in all samples were not significantly different at the end of fermentation process, while the 2% and 3% pea flour increased significantly the CFU values of *L. bulgaricus* in comparison with other samples after fermentation.

9.1.8 Yogurt supplementation with 1-3% pea flour either improved or minimally altered the physical and rheological properties of yogurt which could be due to the higher carbohydrate (fiber) content of lentil flour in comparison with skim milk supplemented and control samples. pH and color in 1-3% pea flour supplemented yogurt was comparable with 1-3% skim milk supplemented and control yogurt after production and during 28 days storage. Pea flour supplementation lowered the syneresis especially for 2% and 3% pea flour supplemented

yogurt in comparison with control sample. 1% and 2% pea flour supplemented yogurt ranked as acceptable as 1% and 2% skim milk supplemented yogurt in terms of smoothness, graininess, flavour and overall acceptance.

9.1.9 Probiotic supplementation with 1-3% pea flour resulted in faster fermentation and rate to pH reduction topH 4.5, in comparison with 1-3% skim milk supplemented and control samples, which may partially be due to the lower buffering capacity of pea flour. Pea flour supplementation resulted in higher viable counts of *L. rhamnosus* in comparison with skim milk supplemented and control sample. The viability of *L. rhamnosus* in 1% and 2% pea flour supplemented probiotic was at least as good as skim milk supplemented probiotic samples and *L. rhamnosus* showed the highest stability in 3% pea flour supplemented probiotic, after production and after 28 days storage.

9.1.10 Probiotic supplementation with 1-3% pea flour increased the viscoelasticity of fermented probiotic milk in comparison with skim milk supplemented and control sample. pH changes in pea flour supplemented probiotic were comparable to skim milk supplemented and control sample after production and 28 days storage. Syneresis decreased in 2-3% pea flour supplemented sample in comparison with control sample. Color was minimally altered in pea flour supplemented probiotic in comparison with skim milk supplemented and control samples after production and after 28 days storage.

9.2 Comparison of the effect of lentil flour and pea flour on acid production, microbial growth, physical and sensory properties of supplemented yogurt and probiotic

In following sections a comparison between pulse fractions (lentil flour and pea flour) as supplemented into yogurt or probiotic is given.

9.2.1 Comparison of the acidification rate of yogurt supplemented by lentil and pea flour

Figure 9.1 compares acid production in supplemented yogurt by lentil flour and pea flour. Our previous results showed that addition of both lentil flour and pea flour improved acid production greater than skim milk powder especially for 2-3% lentil flour and 1-3% pea flour. After 3.5 h incubation, the lowest pH was observed for the 2% and 3% lentil flour followed by 1% and 2% pea flour supplemented yogurt. However, the rate of acidification in both groups of lentil flour and pea flour supplemented yogurt was not significantly different (P<0.05).

9.2.2 Comparison of acidification rate of fermented probiotic milk by lentil flour and pea flour

Figure 9.2 compares the effect of lentil flour and pea flour on acid production of *L. rhamnosus.* Our previous results showed that both lentil and pea flour increased the rate of acid production in fermented probiotic milk, but after 8 h, the 3% pea flour supplemented probiotic beverage showed significantly lower pH in comparison with the other supplemented probiotic. After 13 h fermentation, the 2% and 3% pea flour supplemented samples still had the lowest pH in comparison with the 1-3% lentil flour supplemented samples. Interestingly, although addition of lentil flour could not lower the pH faster than pea flour during the fermentation, after 19-21 h all the lentil flour supplemented samples reached pH 4.5 which was significantly earlier than for the pea flour supplemented samples. It could be concluded that the bio-availability of some nutrient in pea flour such as amino acids, sugar or vitamins facilitated the growth of *L. rhamnosus* especially during the first hours of fermentation and so the acid production improved better in pea flour supplemented probiotic in comparison with lentil flour supplemented samples. But due to lack of nutrients in the later phase of fermentation, pH could not be lowered as fast as in the lentil flour supplemented samples. Thus, pea flour may stimulate *L. rhamnosus* growth faster than lentil flour, but lentil flour could shorten the fermentation process better than pea flour.

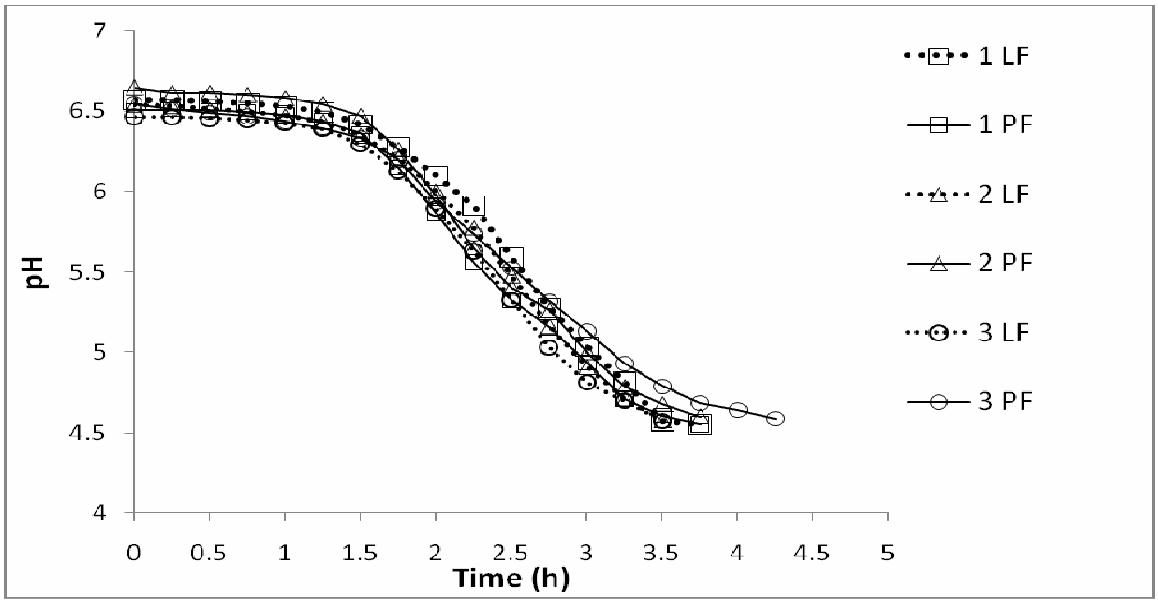


Figure 9.1- Effect of supplementation of skim milk (9.5% solids), with 1 to 3% lentil flour or pea flour (1 LF, 2 LF, 3 LF, 1 PF, 2 PF and 3 PF treatments) on acidification by yogurt starters

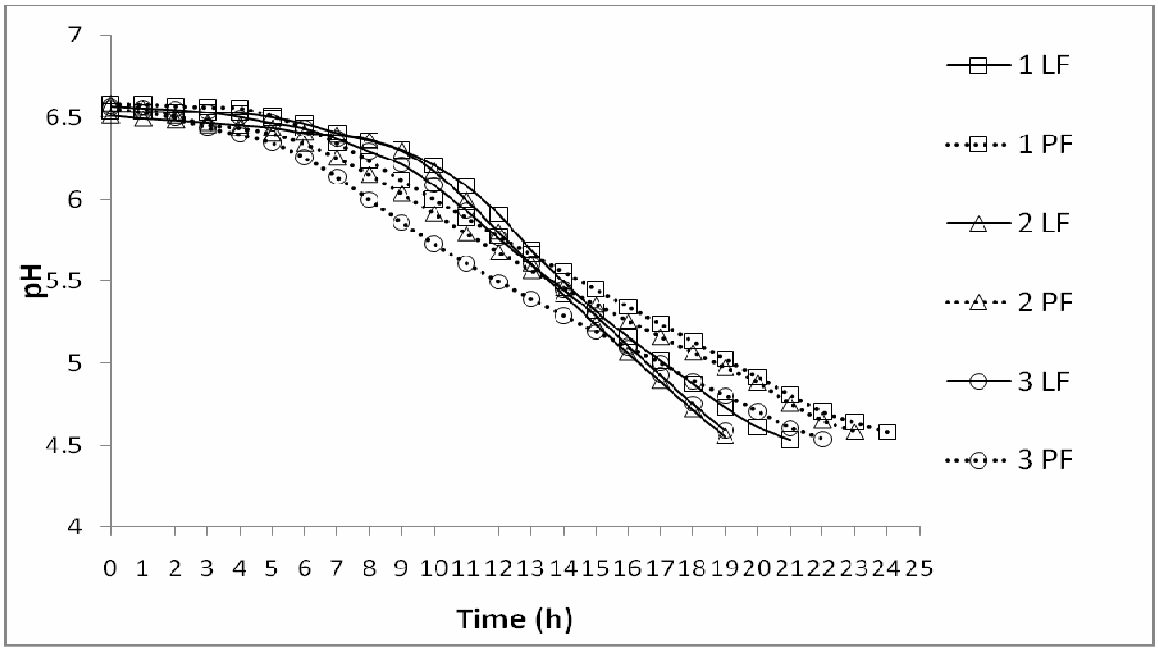


Figure 9.2- Effect of supplementation of skim milk (9.5% solids), with 1 to 3% lentil flour or pea flour (1 LF, 2 LF, 3 Lf, 1 PF, 2 PF and 3 PF treatments) on acidification by *L. rhamnosus*

9.2.3 Comparison of microbial growth and changes in pH of yogurt supplemented with lentil and pea flour after production and during storage

Table 9.1 shows the results of viable count of *S. thermophilus* and *L. bulgaricus* as well as pH changes after production and after 28 days cold storage. After 28 days storage there is a significant difference in pH between the pea flour and lentil flour supplemented yogurts *(P*<0.05), showing that post acidification was greater in pea flour supplemented yogurt. Buffering capacity of lentil flour was also slightly higher than pea flour (Table 5.1 and Table 7.1), so the lower the pH after 28 days in pea flour supplemented samples is not unexpected. The result of the log CFUs of *L. bulgaricus* on day 0 and day 28 shows that the viable count of *L. bulgaricus* was higher in pea flour supplemented yogurt in comparison with lentil flour supplemented yogurt, which suggests greater viability and stability of *L. bulgaricus* in the pea flour supplemented samples (especially 2% and 3%) in comparison with the lentil flour supplemented samples; the pH reduction in pea flour supplemented samples could be attributed to higher microbial growth rather than the lower buffering capacity.

9.2.4 Comparison of microbial growth and changes in pH of probiotic fermented milk supplemented with lentil and pea flour after production and during storage

Table 9.2 shows the effect of lentil flour and pea flour on viable count of *L. rhamnosus* after production and during 28 days storage, as well as pH changes after storage. These results suggest that, after production the log CFUs in 1-3% pea flour supplemented sample was higher than for the 1-3% lentil flour supplemented samples. In both lentil flour and pea flour supplemented samples the viable count of *L. rhamnosus* decreased during storage; the highest stability of *L. rhamnosus* was observed in the sample supplemented with 3% pea flour. The pH reduction was not significantly different in both lentil flour and pea flour supplemented probiotics after production and 28 days storage.

Table 9.1: Effect of milk supplementation with lentil flour (LF) and pea flour (PF), on pH and viable counts of yogurt starters after the fermentation as well as after 28 days of storage at 4°C

Medium	pH		*S. thermophilus* Log CFU/ mL		*L. bulgaricus* Log CFU/ mL	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
1% LF	4.50 ± 0.12 a	4.01 ± 0.01 a	8.33 b	8.01 a	7.91 d	7.76 c
2% LF	4.53 ± 0.03 a	4.03 ± 0.02 a	8.37 b	8.35 a	7.85 de	7.55 d
3% LF	4.56 ± 0.04 a	4.19 ± 0.01 a	8.33 b	8.19 a	7.71 e	7.49 d
1% PF	4.55 ± 0.00 a	3.81 ± 0.01 b	8.61 a	8.35 a	8.38 c	7.66 c
2% PF	4.57 ± 0.02 a	3.80 ± 0.01 b	8.65 a	8.41 a	8.51 ab	8.03 ab
3% PF	4.56 ± 0.01 a	3.75 ± 0.00 b	8.66 a	8.33 a	8.66 a	8.44 a

Means followed by the same letter are not different significantly, for a given column (P<0.05)

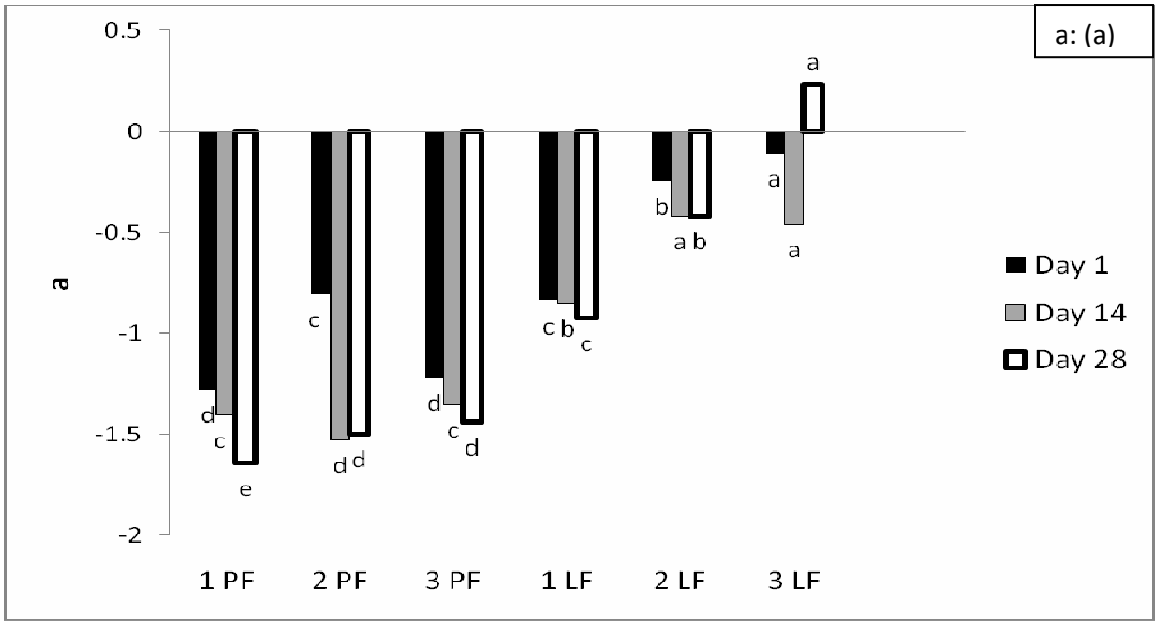
Table 9.2: Effect of milk supplementation with lentil flour (LF) or pea flour (PF), on pH and viable counts of probiotic *L. rhamnosus* after the fermentation as well as after 28 days of storage at 4°C

Medium	*L. rhamnosus* (Log CFU/mL)				pH	
	Day 0	Day 7	Day 14	Day 28	Day 0	Day 28
1% LF	8.15 de	8.03 e	8.00 cd	7.83 d	4.53 a	4.00 c
2% LF	8.21 d	8.14 d	8.03 cd	7.92 c	4.55 a	4.10 ab
3% LF	8.21 d	8.14 d	8.04 cd	7.99 c	4.59 a	4.15 a
1% PF	8.77 c	8.47 c	8.17 c	8.11 bc	4.51 a	4.04 c
2% PF	8.85 b	8.57 a	8.60 b	8.09 c	4.54 a	4.11 ab
3% PF	9.25 a	8.53 ab	8.70 a	8.66 a	4.56 a	4.12 ab

Means followed by the same letter are not different significantly, for a given column (P<0.05)

9.2.5 Comparison of the physical properties of yogurt supplemented with lentil flour and pea flour

Color: Figure 9.3 (a, b, c) shows the a, b and L values of lentil flour and pea flour supplemented yogurt, after production and after 14 and 28 days storage. According to these results, in both lentil flour and pea flour supplemented yogurt ``a`` and ``b`` values decreased during storage but the ``L`` value slightly increased after 14 and 28 days. Also, pea flour supplementation decreased the ``a`` and ``b`` values more significantly in comparison with lentil flour, but there was no significant difference in ``L`` value for both pea flour and lentil flour supplemented yogurt (P<0.05). Supplementation with pea flour appeared to give the yogurt higher redness and yellowness hues in comparison with lentil flour; for lightness, however, both lentil flour and pea flour supplemented yogurt had values in the same range.



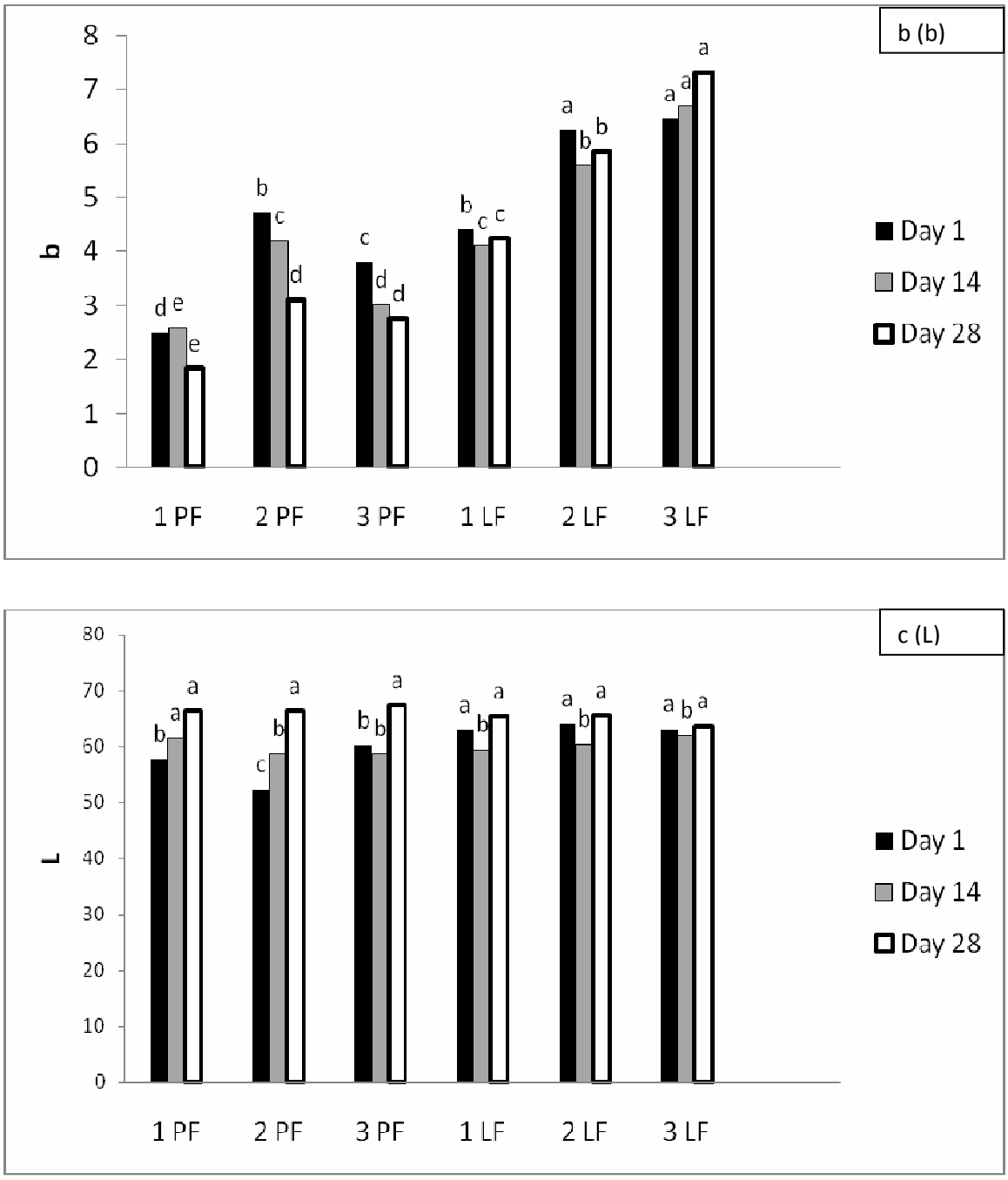


Figure 9.3- Color profile of yogurt supplemented with 1-3% lentil flour and 1-3% pea flour after production and after 14 and 28 days of storage; (LF: lentil flour; PF: pea flour; a (a value) +ve red, -ve green; b (b value) +ve yellow, -ve blue; c (L value): -0 to 100, black to white); for a given storage time, same letter means they are not significantly different (P<0.05)

Syneresis: Figure 9.4 shows the volume of water separation from 100 mL of yogurt supplemented with lentil flour and pea flour after production, 14 and 28 days of storage. Lentil flour had the greatest effect in lowering syneresis especially in 2% and 3% lentil flour supplemented yogurt in comparison with pea flour (P<0.05). Although increasing the level of supplementation decreased the level of syneresis for both lentil and pea flour supplemented samples, after 14 and 28 days storage syneresis increased slightly in all samples. Overall the best results was observed for 3% lentil flour supplemented yogurt followed by 3% pea flour and 2% lentil flour supplemented samples.

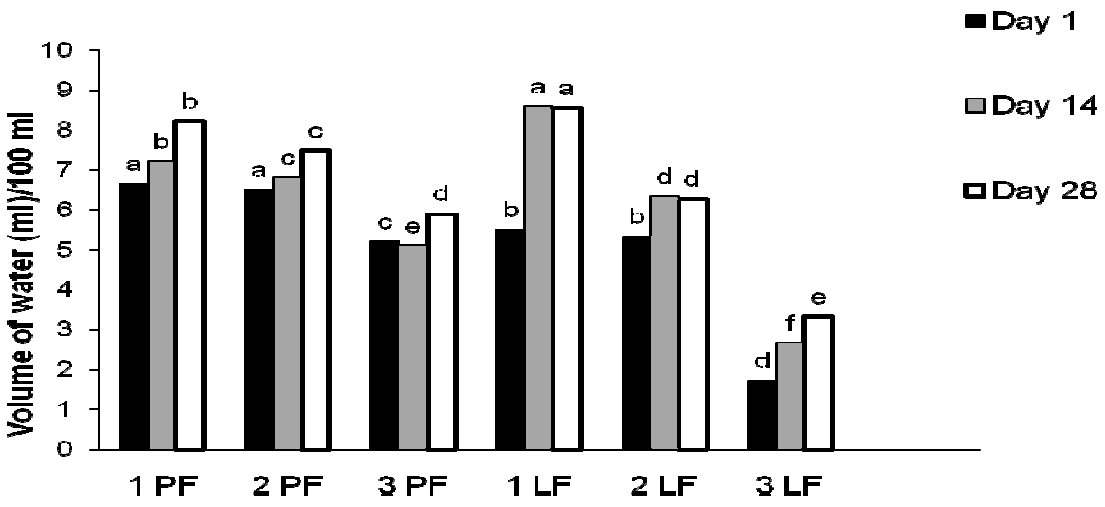
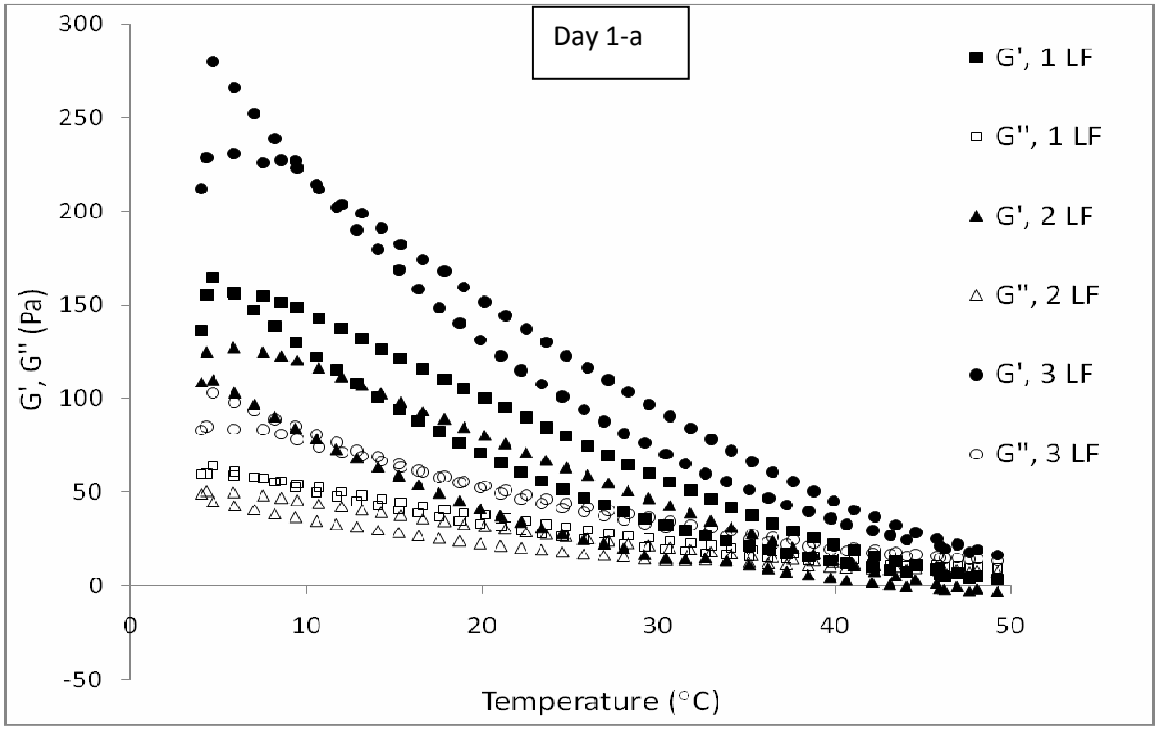
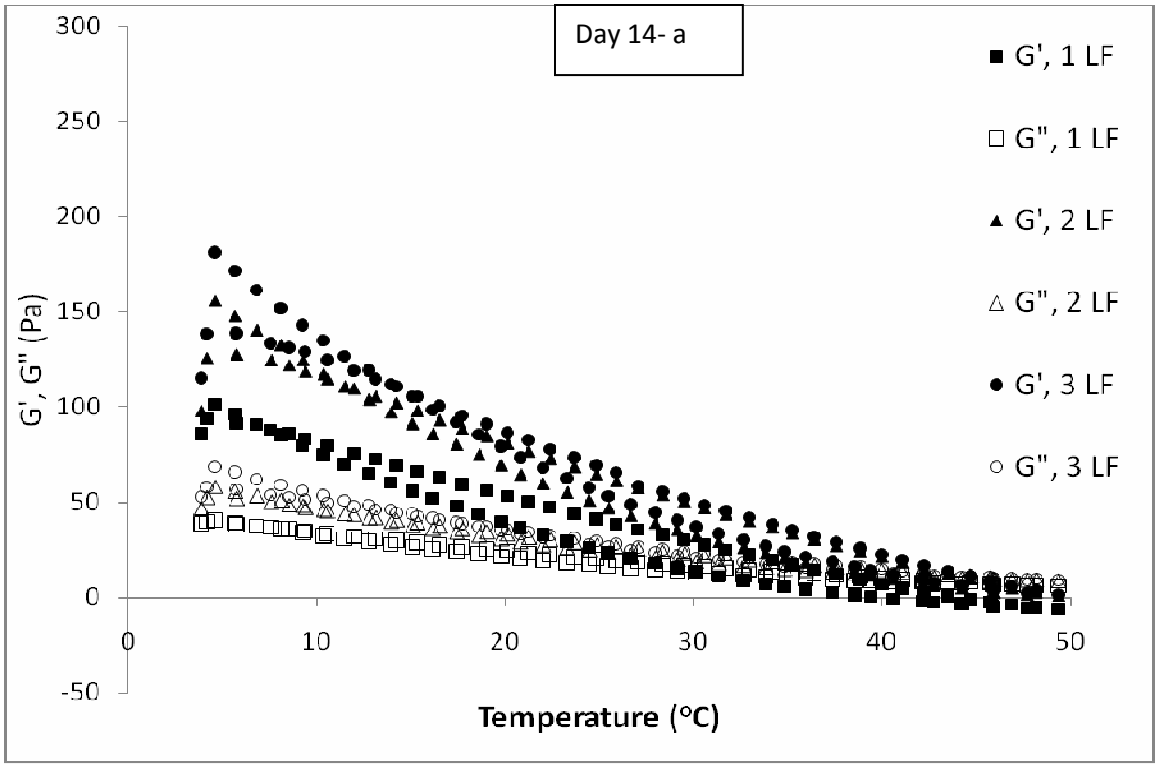


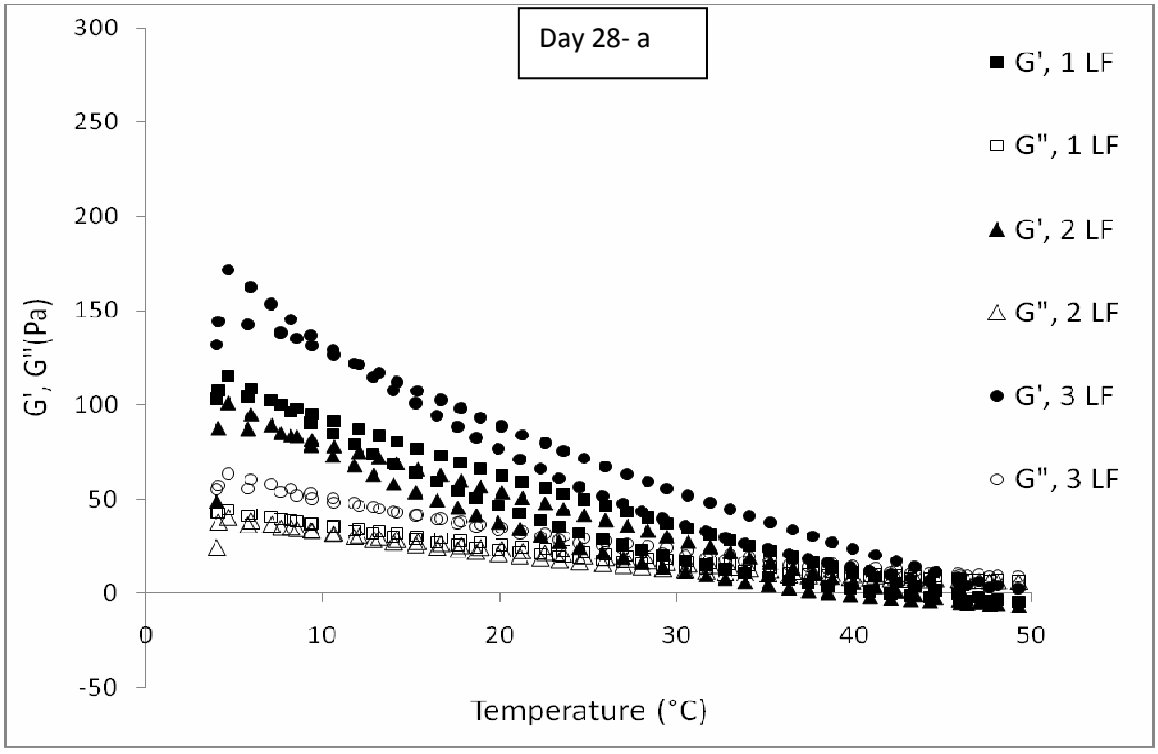
Figure 9.4- Syneresis in 1-3% lentil flour and 1-3% pea flour supplemented yogurt during 28 day storage (LF: lentil flour, PF: pea flour); same letter means they are not significantly different; for a given storage time (P<0.05)

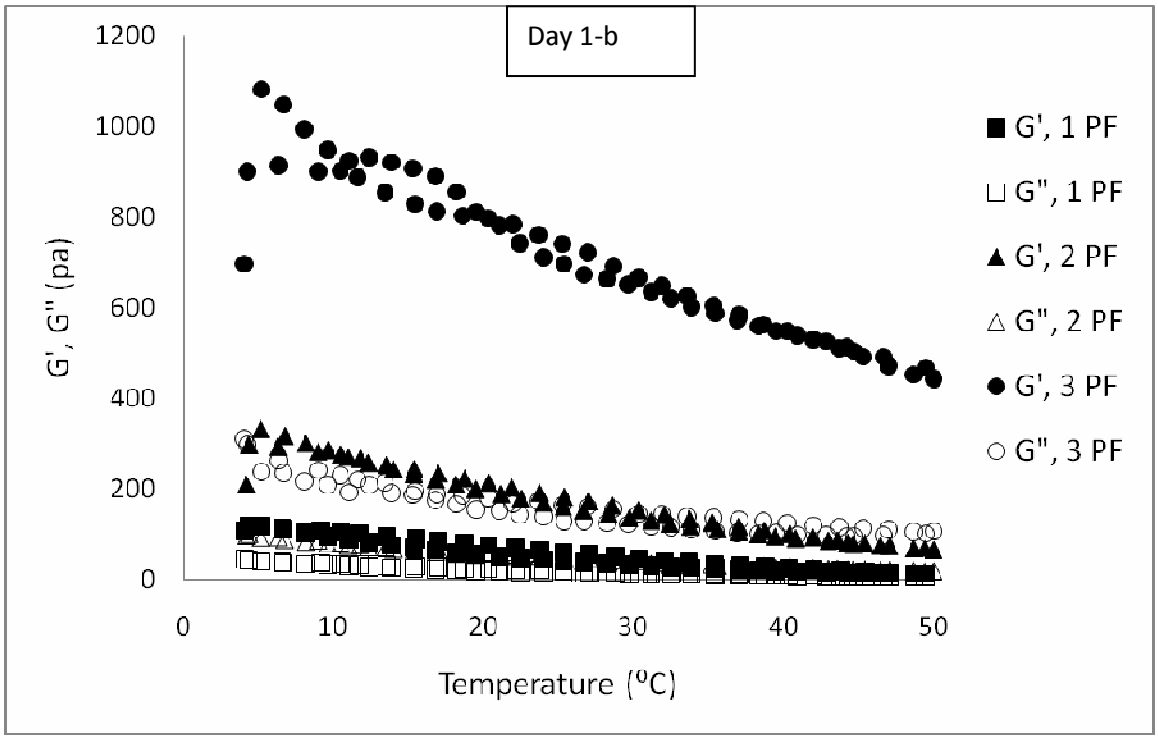
Rheological properties: Figures 9.5 (a, b) present G' and G'' value for 1-3% lentil flour and 1-3% pea flour supplemented yogurt after production and after 14 and 28 days storage. Although both lentil flour and pea flour supplemented samples showed higher storage modulus

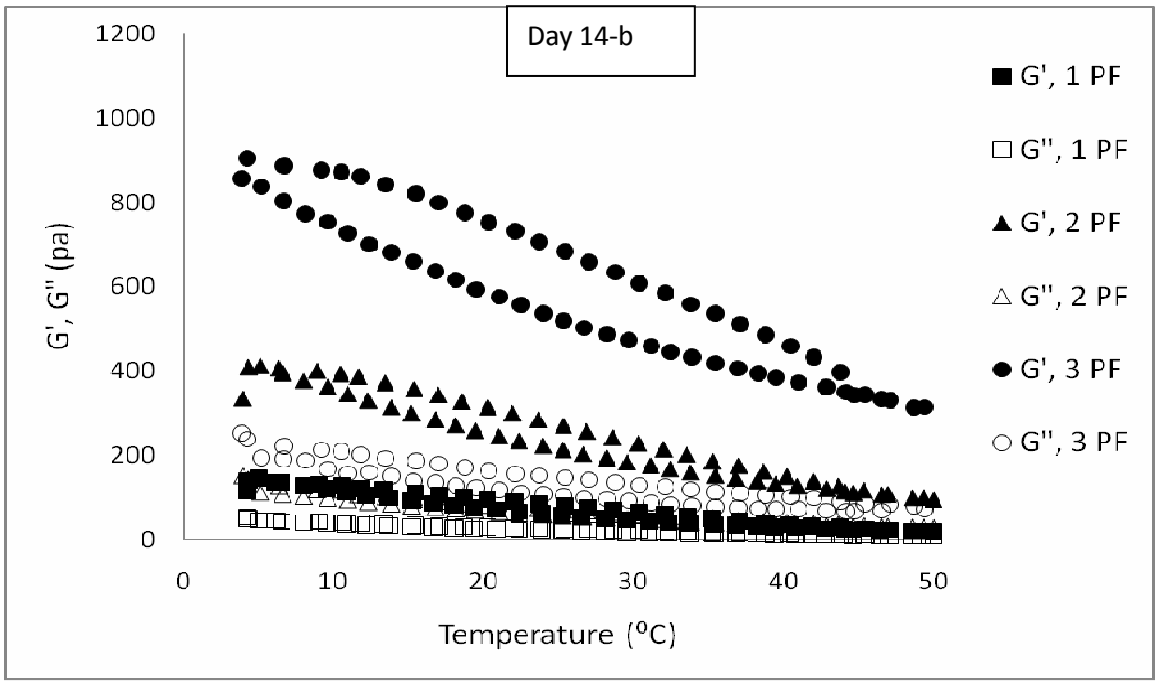
(G′)(elasticity) and loss modulus (G″)(viscosity) at higher levels of supplementation, storage resulted in a lowering of the visco-elastisity of all samples. This result was most significant for the 3% lentil flour and 3% pea flour supplemented yogurt samples. Comparing the G′ and G″ values in lentil flour and pea flour supplemented yogurts, pea flour supplemented yogurt showed greater elasticity after production and during storage. This result is in accordance with the syneresis results shown earlier and indicates that less water binding capacity of the yogurt gel in pea flour supplemented samples could have resulted in a firmer gel in comparison with lentil flour supplemented yogurt.




Day 14- a
G', 1 LF
G", 1 LF
G', 2 LF
G", 2 LF
G', 3 LF
G", 3 LF
G', G" (Pa)
Temperature (°C)
300
250
200
150
100
50
0
-50
0
10
20
30
40
50


Day 28- a
G', 1 LF
G", 1 LF
G', 2 LF
G", 2 LF
G', 3 LF
G", 3 LF
G', G"(Pa)
Temperature (°C)
300
250
200
150
100
50
0
-50
0
10
20
30
40
50


Day 1-b
1200
1000
800
600
400
200
0
G', G'' (pa)
0
10
20
30
40
50
Temperature (°C)
G', 1 PF
G'', 1 PF
G', 2 PF
G'', 2 PF
G', 3 PF
G'', 3 PF


Day 14-b
1200
1000
800
600
400
200
0
G', G'' (pa)
0
10
20
30
40
50
Temperature (°C)
G', 1 PF
G'', 1 PF
G', 2 PF
G'', 2 PF
G', 3 PF
G'', 3 PF

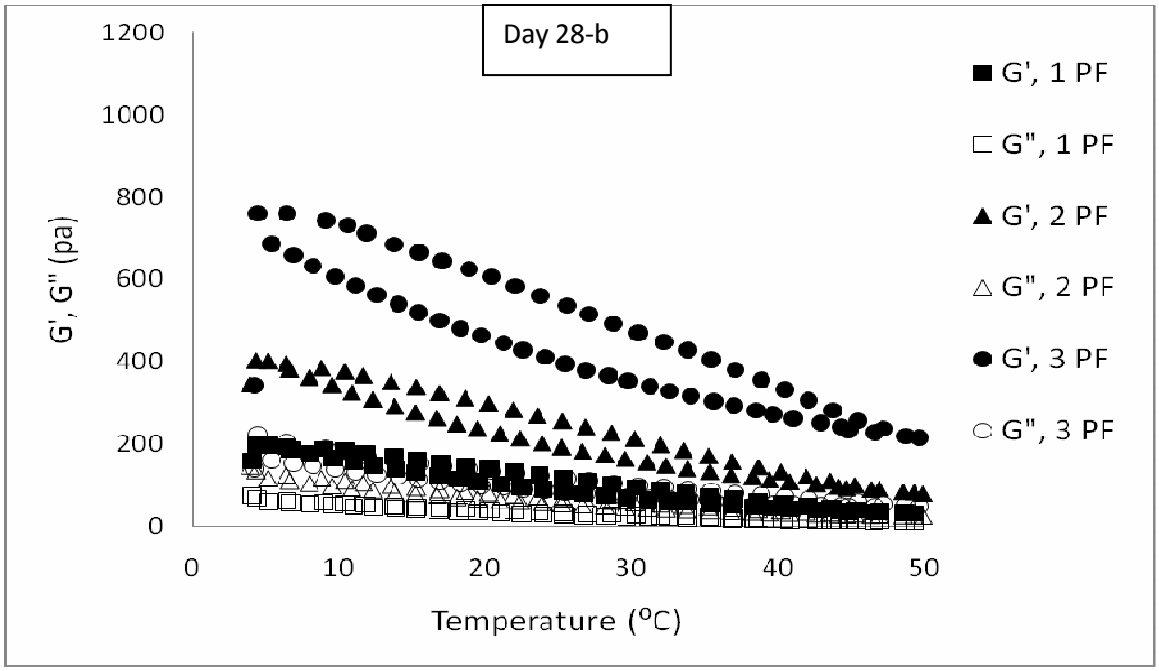
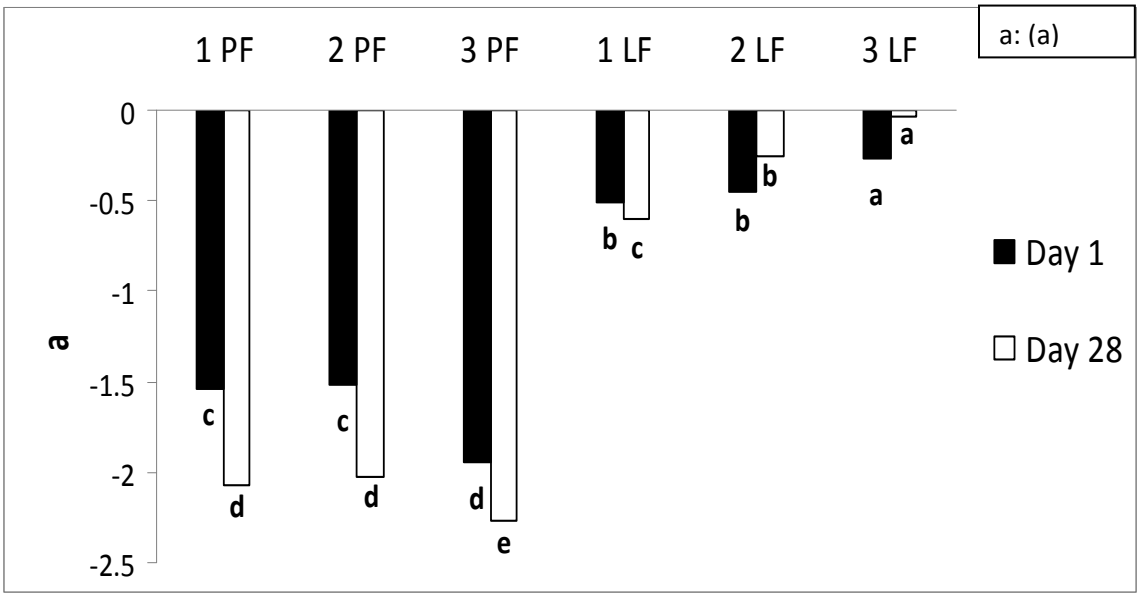


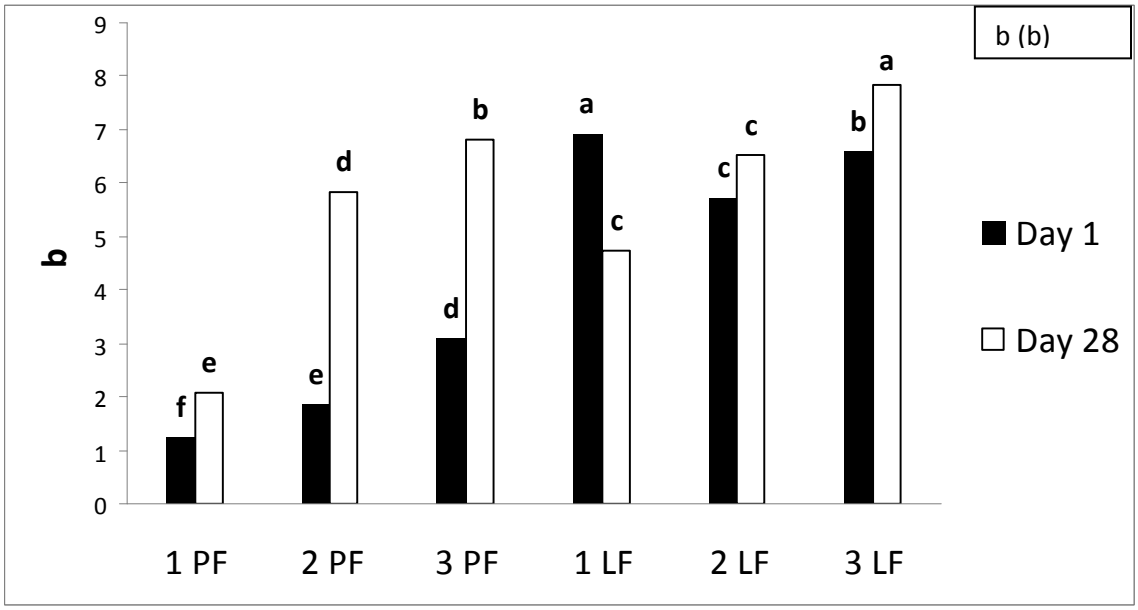
Figure 9.5 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of yogurt supplemented with (a) 1-3% lentil flour and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 ^{o}C after production and 14 and 28days of storage; (LF: lentil flour, PF: pea flour)

9.2.6 Comparison of the physical properties of probiotic fermented milk supplemented by lentil and pea flour

Color: Figure 9.6 (a, b, c) shows “a”, “b” and “L” values for lentil and pea flour supplemented probiotic fermented milk, after production and after 28 days storage. Addition of 1-3% pea flour lowered the ‘’a’’ and ``b`` values more significantly compared to the 1-3% lentil flour immediately after production and after 28 days storage. The ‘’b’’ values increased during storage in pea flour supplemented probiotic, but they were still lower than the ‘’b’’ values of the lentil flour supplemented samples. After production the highest ``L`` value was observed for the 2% lentil flour supplemented sample followed by the 3% pea flour and 3% lentil flour supplemented samples; after 28 days the ‘’L’’ value increased in all pea flour supplemented samples but not in lentil flour probiotic (P<0.05). The results suggest that addition of pea flour imparts more redness and yellowness hues to the probiotic fermented milk products in

comparison with lentil flour; for lightness, however, the results are quite varied and after 28 days storage 3% pea flour supplemented had the lightest color.





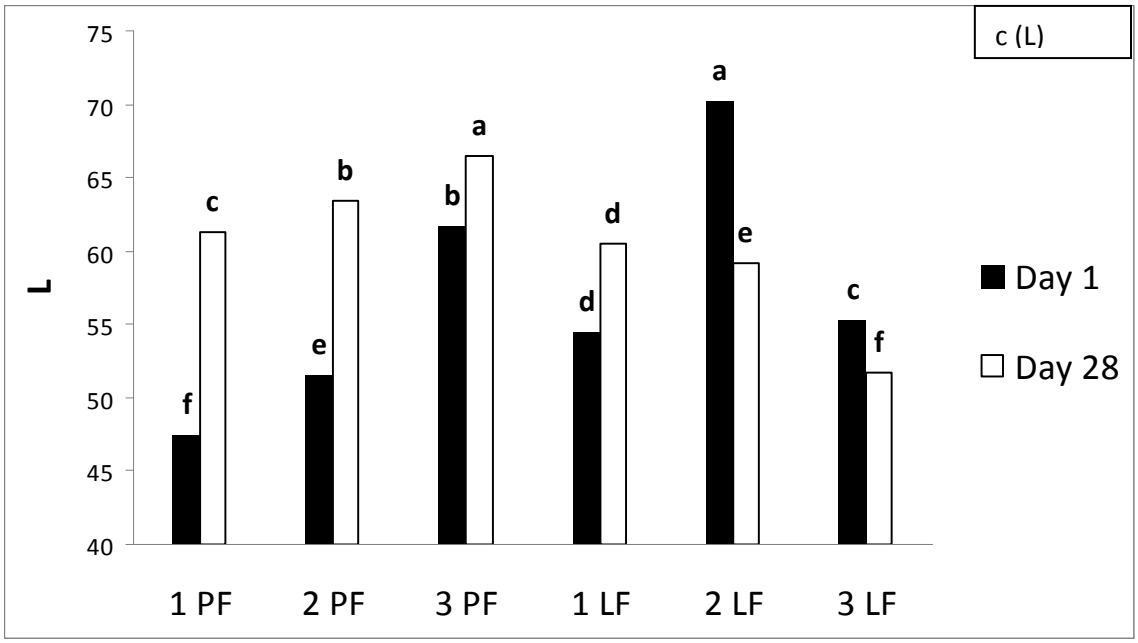


Figure 9.6- Color profile of probiotic fermented milk supplemented with 1-3% lentil flour and 1-3% pea flour after production and after 28 days storage; (LF: lentil flour; PF: pea flour; a (a value) +ve red, -ve green; b (b value) +ve yellow, -ve blue; c (L value): -0 to 100, black to white); the same letter means they are not significantly different, for a given storage time (P<0.05)

Syneresis: Figure 9.7 shows the volume of water separation from 100 mL of supplemented probiotic after addition of lentil flour and pea flour after production and during storage. Lentil flour had a greater effect in lowering syneresis especially in the 2% and 3% lentil flour supplemented yogurt in comparison with pea flour (P<0.05). Although increasing the level of supplementation decreased the level of syneresis for both lentil and pea flour supplemented samples, after 14 and 28 days storage syneresis increased slightly in all samples. Overall the best results was observed for 2-3% lentil flour supplemented probiotic samples followed by 3% pea flour and 1% lentil flour supplemented samples.

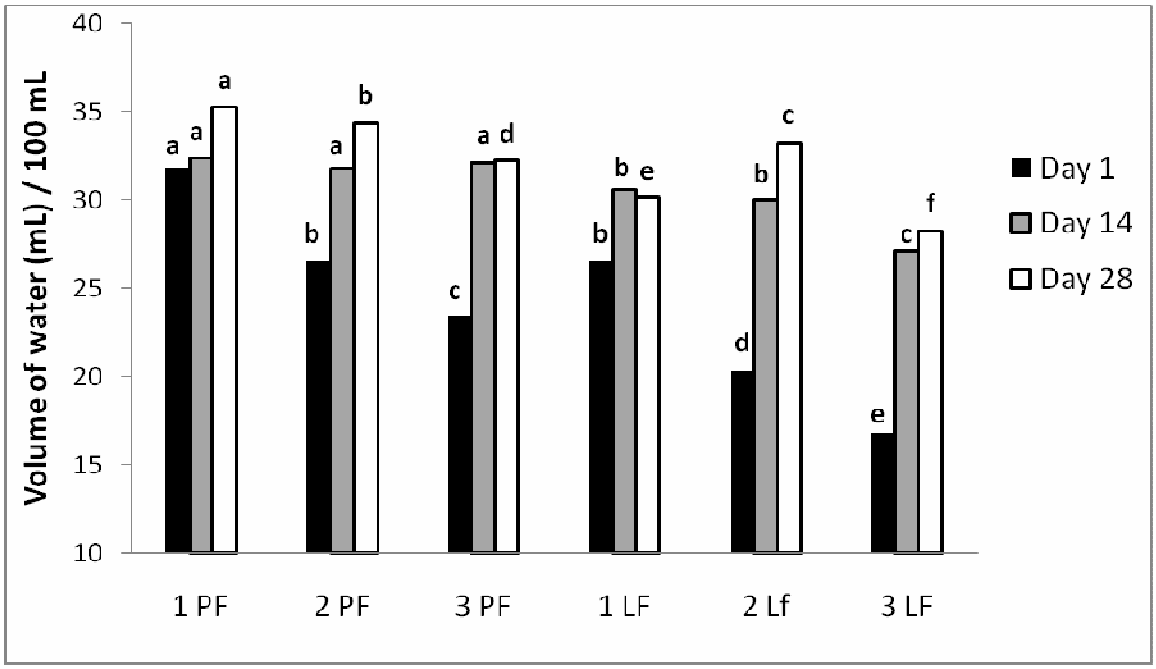
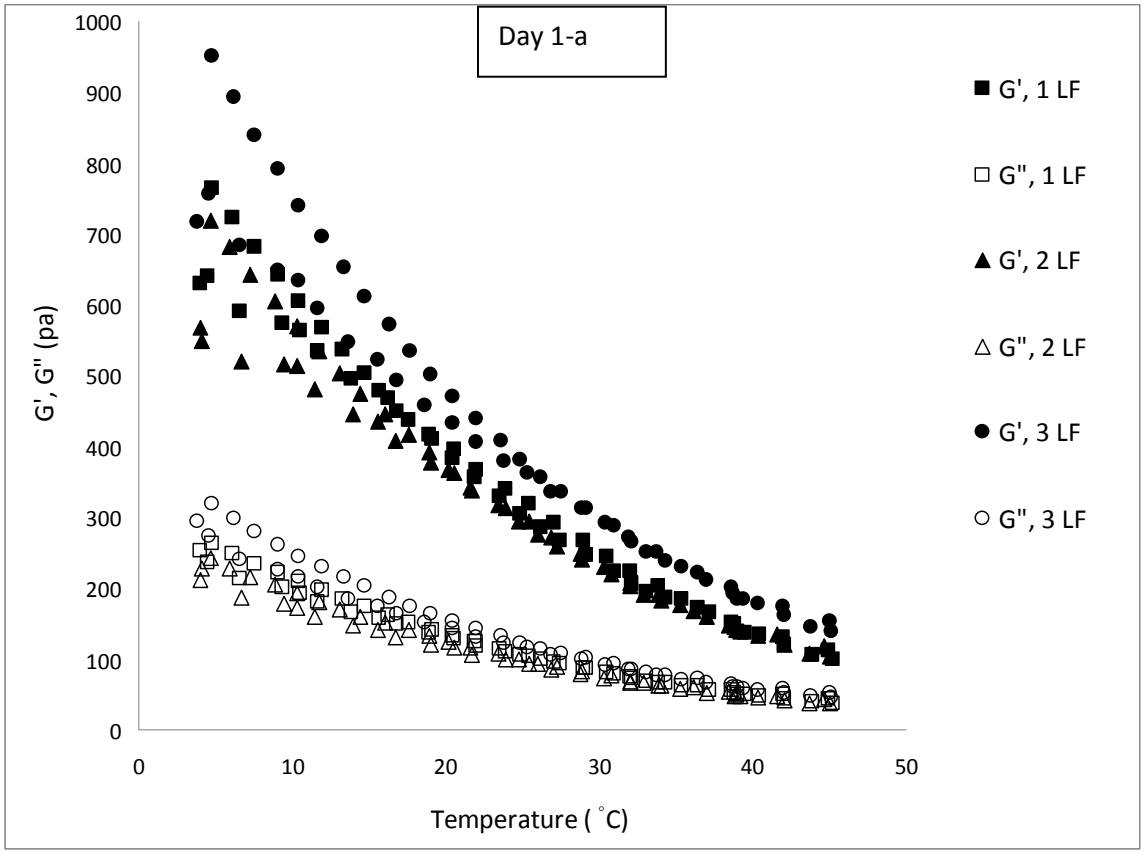
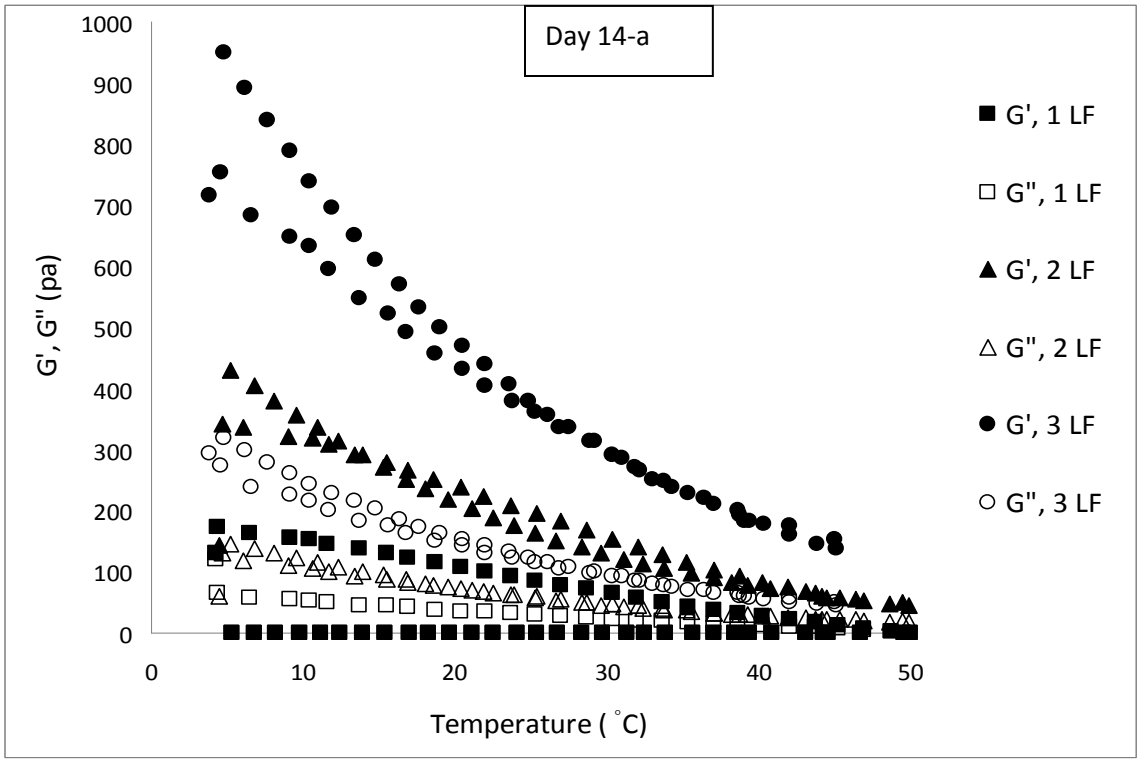
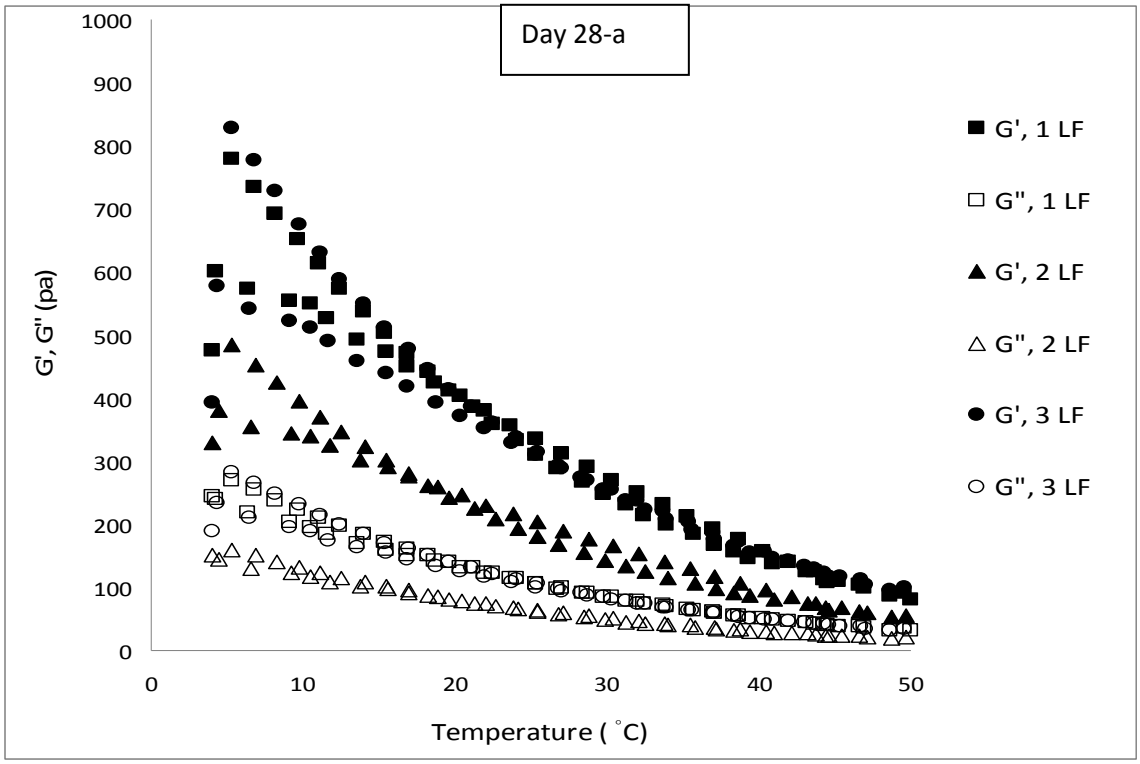


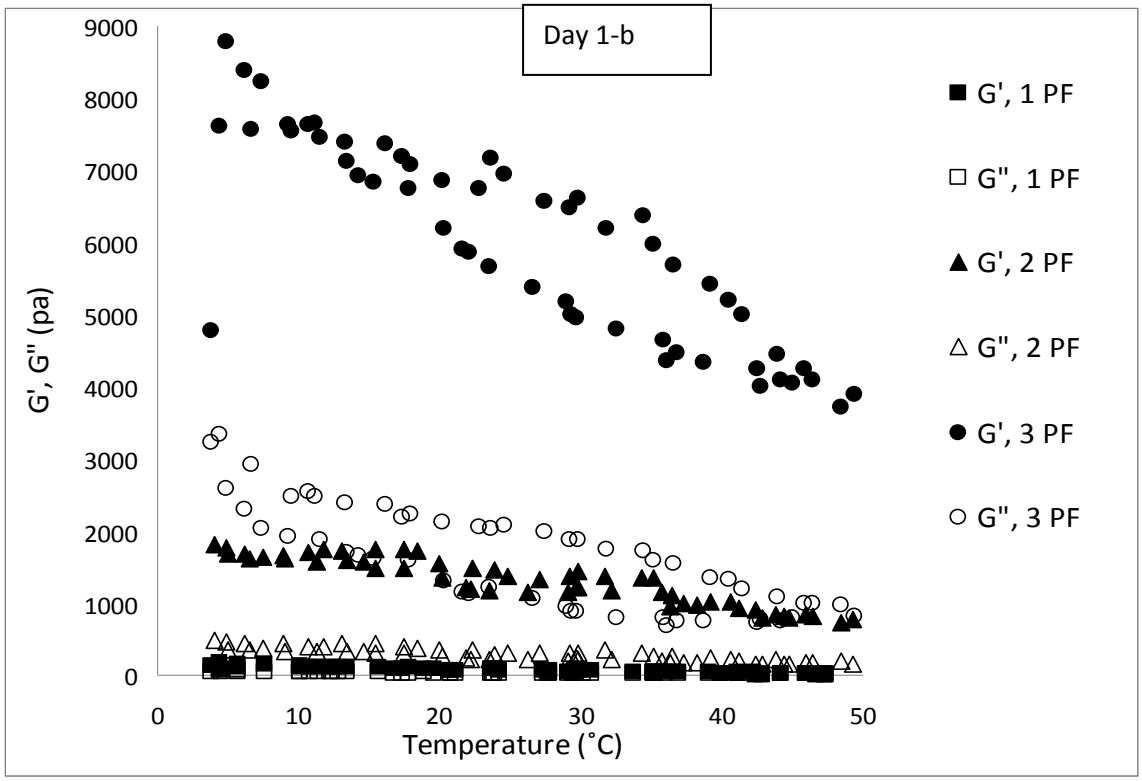
Figure 9.7- Syneresis in 1-3% lentil flour and 1-3% pea flour supplemented probiotic fermented milk during 28 day storage (LF: lentil flour, PF: pea flour); the same letter means they are not significantly different, for a given storage time (P<0.05)

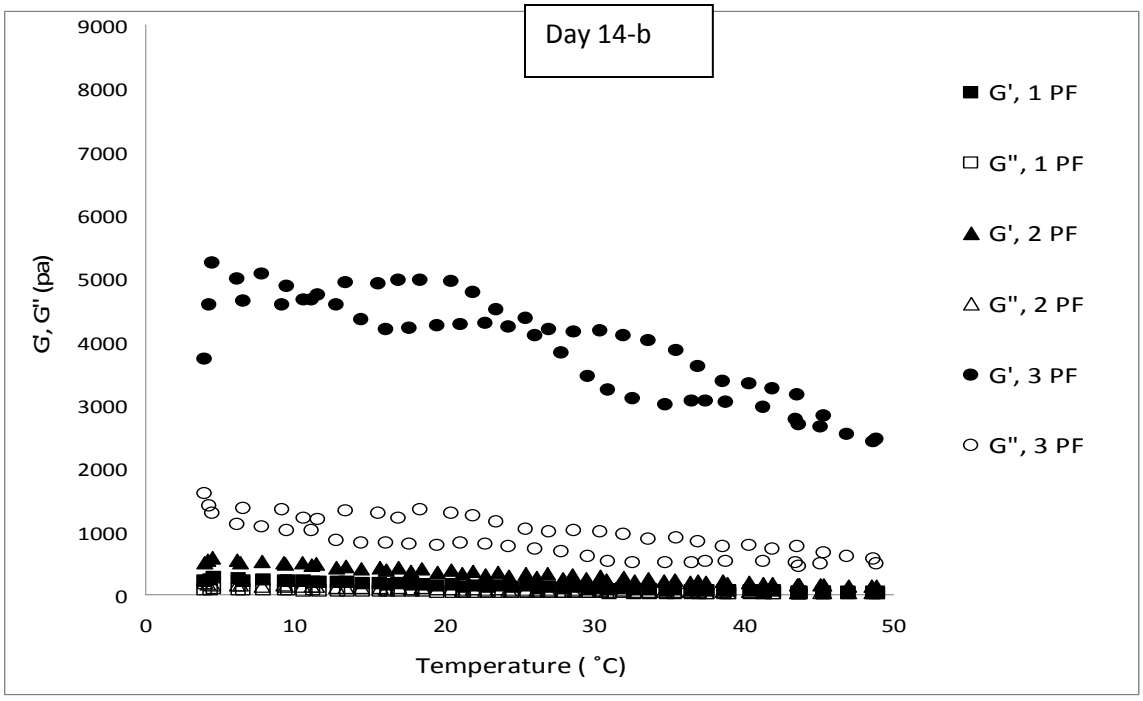
Rheological properties: Figures 9.8 (a, b) present the G' and G'' values for the 1-3% lentil and 1-3% pea flour supplemented probiotic samples immediately after production and during storage. Both the lentil flour and pea flour supplemented samples showed higher storage modulus (G')(elasticity) and loss modulus (G˝)(viscosity) as the level of supplementation was increased, however, storage resulted in a lowering of the visco-elasticity of all samples. Reductions in G' and G'' were greater in 1-3% pea flour supplemented probiotic compared to the 1-3% lentil flour supplemented samples. Comparing the G' and G˝ values for lentil and pea flour supplemented probiotic samples, the pea flour samples had greater G' and G'' after production and during storage, whereas the lentil flour supplemented samples had better gel recovery over the temperature ramping cycle (heating and cooling). This result agrees with the syneresis results reported earlier and indicates that the lower water binding capacity of the probiotic yogurt gel supplemented with pea flour increased the firmness of the gel in comparison with the lentil flour supplemented probiotic yogurt.


Day 1-a
G', G'' (pa)
Temperature (˚C)
G', 1 LF
G'', 1 LF
G', 2 LF
G'', 2 LF
G', 3 LF
G'', 3 LF


Day 14-a
G', G'' (pa)
Temperature (˚C)
G', 1 LF
G'', 1 LF
G', 2 LF
G'', 2 LF
G', 3 LF
G'', 3 LF


Day 28-a
1000
900
800
700
600
500
400
300
200
100
0
G', G'' (pa)
0
10
20
30
40
50
Temperature (˚C)
G', 1 LF
G'', 1 LF
G', 2 LF
G'', 2 LF
G', 3 LF
G'', 3 LF


Day 1-b
G', G'' (pa)
Temperature (˚C)
G', 1 PF
G'', 1 PF
G', 2 PF
G'', 2 PF
G', 3 PF
G'', 3 PF


Day 14-b
G', G'' (pa)
Temperature (˚C)
G', 1 PF
G'', 1 PF
G', 2 PF
G'', 2 PF
G', 3 PF
G'', 3 PF

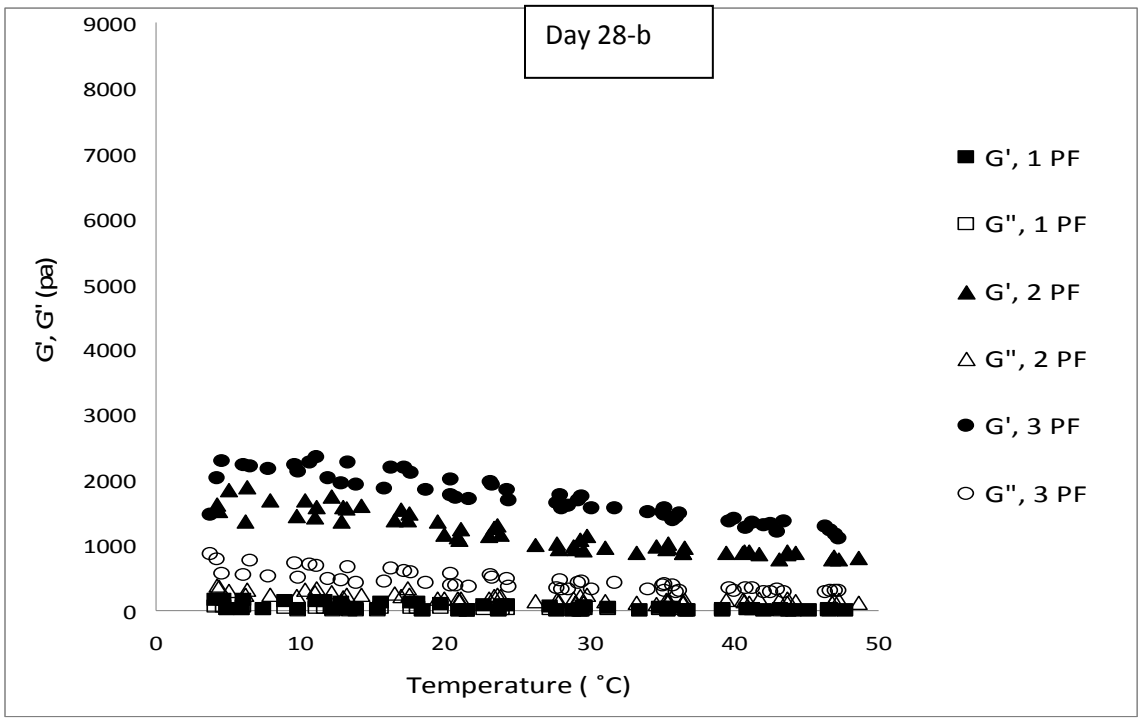


Figure 9.8 - Storage (G′) (elasticity) and loss (G″) (viscosity) moduli of probiotic fermented milk supplemented with (a) 1-3% lentil flour and (b) 1-3% pea flour heated from 4-50 ˚C and from 50-4 ^{o}C after production and 14 and 28days of storage; (LF: lentil flour, PF: pea flour)

9.2.7 Sensory properties of supplemented yogurt by lentil flour or pea flour

Table 9.3 compares the sensory properties of 1-3% lentil flour and 1-3% pea flour supplemented yogurt samples. The lower scores show the more desirable sample for panelists, hence the obtained ranking is, in terms of smoothness and graininess 1% PF, 1% LF, 2% LF and 2% PF; in terms of flavour 1% PF, 1% LF, 2% LF, 2% PF and in terms of overall acceptance 1% PF, 1% LF, 2% PF and 2% LF ranked in lowest score order. Meanwhile, all lentil flour and pea flour supplemented samples ranked with a less than 5 score (neither like nor dislike), except for 3% pea flour supplemented samples. Comparing the overall sensory results for lentil flour and pea flour supplemented yogurt, 1-2% pea flour supplemented yogurt showed a more promising product in comparison with 1-2% lentil flour supplemented sample, especially with consideration of lower color scores.

Table 9.3- Sensory properties of yogurt supplemented with 1-3 % lentil flour and 1-3 % pea flour after production (PF: pea flour, LF: lentil flour; 1 - extremely like to 9 - extremely dislike)

Sample	Average				
	Smoothness	**Graininess**	**Flavor**	**Overall acceptance**	**Color**
1 PF	2.68 a	2.88 bc	3.76 c	3.44 b	2.72 a
2 PF	3.12 a	3.76 b	4.84 b	4.08 ab	2.96 a
3 PF	3.12 a	4.28 a	5.40 a	4.76 a	3.04 a
1 LF	2.72 ab	2.76 bc	3.84 c	3.40 b	3.60 a
2 LF	2.88 ab	3.32 b	4.44 b	4.20 a	3.56 a
3 LF	3.16 a	3.44 b	4.88 b	4.32 a	3.44 a

Means followed by the same letter are not different significantly, for a given column (P<0.05)

9.3 Recommendation for future studies

This research work has demonstrated several important findings and has also helped to identify some areas that could be of interest for future product development which are summarized below.

- Study of the effect of pulse ingredient on viable counts of yogurt starters and probiotic during fermentation process
- Study of the effect of stimulatory nutrient factors in lentil flour and pea flour on the growth of yogurt starters and probiotic
- Study of the bioactive components due to growth of yogurt starters and probiotic in supplemented products with pulse ingredients after fermentation and during storage
- Optimization of the formulation for the supplemented of yogurt with lentil flour or pea flour using sweeteners, coloring and flavoring agents
- Optimization of the formulation for the supplemented of probiotic milk with lentil flour or pea flour using sweeteners, coloring and flavouring agents

- Formulation of drinkable or set yogurt, probiotic or probiotic yogurt product supplemented with lentil flour or pea flour using sweeteners and flavouring agents
- Formulation of drinkable or set yogurt, probiotic or probiotic yogurt product supplemented with lentil flour or pea flour using fruit juice or vegetable juice

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