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The effect of frozen storage on the survival of probiotic microorganisms found in traditional and commercial kefir

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THE EFFECT OF FROZEN STORAGE ON THE SURVIVAL OF PROBIOTIC MICROORGANISMS FOUND IN TRADITIONAL AND COMMERCIAL KEFIR

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DEDICATION

This thesis is dedicated to my friends and family (especially the kefir makers, you know who you are….) and to fermentation enthusiasts everywhere!

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ABSTRACT

Kefir is a fermented milk traditionally made from a unique starter culture, which consists of numerous bacteria and yeast species bound together in an exopolysaccharide matrix produced by certain lactic acid bacteria. Many health benefits are associated with traditionally produced kefir; however, bulging and leaking packaging, caused by secondary yeast fermentation during storage, has limited large scale manufacture traditionally produced kefir. Commercial kefir products have been designed to reduce these effects by using a pure starter culture consisting of a mixture of bacteria and yeast species that give a flavor similar to traditional kefir, but some health benefits may be lost in commercial production due to reduced microbial diversity and lack of beneficial exopolysaccharides. In this study, traditional and commercial kefir was frozen to study the effects of frozen storage on the viability of probiotic bacteria over time. The traditional kefir was prepared by inoculating 1 L of pasteurized whole goats milk with approximately 30 g of kefir grains. Commercial kefir was prepared by inoculating 1 L of full fat, pasteurized goat milk with a commercial kefir starter. The milk was allowed to ferment at room temperature (24-28°C) until pH 4.6 was reached. Samples were frozen (-8 to -14°C.) immediately following the completion of fermentation and were thawed and plated for lactobacilli, lactococci and yeasts on day 0, day 7, day 14 and day 30 of frozen storage. Statistical analysis was preformed by statistical analysis software (SAS®) using the variance analysis (ANOVA) f-test, with a confidence interval of 95% (P<0.05). Means were compared by the least significant difference (LSD) test. Lactobacilli, lactococci and yeasts were significantly (P<0.05) reduced in number during frozen storage; however, the traditionally produced kefir was shown to have significantly

($P<0.05$) higher counts of bacteria and yeast at each sampling. It was concluded that frozen storage and the development of frozen kefir products could eliminate most packaging concerns associated with the large scale manufacture of traditionally produced kefir, resulting in increased production and marketability of this healthful product.

CHAPTER 1. INTRODUCTION

1.1 Milk and Fermentation Throughout History

Fermented milks have been a staple food, or present in some amounts, in the diets of many diverse and geographically widespread cultures throughout history. Peoples who were traditionally associated with herding or keeping livestock, be it cattle, sheep, goats, mares or water buffalo, discovered and subsequently refined the process of fermentation as a method of milk preservation; and the types of fermented milks are as varied as the cultures that produce them, ranging from the traditional sour milks of Eastern Europe to the hard salty cheeses developed throughout the Mediterranean region.

Production of the first fermented milks dates back to 7000 BC with origins in the middle and far-east of Asia, making it one of the oldest methods of long term food preservation. A further spreading east of these traditions, by way of Russia and Eastern Europe, by the Tartars, Mongols and Huns occurred during their conquests (Vasiljevic, et. al., 2008). The expansion of areas that maintained livestock as a source of meat and dairy food by the introduction of herds and traditional production methods, and subsequent industrialization of dairy food production, has led to a total worldwide domestic ruminant population of nearly three billion at the beginning of the twenty-first century (Weimer, 2001).

Although the original fermenters did not take into account the microbiological processes involved, traditions were established that ensured the methodologies and knowledge required to produce the flavors and textures associated with these products were kept

intact. Often, these practices were passed down from generation to generation within local communities, feudal states and monasteries (Caplice, et. al., 1999). Over time, the tastes associated with fermented milk, such as the characteristic acidic flavor, may have also become associated with increased health and longevity, thus furthering its spread and increasing manufacture throughout and within ancient populations.

1.2 Goat Milk

The type of milk originally used in the production of fermented foods was determined by the type of milk producing mammal that was nearest to a group of people or the indigenous species that was domesticated in the region. Goat milk contains approximately 4.5% lipids, with the highest amount being medium chain-length triglycerides and short chain fatty acids (Chen, et. al., 2004). Because of the higher proportion of short and medium chain fatty acids, goat milk fat is thought to have marked benefits in human nutrition such as in the treatment of many malabsorption symptoms associated with intestinal resection, premature infant feeding, gallstones, etc. (Babayan, 1981 and Haenlein, 1992); however, for the most part, these properties have been greatly unexplored. These short and medium chain fatty acids, including hexanoic, octanoic and nonanoic variants, are the primary contributors to the characteristic goat flavor (Rahmat, et. al., 1996).

Other health benefits can be attributed to the affect of these fatty acids, as well as capric and caprylic acids, on functioning of the cardiovascular system, particularly following coronary bypass (Nutting, et. al., 1991). A study by White, et. al. (1991) demonstrated

capric acid to be an important vasorelaxant of human basilar arteries and showed the ability to inhibit and limit the deposition of cholesterol in tissues; and this same study showed that, even in very low doses, capric acid was capable of dissolving cholesterol gallstones in growing children.

1.3 Principles of Milk Fermentation

Despite its lengthy history, it was not until the late nineteenth century that scientists first began to take note that there were factors present in fermented milks, in addition to prolonged shelf life and enhanced sensory qualities, which may provide additional benefits to the consumer. This realization sparked the early microbiological work by numerous scientists, including Nobel Laureate Elie Metchnikoff who correlated a large consumption of fermented milk to an above average life span. In populations of Bulgarian peasants, he noticed that they lived to an average of eighty-seven years old, with one out of four living past one hundred years of age; this was a remarkable life span for the turn of the nineteenth century (Vasiljevic, 2008). And as early as 1905, scientists such as Grigoroff (1905) and Rettger, et. al. (1914), as well as Metchnikoff (1905) were isolating bacteria from fermented milk and demonstrating that certain strains could survive and colonize the intestinal tract.

Fermentation, as it pertains to food manufacture, is defined as the conversion of carbohydrates to organic acids or alcohol and carbon dioxide, using bacteria and yeasts, or a combination thereof, under anaerobic conditions (Kosikowski, et. al., 1999). In milk, these fermentations occur as a result of the action of lactic acid bacteria, and

occasionally, lactose fermenting yeasts, on lactose, a disaccharide and only sugar, found in milk. Lactic acid bacteria prefer lactose as their source of carbon, and the end products can be exclusively lactic acid, or other substances may be produced, such as acetic acid, carbon dioxide and hydrogen (Alfa-Laval, 1987). Yeasts, such as *Saccharomyces cerevisiae*, are also capable of fermenting lactose and other sugars and can be found in some fermented milks (Kwak, et.al., 1996). It also important to note that while the production of lactic acid from lactose contributes to the characteristic acidity associated with fermented milks, many interesting parallel, or post fermentation reactions often occur with other substrates, such as peptones, peptides and fatty acids to produce some of the distinctive flavors associated with certain products.

1.4 Kefir Definition and Origin

Due to a growing consumer awareness and demand for foods with added or naturally occurring probiotics, a type of traditional fermented milk called "kefir" is gaining in popularity and commercial production of kefir-like products has greatly increased over the past few years. Kefir is a naturally fermented milk beverage with a smooth and creamy texture and has an acidic and slightly alcoholic and yeasty taste; the presence of carbon dioxide gives a varying degree of effervescence (Farnsworth, 1999). In fact, the word kefir is derived from the Turkish word 'kef', which means pleasant taste (Kurmann, et. al., 1992).

Health aspects attributed to the consumption of kefir, as similar to other fermented dairy foods supplemented with probiotic bacteria, include, but are not limited to, improved

lactose utilization, anticarcinogenic activity, control of intestinal infections and improved flavor and nutritional quality of the milk (Kaur, et. al., 2002).

Kefir originated in the Caucasus Mountains several centuries ago and was traditionally produced with caprine milk primarily by inhabitants closely associated with the herding of goats and sheep. Kefir has a rich history as it pertains to its genesis and spread throughout the regions of the Balkan and Caucasus regions of Eastern Europe; in fact, the origins of kefir predate written records. Because of its ancient and apparently mysterious origin, kefir was known in antiquity as the "Drink of the Prophet [Mohammad]" and the culture used to prepare it as the "Grains of the Prophet Mohammad"; it was believed that the Prophet of Islam, Mohammad, was given the original kefir grains by the Angel Gabriel to be given to his followers, thus introducing kefir to the Orthodox Christians living in the mountainous regions of modern day Georgia (Rosell, 1932 and Margulis, 1996).

1.5 Kefir Starter Culture

Kefir differs from other fermented milk products in its unique starter culture, which is an aggregation of many different bacteria and yeast species bound together in an exopolysaccharide matrix produced by certain lactic acid bacteria. Farnsworth (1999) describes kefir grains as a mass of bacteria, yeasts, polysaccharides, and other products of bacterial metabolism, together with curds of milk proteins. The starter cultures, termed "grains", grow, propagate and pass their properties along to the following generations of grains (Simova, et. al., 2002).

When describing the perpetuation of kefir grains by certain groups throughout history in an article published in 1932, Rosell says, "One of the things that puzzle investigators in regard to the preparation of these milks [kefir] is that most of the races named are those who have kept kefir in its pure form. The method of their preparation was handed down as a precious inheritance from father to son in the families who concerned themselves with these products of ancient lineage, which, in a certain sense, may be said to constitute the "secret medicine" of many countries." To restate Rosell's observation, it is the production of kefir and the propagation of the starter culture, using the traditional methods that preserve its defining characteristics, which have remained intact due to the preservation of the complex diversity and delicate balance of the microbial communities of the grains.

The fermentation of fresh milk is accomplished by the addition of the kefir grains, which may contain up to 27 bacterial species from genera including lactobacilli, lactococci, leuconostocs, acetobacter, enterococci and micrococci and up to 30 different yeast species from genera such as kluvermyces and sacromyces; the strains are bound together by the exopolysaccharide kefiran, which is produced by the bacterial species *Lactobacillus kefiranofaciens* (Kwak, et. al., 1996). The yellowish, white structures resemble small cauliflower florets and have a firm gel texture; the average kefir grain is approximately the size of a small marble with a weight of 0.5-1.5 grams, although individual grains can vary greatly in size and shape. The grains typically range in size from 0.3 to 3.5 cm in diameter; however some grains have been reported to grow much larger (Garrote, et. al., 1997). They are insoluble in water and ordinary solvents, and when immersed in milk the grains initiate

the dual lactic acid and alcohol fermentations (Kosikowski, et. al., 1999). Zourari, et. al. (1988) reports the chemical composition of kefir grains as 890-900 g/kg water, 2 g/kg lipid, 30 g/kg protein, 60 g/kg sugars and 7 g/kg ash.

The microflora of kefir grains is remarkably stable, retaining its activity for years if preserved and incubated under appropriate physiological conditions (Simova, et. al., 2002). According to Garrote, et. al. (1997), wet kefir grains will only retain activity for only 8-10 days (if not inoculated into fresh milk), while dried grains retain activity for 12-18 months.

Studies have shown that grains from different geographic regions vary widely in composition, which can result in large variance in the finished kefir products (Marshall, et. al., 1984; Pintado, et. al., 1996; Simova, et. al., 2002; Wang, et. al., 2008). In addition, the microbiologic study of kefir is complicated by the constant evolution of identification and the related nomenclature of bacteria, often causing difficulties when comparing data between labs or with previous reports (Farnsworth, 1999).

In studies preformed by Bottazzi, et. al. (1980), using electron microscopy to examine the structure and composition of kefir grains, it was suggested that the yeasts and lactobacilli are not randomly distributed in the grain, with the lactobacilli at the periphery of the grain and the majority of yeasts located inside the grain. The source of the kefir grains for this study were only identified as "obtained from a commercial source", which may pose problems as grains from various regions may vary structurally in terms of distribution of

the microflora as the species present varies between grains sourced from different sources/regions. In a 2005 study examining the microflora of Turkish kefir grains, Guzel-Seydem, et.al. found there to be a ratio of 10^9:10^6 of lactic acid bacteria and yeasts, with lactobacilli species predominating, and no significant fluctuation during storage. However, previous studies on Irish kefir grains, preformed by Rea, et. al. (1996) showed the contents of grains to be (cfu/ml) 10^9 lactococci, 10^8 leuconostocs, 10^6 lactobacilli, 10^5 acetic acid bacteria and 10^6 yeasts.

The weight percentage of kefir grains used to inoculate the milk has been shown to have a significant effect on the count of different microorganisms found in the finished product. When using a one percent by milk weight ratio of grains, lactobacilli and lactococci levels were found at the highest levels at the end of a thirty day storage period; when a five percent inoculate was used yeasts and acetic acid counts were highest (Irigoyen, et. al., 2005). Similar, earlier results by Koroleva (1988) also demonstrated that the number of lactic acid bacteria tended to increase when lesser amounts of kefir grains were inoculated into the milk.

1.6 Microflora

Isolation and identification of the different strains of bacteria and yeasts present in kefir grains has traditionally been performed using culture-dependent methods, meaning that the probiotic species must be grown on selective media with identification being based on morphological and biochemical characteristics (Simova, et. al., 2002 and Wang, et. al.,

2008). However, some studies have shown that many of the strains are very closely related and may pose problems when trying to isolate and identify individual strains (Micheli, et. al., 1999; Guzel-Seydim, et. al., 2005). More recent investigations have attempted to isolate strains based on genotype using polymerase chain reaction (PCR) combined with denaturing gradient gel electrophoresis (DGGE) (Wang, et. al., 2008). This culture-independent identification may be a useful in analyzing complex microbial populations because this type of testing does not require prior separation of individual strains, as in culture-dependent identifications (Ercolini, 2004).

1.6.1 Lactobacilli

Lactobacilli are present in the largest amounts (65-85%) of the microbial population(Witthuhn, et. al., 2004). In a 2011 study examining the microflora of Brazilian kefir and kefir grains, Magalhas, et. al. also found lactobacilli species to be the predominant lactic acid bacteria type (78%) in kefir fermented with kefir grains, with lactococci comprising the majority of the remaining 28 % of lactic acid species. Magalhas, et.al. also identified lactic acid bacteria isolates; *Lactobacillus paracasei* represented the largest and most commonly identified lactic acid bacteria isolate with a total of 89 of a total of 249 isolates. This was followed by *Lactobacilli parabuchneri* (41 isolates), *Lactobacilli casei* (32 isolates) and *Lactobacilli kefiri* (31 isolates).

1.6.2 Lactococci

Magalhas, et. al. (2011) was only able to isolate one species, *Lactococcus lactis*, from Brazilian kefir and kefir grains; this particular species was identified in all 24 lactococci

isolates from the total of 249 lactic acid bacteria isolates. In a 2005 study by Guzel-Seydim, et. al. a microbial enumeration and electron microscopy was performed on Turkish kefir and kefir grains; although long, short and curved lactobacilli and yeasts were found in all samples, lactococci were not observed in any portion of the kefir grain. They postulated that the presence of lactococci in the kefir but not in the grain samples may be caused by the unintentional removal of lactococci from the surface of the grains.

1.6.3 Yeasts

Magalhas, et. al. (2011) showed the majority of Brazilian kefir yeast isolates to be lactose-negative strains; specifically *Saccharomyces cerevisiae,* which made up 41 of the total 110 yeast isolates. Other non-lactose fermenting species isolated included *Kazachstania aerobia* (23 isolates) and *Lachancea meyersii* (15 isolates); these two species had been previously unreported in kefir and kefir grain studies. *Saccharomyces cerevisae* is an important yeast, in terms of the enhancement of the sensory qualities of the kefir beverage, as it promotes a strong and typically yeasty aroma, as well as a refreshing taste (Magalhaes, et. al., 2011). It is also worth noting that the presence of non-lactose fermenting yeasts in kefir and kefir grain is dependent on the presence of other lactose fermenting species of bacteria and yeasts capable of hydrolyzing the disaccharide, lactose (Simova, et. al., 2002).

According to Irigoyen, et. al. (2005), the levels of yeasts and acetic acid bacteria present in kefir are directly proportional to the quantity of grains inoculated. Interestingly, their study also found the levels of lactobacilli and lactococci to be inversely proportional to the amount of inoculate used; therefore, the number of microorganisms was higher when less

kefir grains were used. This might be due to a more rapid initial increase in the amount of lactic acid bacteria in the kefir inoculated with the higher percentage of grains; the higher number of initial bacteria might cause a quick, sharp drop in pH which would kill some of the more acid sensitive strains, thus preventing their growth during storage and allowing for an increased proliferation over time of yeasts and other types of bacteria, such as micrococci and acetic acid bacteria. It has also been shown that lactic acid bacteria multiply less rapidly, and therefore, produce lactic and acetic acids more slowly when incorporated into a mixture containing yeasts than in a pure culture (Collar, 1996).

During refrigerated storage of kefir lactic acid bacteria will begin to decrease, while the numbers of yeasts and acetic acid bacteria will remain fairly consistent. Irigoyen, et. al. (2005) found no significant differences in yeast counts during a thirty day storage period at approximately 5°C; however, lactobacilli and lactococci were shown to be significantly lower after thirty days of storage. This differs from another study by Guzel-Seydim, et. al. (2005) that examined the microbiota of Turkish kefir and kefir grains; the microbial counts of the lactic acid bacteria did not decrease, and actually exhibited continued growth during and after 21 days of refrigerated storage.

1.7 Kefiran

Exopolysaccharides produced by some lactic acid bacteria have been the recent focus of research in various food industries as a beneficial additive for increasing viscosity in products, and the stipulated health benefits associated with bacterial exopolysaccharides provide an added appeal to the consumer. In studies kefiran, a polysaccharide produced

and subsequently excreted by a certain strain of lactic acid producing bacteria found in kefir grains and kefir, was isolated and its composition and chemical structure were determined using methods such as acid and enzymatic hydrolysis. Although, at present, no studies isolating kefiran from the kefir beverage have been reported, Cerning, et. al. (1999) listed the range amount of exopolysaccharides produced by lactic acid bacteria in fermented products as 25 to 890 mg/L.

The structure of kefiran was described by Riviere, et. al. (1967) as a water-soluble glucogalactan containing roughly equal amount of D-glucose and D-galactose residues. They also found *Lactobacillus brevis*, the strain thought to produce kefiran, to comprise at least 24% of the dry material of the kefir grain. Although the chemical analysis preformed by Riviere, et. al. (1967) was accurate, the bacterium has, in recent years been referred to as *Lactobacillus kefir* and currently *Lactobacillus kefiranofacians* (Frengova, et, al., 2002 and Cheirsilp, et. al., 2003). However, there are conflicting opinions regarding the naming of this bacterium and if the *Lactobacillus kefiran* and *Lactobacillus kefiranofacians* are the same strain or two different strains, with one or both producing kefiran in differing amounts (Kandler, et. al., 1983; Frengova, et. al., 2002; Rimada, et. al., 2002; Piermaria, et. al., 2009).

Different conditions such as fermentation time and temperature and storage time and incubation and storage temperature affect the amount of exopolysaccharide produced by certain strains (van Geel-Shutten, et. al., 1998). Mozzi, et. al. (1995) found that by increasing the time of fermentation up to 72 hours, a marked reduction in the

exopolysaccharide synthesis was observed; these results were dependent both on the temperature tested (30, 37 and 42°C) and the strain of lactic acid bacteria employed.

The kefir producing strain, *Lactobacillus kefiranofaciens*, has also been shown to produce kefiran at a significantly higher rate when in a mixed culture containing *Saccharomyces cerevisiae* when compared with those in pure cultures (Cheirslip, et. al., 2003 and Cheirslip, et. al., 2003); some yeast species present are able to metabolize some of the lactic acid produced by the bacteria, therefore enhancing the survivability of the lactic acid bacteria by the reduction metabolic end products.

Exopolysaccharides, similar to kefiran, have also been isolated, although in lesser amounts, from other lactic acid species such as *Lactobacillus reuteri* and *Lactococcus lactis* ssp. *cremoris* (van-Geel Schutten, et. al., 1998 and Yang, et. al., 1999). Other examples of polysaccharide use in the food industry are xanthan produced by *Xanthomonas campestris* and gellan from *Pseudomonas eloda* (Matsukawa, et. al., 2007).

1.8 Fermentation and Production of Flavor Compounds

During kefir manufacture with the grains, the lactic acid fermentation slows considerably or stops as the pH declines, but the yeast fermentations continue allowing for an increase in ethanol production during storage. The secondary alcohol fermentations can lead to substantial changes in flavor as well as bulging or leaking packaging due to the continued production of carbon dioxide gas (Kwak, et. al., 1996).

The major end products, according to Kooman (1968), are approximately 0.8% lactic acid, 1.0% ethyl alcohol and carbon dioxide. Also present in smaller amounts are acetic acid, numerous volatile flavor compounds such as diacetyl and acetylaldehyde, exopolysaccharides, organic acids, and various vitamins and minerals. The optimum taste profile in commercial kefir has a 3:1 diacetyl to acetylaldehyde ratio with a pH of 4.6 using milk with an initial fat content of no less than 3.0% (Kosikowski, et. al., 1997). The typical flavor of kefir can be attributed to an optimum ratio of 3:1 diacetyl to acetaldehyde, and although complex alcohols and acetone have also been identified as end products, they are not thought to be predominant factors in the flavor profile (Kosikowski, et. al., 1999).

The fat content of kefir may range from 0.5 to 3.0 percent, with solids not from fat from 8.0 to 11.0 percent (Kosikowski, et. al., 1999). The fat content will vary depending on the original fat content of the milk used (whole vs. skim, bovine vs. caprine) as well as the storage time of the finished kefir. In a study involving the physiochemical analysis of milk, Irigoyen, et. al. (2005) found that the fat content of the finished kefir did not differ significantly from the fat content of the milk that the kefir was made from. Oxidation of the fat molecules and the off-flavors associated with lipid oxidation can be counteracted to some degree by the microorganisms in kefir (as well as other fermented milks). The lactic acid bacteria consume oxygen providing a reducing effect (Dairy Handbook).

During storage lipolysis, the breakdown of fats into glycerol and free fatty acids will also occur, contributing to an increase in free low-molecular weight free fatty acids (Dairy Handbook). The release of capric acid, the most abundant saturated fatty acid found in

goat milk, from the triglyceride will result in a distinctive goat milk flavor, often characterized as a "goaty" or "musky" flavor.

Saccharomyces cerevisiae is an important yeast to note, in terms of the enhancement of the sensory qualities of the kefir beverage, as it promotes a strong and typically yeasty aroma, as well as a refreshing and pungent taste (Magahlaes, et. al., 2010). The reduction of excess lactic acid, the removal of hydrogen peroxide and the production of compounds that can help stimulate bacterial growth, and possibly increase kefiran production, has been demonstrated by *Saccharomyces cerevisae* (Cheirsilp, et. al., 2003).

1.9 Health Benefits

In order for a probiotic to benefit human health it must have good technological properties, survive through the upper gastrointestinal tract and be able to function in the gut environment (Mattila-Sandholm, et. al., 2002). These properties, as well as many health benefits, have been examined, and kefir has demonstrated a wide array of positive effects such as antitumor and immunostimulating activity in animals (Quiros, et. al., 2005); these effects are only seen when the probiotics ingested are functioning properly in the intestinal mucosa. Antioxidant action, antibacterial and antifungal properties have been also been observed (Zacconi, et. al., 2003; Rodrigues, et. al., 2004, Medrano, et. al., 2006; Ismaiel, et. al., 2011). Both prebiotic and probiotic benefits are incurred by the consumer, including: competitive exclusion of pathogenic bacteria, increased absorption of nutrients, and immunomodulating effects such as the modification of the balance of

immune cells in the intestinal mucosa (Vinderola, et. al., 2006; Maalouf, et. al., 2011; Medrano, 2011).

The probiotic organisms can exert their beneficial properties through two mechanisms: direct effects of the live microbial cells (probiotics) or indirect effects via metabolites of these cells (biogenics) (Vinderola., et. al., 2004). Biogenics are defined as food components that are derived from microbial activity which provide health benefits without involving the intestinal microflora (Takano, 2002).

Several strains of *Lactobacillus delbrueckii* and *Streptococcus thermophilus* produce extracellular polysaccharides (Hong and Marshall, 2001). In kefir, these loosely bound exopolysaccharides can act as a stabilizer, preventing syneresis and graininess and provides a natural thickening effect (Cerning, 1990), and, in regards to health can provide benefits such as aiding in bacterial adhesion to the lining of the gut and protection of probiotics during transit though the gastrointestinal tract.

There are also the beneficial effects on the properties of the milk due to the fermentation by the probiotic organisms, such as an increased protein content (Magalhaes, et. al., 2011). The increased biomass of the microbes and the production of cellular proteins, peptides, as well as free amino acids, that are subsequently released into the kefir beverage, makes this fermented beverage great source of nutrients required for muscle synthesis and regeneration, and could be used as a natural, minimally processed, protein rich supplement.

Kefir exhibits numerous biological activities that include antibacterial and antifungal properties, in addition to other immunostimulating benefits of probiotics (Farnsworth, et. al., 2003). In a 2004 study, Rodrigues, et. al. demonstrated the antimicrobial and healing activity of kefir and kefiran extract; they showed that successful and faster wound healing occurred in rats when a topical kefiran mixture was used as alternative to antibiotics. Numerous studies have demonstrated that antibacterial, antimycotic and antitumor activity of cells increases when exposed to kefir and kefiran (Garrote, et. al., 2004; Micheli, et. al., 1999; Frengova, et. al., 2002).

In addition to these healthful properties acquired by kefir during fermentation, there are also many beneficial properties intrinsic to the milk itself. For example, the large amounts of medium and short chain fatty acids found in goat milk are known to aid in digestion and utilization of lipids (Kalser, 1971 and Babayan, et. al., 1981).

1.10 Commercial Manufacture

Commercial kefir production utilizes a dry starter culture usually consisting of up to 12 species isolated from lyophilized kefir grains. A new commercial starter, whether dry or from a liquid mother culture, must be added to each new batch of milk for kefir production.

Studies preformed by Simova, et. al. (2002) demonstrated that traditionally produced kefir (with the starter grains removed) could not be used as a starter culture for kefir.

The primary drawback, other than increased labor costs, for the large scale manufacture and marketing of traditionally produced kefir is that secondary alcohol fermentation often occurs at the distribution and storage phases, resulting in changes in flavor and taste because of the continued formation of ethanol and carbon dioxide gas (Kwak, et. al., 1996). This can result in swollen and leaking containers as the excess gas increases the internal pressure of the container. However, the most significant difference in the compositional and sensory aspects of the commercial and traditionally produced kefir is an increase in ethanol and CO_2 production in the commercial kefir.

A study by Beshkova, et. al. (2002) showed and increase in ethanol from 0.25% in traditional to 0.48% in commercial kefir, and an increase in CO_2 of 1.05g/l to 1.98 g/l. Kwak, et. al. showed that biostabilization of kefir can be achieved by using a starter culture in which a non-lactose fermenting yeast, such as *Saccharomyces cerevisiae,* is present in substantial numbers. In fact, the standardization of the production of flavor compounds, including lactic acid and ethanol, has been achieved, for the most part by the utilization of pure cultures as a starter for the commercial production of kefir. Gronnevik, et. al. (2011) showed that the levels of lactic acid and ethanol in Norwegian commercial kefir remained consistent during an 8 week storage period, with some volatile compounds fluctuating slightly; this was probably due to an increase in the yeast metabolism of bacterial intermediate or end products. This can be compared to studies preformed on the physicochemical changes in milk during the production and storage of traditional kefir, which were found to be in flux and somewhat inconsistent between kefir

made from differently sourced grains (Wszolek, et. al., 2001; Chen, et. al., 2005; Irigoyen, et. al., 2005; Magalhaes, et. al., 2011).

The development of commercial starter cultures has allowed for widespread distribution of kefir and kefir products; however, the demand for traditionally produced kefir is rising, and methods for producing a consistent product with an adequate shelf life are being developed. In fact, the optimum taste profile in commercial kefir has a 3:1 diacetyl to acetylaldehyde ratio with a pH of 4.6 using milk with an initial fat content of no less than 3.0% (Kosikowski, et. al., 1997). This indicates that the consumer preference might be for a product with a flavor akin to traditionally produced kefir.

1.11 Frozen Dairy Products

Organic acids and volatile flavor compounds produced during the fermentation and storage of kefir can have a profound effect on the flavor profile and can greatly affect the consumer. Because taste preferences are met by traditionally produced kefir and because of possible added health benefits of traditional over commercial kefir, frozen storage and transport could serve as an alternative solution to the problems typically associated with traditionally produced kefir.

Microorganisms present in a cultured dairy product have a high survivability rate, especially when the fermented product is incorporated into a mixture containing 10% sucrose (Miles, et. al., 1981); however, numerous reports have observed structural damage to living lactobacilli cells when subjected to freezing and thawing (Breunan, et.

al., 1986; Valdez, et. al., 1993; Lopez, et. al., 1998). Therefore, other considerations, such as the freezing method, must also be taken into account when attempting to provide a product with the highest number of surviving probiotics. For example, freezing in a soft serve freezer, with the added agitation and scraping action has shown to decrease the number of viable bacteria in soft serve frozen yogurt by a reduction of at least 1.5 log; this can be compared to freezing without the soft serve machine, after which no effects on cell numbers were observed after 3 months of frozen storage (Thompson, et. al., 1994).

Exopolysaccharides, such as kefiran, might also serve to enhance the survival of probiotic organisms in a frozen dessert by providing a protective coat that may help to ameliorate the harsh conditions associated with freezing and thawing. In fact, a study by Monnet, et. al. (2003) showed a significantly higher cryotolerance during freezing of *Lactobacillus delbrueckii* strains with a mutation causing an excess production of exopolysaccharide. Another study by Shah, et. al. (2000), reported counts of *Lactobacillus acidophilus* increased from $<10^3$ cfu/g in the control batch (non-encapsulated) to $>10^5$ cfu/g (encapsulated cells).

A major determination in the purchase of fermented dairy products, especially frozen desserts, is the ability of the consumer to digest the milk sugar lactose. In any fermented product there is still likely to be a percentage of lactose present; and in frozen yogurt products that add unfermented milk to the mix (mainly to reduce perceived acidity), this can be a major problem for those seeking a lactose free product. Fortunately, viable lactic acid producing bacteria can be a substantial source of β-galactosidase, the enzyme

responsible for the degradation of lactose into in monomers. A 2001 study by Hong, et. al. showed that cultures having the highest β-galactosidase activity were better able to reduce to amount of lactose in the final product; this enzyme retained some activity even during frozen storage.

Consumer acceptability of acidified dairy foods is typically high in sensory tests conducted on other frozen dairy desserts, such as frozen yogurt (Guinard, et. al., 1994). This same study also showed that the most preferred samples of frozen yogurt were the ones with the lowest acidity; these results suggest that an ideal frozen dairy dessert, for most consumers, should combine the sensory properties of ice cream and the nutritional benefits of yogurt (Guinard, et. al., 1994). However, there are many consumers who enjoy the pronounced acidity and complex flavor profile, described as "yeasty" and "prickling", associated with traditional kefir. Flavored traditional kefir, which scored high during sensory studies, might be more acceptable to the Western palate than unflavored kefir (Muir, et. al., 1999).

The objective of this project was to quantify viable probiotic bacteria and yeasts in traditionally and commercially produced kefir following various periods of frozen storage.

CHAPTER 2. MATERIALS AND METHODS

2.1 Experimental Design

Two types of kefir were made; one was traditionally produced by inoculation of milk with kefir grains, and the second was made by inoculating milk with a commercial kefir starter. Once fermented, the kefir was frozen in several aliquots, which were removed and thawed at multiple time intervals. One sample for each kefir type was left unfrozen at room temperature and served as the experimental control. The samples were then tested for three different types of probiotics: lactobacilli, lactococci and yeasts. The entire experiment was repeated in triplicate.

2.2 Sample Preparation

Traditional kefir was be prepared by inoculating one liter of full fat (four to five percent), pasteurized goats' milk (Ryals Goat Dairy, Tylertown, MS) with kefir grains in a liter sized glass jar. The kefir grains used in this research were obtained from a household in Louisiana, USA; approximately thirty grams of kefir grains were added to one liter of milk to give a three to five percent ratio of kefir grains to milk as described by Chen, et. al. (2005). The grains were cultivated, using this method and with the addition of fresh milk weekly, in the Louisiana State University Dairy Science Building for several months before experimental use.

Commercial kefir was prepared by inoculating one liter of full fat, pasteurized goat milk with a commercial kefir starter (Lifeway Foods, Morton Grove, IL, USA) in a liter sized glass jar. The commercial culture used in this study contained the following twelve

microorganisms: *Lactobacillus lactis*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus casei, Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Streptococcus diacetylactis*, *Leuconostoc cremoris*, *Bifodobacterium longum*, *Bifodobacterium breve, Bifidobacterium lactis* and *Saccharomyces florentinus.*

The milk was allowed to ferment at room temperature (24-28°C) and was agitated by manually shaking every few hours for approximately 24 hours to ensure proper mixing of the grains and milk. The kefir fermentation was considered complete when a pH of 4.6 was reached. The grains used to ferment the traditional kefir were recovered by straining the kefir through a fine mesh sieve.

Three 50 g samples of both the traditional and commercial kefirs were collected in separate clear, food-grade plastic containers before storage at -14±6°C, the temperature range that includes most household freezers. The samples were frozen immediately following the completion of fermentation (approximately 24 hours). The samples were thawed and plated on day 7, day 14 and day 30 of frozen storage. One additional sample was not frozen and was used as the control for each replication of the experiment; this sample was plated for probiotic microorganisms immediately following fermentation. The frozen samples were allowed to thaw at room temperature for 4 hours and were incubated at 37°C for 1 hour before plating (Hong, et. al., 2001).

2.3 Microbiological Enumeration

In order to quantify the amounts of probiotic bacteria and yeasts in each sample, serial dilutions were made using 0.1% peptone water (Becton, Dickinson and Company, Sparks, MD, USA) (Mian, et. al., 1997). The peptone water was sterilized by autoclaving at 121°C for 15 minutes; the peptone was cooled to approximately 27°C and was inoculated with 1% v/v kefir and further diluted to 10^{-10}.

The kefir samples were plated for *Lactobacilli* and *Lactococci* using deMan, Rogasa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) and M17 (Becton, Dickinson and Company) agars (Witthuhn, et. al., 2005 and Garcia Fontan, et. al., 2006). To prevent the growth of yeasts on the bacterial plates 200 mg L^{-1} cycloheximide (Acros, Geel, Belgium) was added to the MRS and M17 agars (Chen, Wang and Chen, 2008). Several dilutions of each sample were plated and each dilution was plated in triplicate. The MRS and M17 plates were incubated anaerobically for 72 and 48 hours at 32°C (Irigoyen, et. al., 2005).

Yeasts were grown on Yeast Extract Glucose Chloramphenicol (YGC) agar (Merck) for 5 days at 25°C under aerobic conditions (Gronnevik, et. al., 2011). Following incubation, growth was determined by counting the number of bacterial and yeast colonies on each plate; colony totals were presented as colony forming units per milliliter of kefir (CFU/mL).

2.4 Statistical Analysis

Statistical analysis was preformed using the analysis of variance (ANOVA) f-test, with a confidence interval of 95% (P<0.05). Means were compared by the least significant difference (LSD) test. A regression analysis was used to determine the rate of microbial reduction as represented by the slope of the regression. Statistical analysis was performed using the Statistical Analysis Software (SAS®) Software Package Version v9.3 (SAS® Institute, 2010).

CHAPTER 3. RESULTS

The overall rate of reduction for lactobacilli in the traditional and commercial kefir was found to be significantly (P<0.05) different (Table 1). The lactobacilli populations found in the traditional kefir decreased at a slower rate than the lactobacilli populations in the commercial kefir during frozen storage (Table 2). The rate of decline of yeasts in the traditional kefir was also significantly (P<0.05) lower than the rate of decline in the commercial kefir; the reduction rates of the lactococci in the traditional and commercial kefirs were not found to be significantly (P<0.05) different.

Table 1 shows that the lactococci populations in the traditional and commercial kefir were found to decrease at rates that were not significantly (P<0.05) different. Because no interaction was observed between the two kefir type; however, the lactococci population the traditional kefir was still found to be significantly (P<0.05) higher after 30 days of frozen storage, when compared to lactococci in commercial kefir.

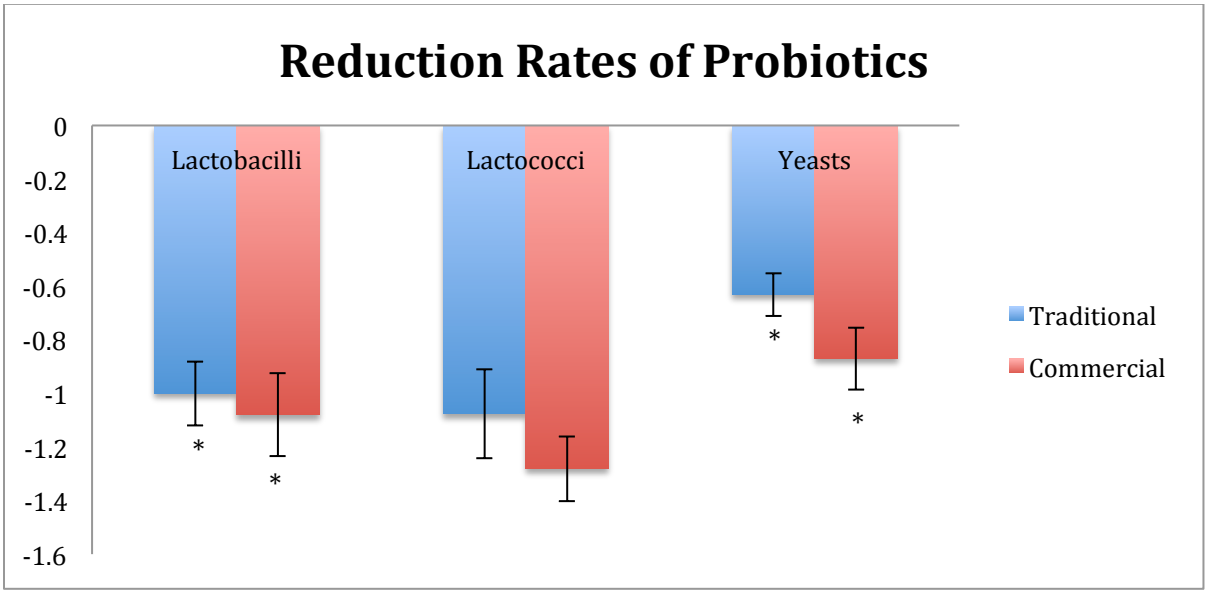
The reduction rate of yeasts found in the traditional kefir was found to be significantly (P<0.05) different from the reduction rate of yeasts in commercial kefir. This can be seen in Table 1 by the interaction of the Type*Storage treatments of two kefir types.

Table 1. The effects of type and storage treatments on the reduction rate of microorganisms

EFFECT	Lactobacilli	Lactococci	Yeasts
	Pr > F	Pr > F	Pr > F
Type	0.0191	0.0188	<0.0001
Storage	<0.0001	<0.0001	<0.0001
Type*Storage	<0.0001	0.5800	0.0037

Type = traditional and commercial kefir
Storage = 30 days

Once the interactions between the effects of storage period and the type of kefir were determined, the individual rates of microbial reduction all three probiotic types between the traditional and commercial kefir were compared. Figure 1 presents rates of microbial reduction (slope) during the entirety of frozen storage.



* indicates values that are significantly (P<0.05) different

Figure 1. Reduction rates of lactobacilli, lactococci and yeast populations in traditional and commercial kefir during 30 days of frozen storage

3.1 Lactobacilli

When subjected to frozen storage conditions, the lactobacilli in the traditionally prepared kefir showed a significant decrease in number after storage for 30 days, and significant (P<0.05) differences were found between bacterial counts in the traditional kefir at all time intervals tested (Table 3). The lactobacilli in the commercial kefir was also significantly (P<0.05) reduced at the end of the storage period and significant differences were found at all time intervals.

Table 2. Mean counts (log cfu/ml) of viable lactobacilli following storage periods

Treatment (kefir type)	Lactobacilli			
	Control	Day 7	Day 14	Day 30
Traditional	$10.41^{A,a}$	$8.48^{B,a}$	$8.00^{C,a}$	$7.24^{D,a}$
Commercial	$9.15^{A,b}$	$8.95^{B,b}$	$6.61^{C,b}$	$6.33^{D,b}$

All values evaluated at confidence interval P<0.05

^{ABCD}Values with the same letter within the row are not significantly different

^{ab}Values with the same letter within the column are not significantly different

In a 2005, Guzel-Seydem, et.al. found there to be 10^9 of lactic acid bacteria in Turkish kefir, with lactobacilli species predominating. However, previous studies on Irish kefir grains, preformed by Rea, et. al. (1996) showed that the grains, as well as the fermented milk, contained (cfu/ml) 10^6 lactobacilli.

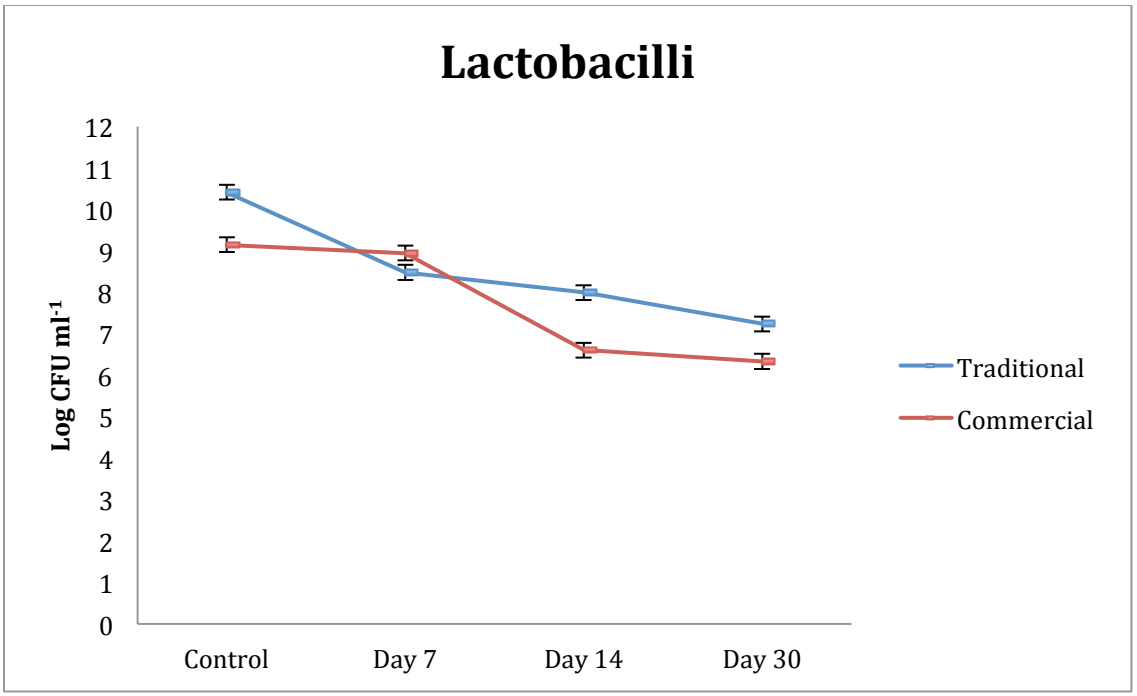


Figure 2. Reduction of lactobacilli in traditional and commercial kefir during 30 days of frozen storage

The findings presented in this study show the amount of lactobacilli present in the commercial kefir is consistent with several previous studies (Marshall, et. al., 1985; Garrote, et. al., 1998; Fontan, et. al., 2006); they also found the counts of presumptive lactobacilli obtained from kefir produced with grains were on the order of one log higher than lactobacilli from kefir produced with a commercial starter. Witthuhn et. al. (2005) reported varying lactobacilli numbers during kefir production between $4.6x10^3$ and $2.6x10^8$. These numbers reflect kefir produced commercially and traditionally, with the

traditional kefir consistently representing the larger values, meaning that traditional kefir was consistently found to have an higher overall microbial load when compared to commercial kefir.

3.2 Lactococci

When subjected to frozen storage conditions, the lactococci in the traditionally prepared kefir showed a significant (P<0.05) decrease in number after storage for 30 days, and significant (P<0.05) differences were found between all time intervals tested (Table 3). Significant (P<0.05) differences in both the overall reduction at the end of the 30 day storage period and the reduction between each storage interval were also observed in the commercial kefir (P<0.05).

Table 3. Mean counts (log cfu/ml) of viable lactococci following storage periods

Treatment (kefir type)	Lactococci			
	Control	Day 7	Day 14	Day 30
Traditional	$9.32^{A,a}$	$8.87^{B,a}$	$7.36^{C,a}$	$6.24^{D,a}$
Commercial	$9.00^{A,b}$	$8.71^{B,b}$	$6.55^{C,b}$	$5.44^{D,b}$

All values evaluated at confidence interval P<0.05

^{ABCD}Values with the same letter within the row are not significantly different

^{ab}Values with the same letter within the column are not significantly different

Results presented by Rea, et. al. (1996) showed the contents of Irish kefir grains to be (cfu/ml) 10^9 lactococci. This is consistent with the numbers of lactococci observed in this study, but the counts reported here (10^9 cfu/g) were two logs lower than in studies by Beshkova, et. al. (2002) who reported cells counts of 10^{11} cuf/g for both traditional and commercial kefir immediately following fermentation. In a 2005 study by Guzel-Seydim, et. al., a microbial enumeration and electron microscopy was performed on Turkish kefir and kefir grains and lactococci were not observed in any portion of the kefir grain; however, lactococci was enumerated to 10^8 in the traditional kefir beverage.

The absence of lactococci in the kefir grain, and its subsequent growth in fermenting kefir, may be caused by the unintentional removal of lactococci from the surface of the grains during manufacture into the milk medium where it is able to proliferate; this would most likely occur due to agitation during the manufacture process (Guzel-Seydim, et. al., 2005). Because the lactococci present in kefir are not known to produce any exopolysaccharides that may help with adhesion to the grain, they may not be able to attach and become incorporated into the kefir grain.

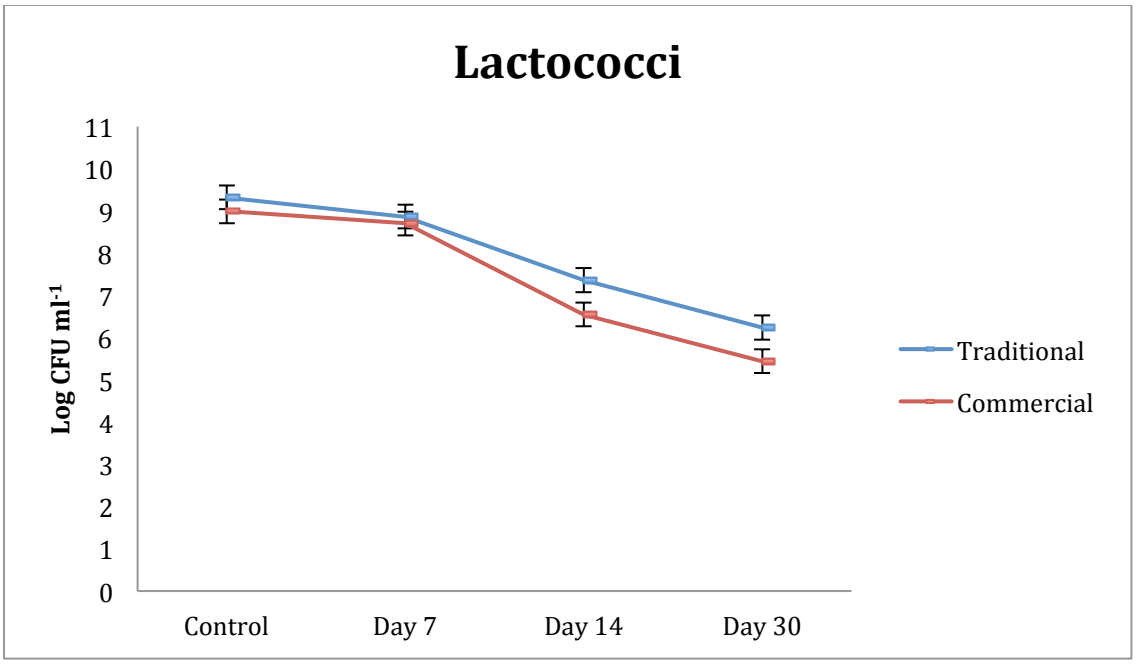


Figure 3. Reduction of lactococci in traditional and commercial kefir during 30 days of frozen storage

The rate of decrease of lactococci bacteria in the traditional kefir was not significantly (P<0.05) different from the rate of decrease of lactococci in the commercial kefir. Although the traditional kefir ultimately contained more lactococci at the end of the 30 days of frozen storage, the rate of lactococci decline in the commercial kefir was not significantly (P<0.05) higher than the rate of decline in the traditional kefir; it can, therefore, be inferred that there were no benefits incurred by the lactococci, in terms of overall survivability, by any intrinsic protective agent, such as an exopolysaccharide, that might be present the traditional kefir.

3.3 Yeasts

When subjected to frozen storage conditions, the yeasts in the traditionally prepared kefir showed a significant ($P<0.05$) decrease in number after storage for 30 days, and significant ($P<0.05$) differences of yeast numbers in traditional kefir were found between all time intervals tested (Table 5). The commercial kefir was also shown to have significant ($P<0.05$) reductions in viable yeasts between intervals and following the full 30 days of frozen storage.

Table 4. Mean counts (log cfu/ml) of viable yeasts following storage period

Treatment (kefir type)	Yeasts			
	Control	Day 7	Day 14	Day 30
Traditional	$8.83^{A,a}$	$8.40^{B,a}$	$8.13^{C,a}$	$6.82^{D,a}$
Commercial	$7.20^{A,b}$	$5.56^{B,b}$	$5.32^{C,b}$	$4.38^{D,b}$

All values evaluated at confidence interval $P<0.05$

^{ABCD}Values with the same letter within the row are not significantly different

^{ab}Values with the same letter within the column are not significantly different

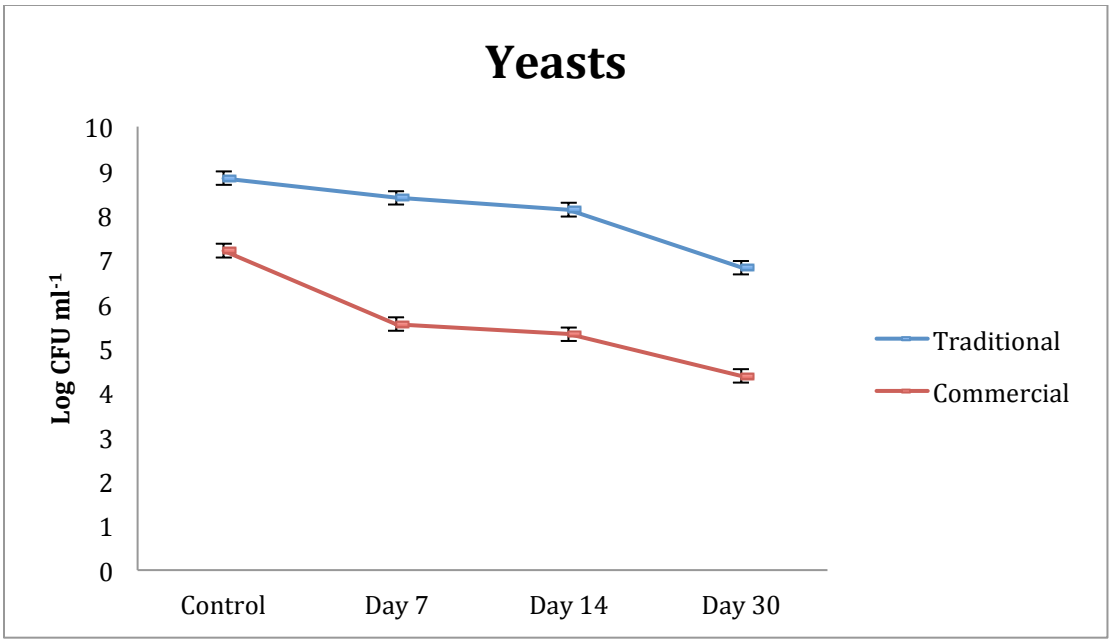


Figure 4. Reduction of yeasts in traditional and commercial kefir during 30 days of frozen storage

The population of yeasts was lower than the lactic acid populations in both the traditional and commercial kefir. The initial counts of 10^8 cfu/g in traditional kefir observed were higher than the 10^7 cfu/g found by Wang, et. al. (2008); however, these results are lower than previous yeast counts in traditional kefir of 10^5 cfu/g, as reported by Beshkova, et. al. (2002). Fermentation conditions, most likely a fluctuating decrease in ambient temperature, could have resulted in a more favorable environment for the yeasts and caused the high numbers in this study. However, the commercial kefir is consistent with findings by Beshkova, et. al. (2002), who report the total number of yeasts as 10^6-10^7 cfu/g in kefir made with pure cultures. During storage under refrigeration the yeast populations see a marked growth in traditional kefir due to their increased consumption of bacterial metabolites (Guzel-Seydim, et. al., 2000). The quantity of yeasts in this study correlate more directly with the yeast counts observed directly after

fermentation; therefore, it would be expected that the amount of yeasts in frozen kefir would be lower than an unfrozen, stored kefir.

CHAPTER 4: DISCUSSION

The concentration of probiotics in commercial dairy products is usually in the range of 10^8-10^9 cfu/ml (Rokka, et. al., 2010), which is somewhat higher than 10^5-10^7 cfu/ml, the range thought to be beneficial as a human health supplement (Kurmann, et. al., 1991). However, the actual amount of probiotic cells viable in a fermented dairy product, at the time of consumption and after transit through the digestive tract, is quite difficult to predict due the many variables and conditions associated with processing and digestion, but because kefir, especially traditionally produced, has a wide range of different bacterial strains and species, the adhesion to the intestinal mucosa might be improved over probiotic products with only one or two bacterial strains. Findings by Collado, et. al. (2007) have suggested that different probiotic combinations may enhance beneficial health effects due to synergistic adhesion effects; the combination of complimenting probiotic system and the protective nature incurred by an exopolysaccharide encapsulated cell could greatly increase the efficacy of a probiotic product, such as kefir.

Kefiran, in regards to survivability and its ability to infer positive intestinal health benefits, may aid in the ability of certain probiotic species to flourish due to a protective factor incurred by this this exopolysaccharide. The presented results showed a higher survivability of both the lactobacilli and the yeasts in traditional kefir during freeze thaw conditions when compared to the commercial kefir; the lactobacilli that secrete the polysaccharides are in close proximity with the yeasts, and they together make up the majority of the permanent microflora of the kefir grain; and could help to explain the enhanced the survivability of the yeasts during freezing in the traditional kefir. The

survival of bacteria during frozen storage and subsequent exposure to the harsh conditions of the upper digestive tract, as well as the ability to adhere to and exclude enteropathogenic strains from the intestinal wall is greatly enhanced by the presence of naturally produced exopolysaccharides (Ruas-Madiedo, et. al., 2006).

Because the origins of lactic acid bacteria and yeasts are not human associated, they can be sensitive to the harsh conditions of the stomach and bile acids. This aspect becomes president when considering the health benefits incurred by the consumer of probiotic containing products. It has been shown that both the strain and the amount of probiotic species play a role in determining survival in the human gut (Elli, et. al., 2006 and Conway, et. al., 1987); and the adhesion to intestinal epithelial cells may play a critical role in the immunostimulating and immunomodulating effects of certain probiotic species. However, in vivo adhesion will most likely be influenced by both the normal microbiota and the specific probiotics included, but only few studies to date have directly examined the adhesion interactions of the probiotics and the intestinal mucosa (Ouwehand, et. al., 2000 and Collado, et. al., 2007).

During preliminary laboratory work in the process of designing this study, several attempts at isolation and purification of kefiran from both traditional and commercial kefir beverages were made. Precipitation of the exopolysaccharide with ethanol followed by distillation (further purification) as described by Rimada, et. al. (2003) yielded no quantifiable amount of exopolysaccharide. This may be explained by the low inoculation ratio in regards to achieving maximum amounts of kefiran production. In the 2003 study

by Rimada, et. al., the greatest amount of kefiran production was achieved by inoculating 1 L of milk with 100 g of kefir grains; the present study used only the standard inoculation ratio of 3% w/w. The amount of kefiran produced in the traditional kefir might not have been enough to quantify, but enough exopolysaccharide is being produced to maintain grain integrity and provide some advantage to the certain bacteria and yeast populations.

The activity of the grains, or more precisely, the activity of the kefiran producing strains also plays a major role in the amount of kefiran present in the product. Schoevers, et. al. (2003) has reported a lowering of lactobacilli species present, as well as cellular activity, in some the kefir grains that have been subjected to prolonged storage without fresh milk or exposure to widely varying environmental conditions, such as fluctuating incubation temperatures or a change in milk type.

There are several materials used commercially in the encapsulation of probiotic bacteria such as carrageen and alginate derived polysaccharides, starch, gum Arabic and other plant derivatives, gellan and xanthan produced from bacteria and animal proteins like casein and gelatin (Rokka, et. al., 2010). A study by Sheu, et. al. (1993) showed that when encapsulated in an alginate gel, two strains of *Lactobacilli bulgaricus* maintained viable counts that were forty five percent higher than the bacterial strains without the gel, following two weeks of frozen storage. Another study examining encapsulated *Streptococcus thermophilus* cells showed them to be more resistant to freezing and frozen storage than non-encapsulated strains (Hong, 1995). The kefiran could be acting in a

similar manner to the alginate gel and other exopolysaccharides by encapsulating the individual cells thereby limiting the susceptibility of the lactobacilli to freeze damage.

It could be suggested that the lactobacilli also aids in the protection of the yeasts to freeze thaw conditions due to the close proximity of the yeasts to the kefiran producing lactobacilli in the grain; the exopolysaccharide might attach to and coat the bacteria and yeasts and allow a greater number to survive in a frozen dairy product and subsequently through the gastrointestinal tract of the consumer. The pure strains of lactobacilli used in the commercial kefir starter culture mixtures have not been able to produce kefiran and this exopolysaccharide cannot be isolated from commercial kefir; therefore, a protective factor for these probiotic species may be lacking.

Sheu, et.al. (1995) also showed that when subjected to freeze thaw conditions, the larger of two *Lactobacillus bulgaricus* strains demonstrated less resistance and declined at a more rapid rate than the smaller cells of the second *Lactobacillus bulgaricus* strain, suggesting that the stresses of freezing are more damaging to larger cells. This means that yeast cells are more likely be damaged during freezing than the relatively small bacteria (0.75-1.5μm) (Kokkinosa, et. al., 1998), due to their larger size (2-10 μm) (Hill-MaGraw, 1997). These finding may help to further explain why the yeasts, which are typically much larger than bacterial cells, found in the traditional kefir had higher survivability during frozen storage as compared to the commercial kefir; the yeasts in the traditional kefir may have incurred some of the protective benefits seen the lactobacilli populations.

The lactococci make up a smaller percentage due to the fact that they are more readily expelled from the grain and reproduce primarily in the liquid medium (Witthun, et. al, 2005; Guzel-Seydim, et. al., 2005). This may be the primary reason that the rates of reduction of lactococci populations in the traditional and commercial kefirs did not differ significantly during any of frozen intervals and at final 30 day sampling.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

Research in the field of microencapsulation of lactic acid bacteria has been steadily increasing as more and more food manufactures are looking for a way to add and enhance the viability of probiotics to many types of products. The cost of growth, isolation and purification of naturally (bacterially) produced exopolysaccharides is very high and has thus far been the limiting factor in their addition into products on a large scale. However, a naturally occurring polysaccharide, that is produced in enough quantity to provide a protective benefit to the probiotic species and is already present in the fermented milk, would greatly enhance, at no extra cost to the producer, the viability of the product's microflora. This would make a frozen kefir dessert, made with traditionally produced kefir, an inexpensive way to improve dairy probiotic consumption and increase the marketability of probiotic dairy products.

Future studies examining the association of the kefiran producing lactobacilli with the yeasts found in kefir grains and in kefired milk would be helpful in determining how the microbiota in the grain function synergistically to adapt and survive in a relatively wide range of environmental conditions. The effects of freezing kefir that has been formulated into a mix, containing sugar and other flavorings, and freezing method must also be examined to more accurately predict the probiotic counts that will be present in the finished product and available to the consumer. The added agitation and scraping of freezer barrel walls needed to achieve proper overrun in frozen dairy desserts may lower the rate of survival, and cellular exposure to oxygen during whipping might also increase cell death due to exposure to free radicals (Marshall, 2001). These variables must be

considered when determining the full protective potential of naturally occurring exopolysaccharides on the probiotics.

The constantly shifting ecology that is unique to fermented milk further enhances the total numbers viable bacteria by ensuring, with a very wide range of species, that a high percentage of diverse populations will survive conditions such as freezing, thawing and exposure to acids and bile salts required for digestion.

Numerous studies have found that the total numbers of viable bacteria found in milk fermented with kefir grains to be greater than kefir made with isolated starter cultures (Marshall, et. al., 1985; Duitschaever, et. al., 1988; Marshall, 1993); this would provide advantageous during periods of cold storage, where the microbial counts are likely to be reduced. However, lactic acid bacteria has been shown to be remarkably stable during long periods of frozen storage; in a study by Lopez, et. al. (1998), lactic acid bacteria did not suffer any significant reduction in lactic acid bacteria during four months of storage at -23°C and retained a log count of around 10^7 cfu/g for the entire period.

The exopolysaccharide, kefiran, produced by a strain specific to traditionally manufactured kefir, has been shown to aid in the colonization of the gut with beneficial bacteria and yeasts by providing adhesion of probiotic species to the epithelium. For these reasons, the consumption of traditionally produced kefir might be preferred over kefir produced from isolated starter cultures, as a way to ensure greater survival and wider range of probiotic species. A frozen product made from traditional kefir would

provide a microbial load great enough to be considered a beneficial supplement to the consumer, and the distribution problems typically associated with refrigerated transport and storage would be eliminated.

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VITA

Keely Virginia O'Brien graduated *Magna Cum Laude* from the University of Tennessee at Chattanooga in 2007 earning a bachelor's degree in biological sciences with a minor in chemistry. At UTC Keely served as the president of the Biology Honor Society, Beta Beta Beta and was a member of several academic and service honor societies including Mortar Board and Greenspaces. In 2006 and 2007 she was awarded Outstanding Chemistry Laboratory Teaching Assistant. After completing this degree Keely owned and operated a small business manufacturing cultured food products such as sauerkraut, kim chi, kombucha and kefir. Outreach was a major part of this endeavor, and community workshops on cooking, composting, rainwater collection and backyard gardening were held frequently. A passion for food and nutrition led to the study of fermented diary products in the master's program in The School of Animal Sciences at Louisiana State University. She was awarded a Graduate Assistantship during her years of study at LSU. When not studying or working, Keely enjoys playing tennis in several Baton Rouge women's and mixed doubles leagues, riding her bicycle, sourcing and cooking locally raised foods and spending time in her yard tending to her garden, five hens and dog, Martha.