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THE EFFECT OF FROZEN STORAGE ON THE SURVIVAL OF MICROORGANISMS AND THEIR PRODUCTS FOUND KEFIR

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ABSTRACT

Kefir is 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. It was conclude 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.

KEYWORDS: Kefir, frozen, Exopolysaccharides, fermented milk.

INTRODUCTION

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. 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).

1.1.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. 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).

1.2.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).

Kefir originated in the Caucasus Mountains several centuries ago and was traditionally produced with caprice 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.

1.3.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 o f 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 milk s [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 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).

1.4.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).

1.4.1.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 b e 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.

1.4.2.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.

1.4.3.Yeasts

According to Irigoyen, et. al. (2005), the levels of yeasts and acetic acid bacteria present in kefir are direc tly 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; howeve r, 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.

1.5.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 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).

1.6.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 ga s (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 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.

Kefir exhibits numerous biological activities that include antibacterial and antifungal properties , in addition to other immuno-stimulating 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).

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).

CONCLUSION

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.

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 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.
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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