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FERNANDA ASSUMPÇÃO FIORDA

# DESENVOLVIMENTO DE UMA NOVA BEBIDA DE MEL FERMENTADA COM GRÃOS DE KEFIR POTENCIALMENTE PROBIÓTICA: PROPRIEDADES FUNCIONAIS, CARACTERÍSTICAS MICROBIOLÓGICAS MOLECULARES E ASPECTOS TECNOLÓGICOS

Curitiba
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FEDERAL UNIVERSITY OF PARANA
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Thesis submitted to the Graduate Program in Food Engineering of Federal University of Paraná in fulfillment of the requirements for the Degree of Doctor of Food Engineering

**Supervisor:** Prof. Dr. Carlos Ricardo Soccol

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# DEDICATION

This thesis is dedicated to God, who has been my eternal rock and source of refuge and for His Word that kept me all through the journey of completing this work. I also dedicate this work to my Family and friends for being great pillars of support.

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## RESUMO

O kefir é tradicionalmente uma bebida produzida a partir de leite através da inoculação de grãos de kefir, uma associação microbiana complexa entre leveduras e bactérias. No entanto, a adaptação de grãos de kefir em diversos outros substratos não-lácteos levou à produção de diferentes bebidas com propriedades funcionais. O objetivo desta tese foi avaliar o uso de diferentes substratos funcionais (extrato de soja hidrolisado, colostro e mel) para o desenvolvimento de novas bebidas probióticas, utilizando grãos de kefir como cultura iniciadora e avaliar a sua capacidade antioxidante e composição físico-química. Além disso, explorar o processo de fermentação de mel com grãos de kefir através de um estudo abrangente de suas propriedades reológicas, cinética em condição de biorreator (fermentação e processo de armazenamento), composição microbiana, potencial antimicrobiano e probiótico, efeito de proteção em danos causados ao DNA e análise sensorial, comparando-a com a bebida tradicional de kefir. A bebida de kefir a base de mel teve maior atividade antioxidante, quando comparada com os substratos extrato de soja hidrolisada e colostro. Altos níveis de bactérias ácido lácticas e populações de levedura (acima de $10^6$ CFU/mL) foram encontrados no produto, compostas principalmente de potenciais estirpes probióticas de *Lactobacillus statsumensis*, *Leuconostoc mesenteroides*, *Bacillus megaterium*, *Saccharomyces cerevisiae* e *Lachancea fermentati*. Além disso, a bebida à base mel fermentada com kefir apresentou efeito de proteção contra danos no DNA, com elevada qualidade sensorial quando comparada à bebida tradicional de kefir. Os grãos de kefir foram bem adaptados às condições do biorreator, atingindo altos níveis de viabilidade celular (acima de $10^6$ UFC / mL para levedura e bactérias totais), tendo considerável produção de compostos fenólicos (190 GAE / 100g). Luminosidade L * e croma a * não sofreram grandes alterações e croma b * decresceu durante o tempo de fermentação. Após 35 dias de armazenamento, a bebida de mel fermentada com grãos de kefir manteve as suas características químicas e viabilidade microbiana necessária para ser classificado como um produto probiótico. Os modelos de Ostwald-De Waele ($R2 \geq 0,98$) e de Herschel-Bulkley ($R2 \geq 0.99$) podem ser utilizados para predizer o comportamento da bebida desenvolvida. Os isolados estudados (*L. satsumensis, L. mesenteroides* e *S. cerevisiae)* demonstraram resistência a condições ácidas (pH 2.0, 3.0, 4.0 e 7.0) e aos sais biliares (0.3% e 0.6%), apresentando habilidade de sobrevivência na presença de suco gastrointestinal, não demonstrando atividade hemolítica. Todos os isolados apresentaram atividade antagônica frente a *E. coli* e *S. aureus* (acima de 7.0 mm). *L. satsumensis* foi a cepa mais resistente. A bebida de mel fermentada com kefir teve alta atividade antimicrobiana (19.5 a 27.5 mm). *L. satsumensis, L. mesenteroides* e *S. cerevisiae* podem ser classificadas como potenciais probióticos. Bebidas à base de kefir têm se apresentado como uma forma alternativa para a produção de bebidas funcionais com atividades probióticas, especialmente para pessoas com necessidades especiais (intolerância à lactose) e para consumidores veganos. O mel pode ser um substrato alternativo ideal para a produção de bebidas de cultura funcional, especialmente para os vegetarianos e consumidores intolerantes à lactose. Os parâmetros analisados durante o processo de bebida a base de mel fermentada com grãos de kefir podem ser considerados relevantes para a produção de uma nova bebida, auxiliando na industrialização deste bioprocesso.

**Palavras-chave:** bebidas de kefir, fermentação, probióticos, bebidas funcionais não-lácteas, cinética, biorreator.

## ABSTRACT

Kefir is traditionally a beverage produced from milk by inoculating kefir grains, a complex microbial association between yeast and bacteria. However, adaptation of kefir grains in many other non-dairy substrates has led to production of different beverages with functional properties. The aim of this thesis was to evaluate the use of different functional substrates (soybean hydrolyzed extract, colostrum and honey) to design a novel probiotic beverages using kefir grains as starter culture and evaluate its antioxidant capacity and physical-chemical composition. In addition, explore the fermentation process of honey with kefir grains through a comprehensive study of its rheological properties, kinetic in bioreactor condition (fermentation and storage process), microbial composition, antimicrobial and probiotic potential, protection effect on DNA damage and sensory analysis when compared with traditional kefir beverage. The probiotic potential and antimicrobial properties of *Lactobacillus satsumensis*, *Leuconostoc mesenteroides* and *Sacharomyces cerevisiae,* isolated from honey kefir beverage, was also investigated. Honey-based kefir beverage had higher antioxidant activity when compared with soybean hydrolyzed extract and colostrum substrates. High levels of lactic acid bacteria and yeast populations (over $10^6$ CFU/mL) were found in the product and were mainly composed of potential probiotic strains of *Lactobacillus statsumensis, Leuconostoc mesenteroides, Bacillus megaterium, Saccharomyces cerevisiae* and *Lachancea fermentati.* In addition, the honey-based kefir beverage showed protection effect on DNA damage and had a high sensory quality compared to traditional kefir beverage. Kefir grains were well adapted to bioreactor conditions, reached high levels of cell viability (over $10^6$ CFU/mL for total yeast and bacteria), had considerable production of phenolic compounds (190 GAE/100g). Color *L** and *a** did not highly changed and b* decreased during fermentation time. After 35 days of storage process, honey kefir beverage (HKB) maintained its chemical characteristics and microbial viability as required to be classified as a probiotic product. The models Ostwald-de Waele ($R^2 \geq 0.98$) and Herschel-Bulkley ($R^2 \geq 0.99$) can be used to predict the behavior of HKB. The isolates showed resistance to acid conditions (pH 2.0, 3.0, 4.0 and 7.0) and bile salts (0.3% and 0.6%), showing ability to survive in the presence of simulated gastric and intestinal juice and did not show hemolytic activity. All the isolates exhibited antagonistic activity against *E. coli* and *S. aureus* (up to 7.0 mm). The isolate *L. satsumensis* showed resistance against the studied pathogens and was the most powerful antagonistic isolates. Honey kefir beverage had high antagonistic activity (19.5 to 27.5 mm). *L. satsumensis, L. mesenteroides* and *S. cerevisiae* isolated from honey kefir beverage could be classified as potential probiotics.
Kefir-based beverages have shown an alternative way to produce functional beverages with probiotic activities, especially for people with special needs (lactose intolerance) and vegan consumers. Honey could be an ideal alternative substrate for the production of functional cultured beverage, especially for vegans and lactose intolerant consumers. The parameters analyzed during HKB process can be considered for production of a novel beverage product, assisting in the industrialization of this bioprocess. In addition, the investigation of the potential probiotic features of these kefir strains should be useful for the development of novel functional beverage.

**Keywords:** kefir beverage, fermentation, non-dairy functional beverage, kinetic, bioreactor

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## ABREVIATIONS AND UNITS LIST

| | |
|---|---|
| **cm** | Centimeter |
| **CKB** | Colostrum-based Kefir Beverage |
| **CMKB** | Cow Milk-based Kefir Beverage |
| **EPS** | Exopolysaccharides |
| **g** | Gram |
| **GAE** | Gallic acid equivalent |
| **h** | Hour |
| **HKB** | honey-based kefir beverage |
| **kg** | Kilogram |
| **LAB** | Lactic acid bacteria |
| **L** | Liter |
| **min** | Minute |
| **mL** | Milliliters |
| **mm** | Millimeters |
| **ND** | Not detected |
| **nm** | Nanometers |
| **Pa** | Pascal |
| **rpm** | Rotates per minute |
| **s** | Seconds |
| **SMKB** | Soybean-Milk Kefir Beverage |
| **T** | Temperature |
| **t** | Tonelade |
| **TKB** | Traditional kefir beverage |
| **CFU** | Colony-forming unit |
| **v** | Volume |
| **w** | Weight |

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## INTRODUCTION

Kefir grains is a mixed culture of various species of yeasts (e.g., *Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia*) and lactic acid bacteria (e.g., *Lactobacillus*, *Lactococcus, Leuconostoc* and *Streptococcus*) which form granules during cell growth under aerobic condition (Athanasiadis et al., 2002). The most common kefir beverages are developed using dairy substrates, limited basically to cow milk (Wojtowski et al., 2003). But kefir grains can also be applied to ferment different substrates besides milk and furthermore, other non-dairy substrates, such as honey, vegetables, soybean, tea and juices and have been tested for adaptation of kefir grains microorganisms and production of different functional beverages. (Fiorda et al., 2016; Mousavi et al., 2011; Peres et al., 2012; Prado et al., 2015; Prado at al., 2008; Schrezenmeir and De Vrese, 2001). The development of alternative substrates used in production of fermented kefir beverage is an ideal way for the conversion of sugars to produce organic acids and alcohol. It is considered a simple and valuable biotechnology based method for maintaining and/or improving the safety, nutritional, sensory and shelf-life properties of fermented beverages (Prado et al., 2008).

Kefir is used as an excellent source of probiotics and beneficial health effects. Kefir was used for the treatment of tuberculosis, cancer and gastrointestinal disorders when modern medical treatments were not available and it is also associated with longevity in Caucasus, mountain region where it originated (Cevikbas et al., 1994; Zourari & Anifantakis, 1988). Nowadays, there is a renewed interest for this product (Shavit, 2008). Most of kefir research are focused in milk substrate from cow, ewe, goat or other type of milk (Bensmira & Jiang, 2012; Kabak & Dobson, 2011).

However, the consumption of kefir beverage is limited for vegan and lactose intolerant consumers. Thus, an alternative way to intake of probiotic from kefir is through of its adaptation in non-dairy substrates.

For centuries, fermentation has been used to preserve, improve the quality or modify the flavor of cereals, fruits, vegetables, legumes and meat. However, research of these matrices as raw material for probiotic microorganisms is still scarce compared to their dairy counterparts. There is little information available on traditional fermented foods and scientific research could help develop new probiotic products for the food industry. This could certainly help problems with lactose intolerance and cholesterol selected issues or when people refuse to ingest dairy product for specific reasons or when the milk products are inaccessible to them (De Dea Lindner et al., 2013; Rivera-Espinoza & Gallardo-Navarro, 2010).

In this context, the aim of this study was to evaluate the use of different functional substrates (soybean hydrolyzed extract, colostrum and honey) to design novel probiotic beverages using kefir grains as starter culture and evaluate its antioxidant capacity and physical-chemical composition. In addition, explore the fermentation process of honey with kefir grains through a comprehensive study of its rheological properties, kinetic in bioreactor condition (fermentation and storage process), microbial composition, antimicrobial and probiotic potential, protection effect on DNA damage and sensory analysis when compared with traditional kefir beverage. The probiotic potential and antimicrobial properties of *Lactobacillus satsumensis*, *Leuconostoc mesenteroides* and *Sacharomyces cerevisiae,* isolated from honey kefir beverage, was also investigated.

# OBJECTIVES

## GENERAL OBJECTIVE

The aim of this study was to evaluate different functional substrates for the production of non-dairy probiotic beverages using kefir grains as starter culture.

## SPECIFIC OBJECTIVES

- Evaluate the functional characteristics and physical-chemical composition of different functional products as raw material (soybean hydrolyzed extract, colostrum and honey) using kefir grains and analyze the microflora, sensory quality and DNA protection effect of honey kefir beverage.

- Explore the fermentation process of honey kefir beverage through a comprehensive study of its rheological properties, probiotic cell viability, instrumental color parameters and kinetic aspects in a batch bioreactor and during storage.

- Characterize the probiotic potential of *Lactobacillus satsumensis*, *Leuconostoc mesenteroides* and *Sacharomyces cerevisiae,* isolated from honey kefir beverage, through acid and bile salts resistance, hemolytic acitivy, survival in simulated gastrointestinal tract conditions, and also to evaluate its *in vitro* antimicrobial properties against growth of two strains of pathogenic microorganisms conveyed by foods.

# CHAPTER 1 (LITERATUTE REVIEW)

## NON-DAIRY KEFIR BEVERAGES: NEW ALTERNATIVES AS CARRIERS AND SOURCES OF POTENTIALLY PROBIOTIC MICROORGANISMS

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**Abstract**

Kefir is a fermented beverage produced traditionally by adding kefir grains — constituted by a complex microbial consortium between yeasts and bacteria — to milk. Alternatively, kefir grains can be also cultivated in a solution of raw sugar and water, known as sugary kefir. This paper reviews the microbiological aspects, grain composition and functional properties of sugary kefir beverage. This survey demonstrated that sugary kefir possess a similar microbial association compared to milk fermentation, especially among yeasts and lactic acid bacteria species such as *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Lactobacillus*, *Lactococcus* and *Leuconostoc*. However, a selective pressure at species level is generally observed, as for example the stimulation of *Saccharomyces* species metabolism, leading to a higher content of alcohol in the final product. A range of other, alternative non-dairy substrates, such as honey, vegetables, tea and juices, have also been tested for adaptation of kefir grains and production of functional beverages with distinct sensory characteristics. This diversification is of crucial importance for the production of new probiotic products in order of achieving people with special needs (i.e., lactose intolerance) and vegan consumers. Thus, further studies are needed to better understanding the microbiological aspects, storage process and functional properties for industrial implementation of these beverages.

**Keywords**: water kefir, microbial diversy, community dynamics, symbiosis, lactic acid bacteria

## 1. INTRODUCTION

For centuries lactic acid fermentation have been used as method to preserve, improve the quality or modify the flavor of dairy products. Lactic acid bacteria (LAB), such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, are the mainly agents of milk fermentation converting sugar into lactic acid. An alternative method for fermentation of dairy matrices is through use of kefir grains as starter culture. Kefir grain consists of a polysaccharide composed by a complex microbial association among bacteria and yeasts, which works as a starter culture for milk fermentation. The result is a naturally carbonated beverage (associated with yeast metabolism), with acid taste and creamy consistency due to LAB metabolism (Magalhães et al., 2010).

Kefir is used as an excellent source of probiotics and beneficial health effects. Kefir was used for the treatment of tuberculosis, cancer and gastrointestinal disorders when modern medical treatments were not available and it is also associated with longevity in Caucasus, mountain region where it originated (Cevikbas et al., 1994; Zourari & Anifantakis, 1988). Nowadays, there is a renewed interest for this product (Shavit, 2008). Most of kefir research are focused in milk substrate from cow, ewe, goat or other type of milk (Kabak & Dobson, 2011; Bensmira & Jiang, 2012). However, the consumption of kefir beverage is limited for vegan and lactose intolerant consumers. Thus, an alternative way to intake of probiotic from kefir is through of its adaptation in non-dairy substrates. Sucrose solution is the main alternative substrate used for kefir fermentation, known as sugary kefir beverage (Schneedorf, 2012). Studies have shown that sugary kefir fermentation is carried by the consortium of yeasts, mainly *Kluyveromyces*, *Pichia* and *Saccharomyces*, and LAB, including the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc*. All these microorganisms are embedded

in a resilient water-soluble branched glucogalactan matrix named kefiran (Rodrigues et al., 2005, Gulitz et al., 2011; Magalhães et al., 2010). Furthermore, other non-dairy substrates, such as honey, vegetables, soybean, honey, tea and juices, have been tested for adaptation of kefir grains microorganisms and production of different functional beverages. (Fiorda et al., 2016; Mousavi et al., 2011; Peres et al., 2012; Prado et al., 2015; Prado at al., 2008; Schrezenmeir & De Vrese, 2001).

Research of others matrixes as raw material for kefir fermentation is still scarce compared with their dairy counterpart. As a wide microbial diversity is found in kefir grains, its adaptation in different substrates can be easier compared to fermentation using single-species starter cultures. The different species from kefir grains can easily adapt to different substrates and lead to production of new probiotic products. Thus, more research is needed on microbiological aspects, chemical and sensory properties for adaptation of kefir grains in these new matrixes. This review promotes an update of the main types of kefir-base beverages products, their microbiological aspects and benefits associated with consumption.

## 2. ORIGIN AND DISTRIBUTION OF SUGARY KEFIR

When kefir grains are applied to ferment fruit juice, molasses or sugary solution, it is referred to as sugary kefir or water kefir (Koutinas et al., 2009; Magalhães et al., 2010). Sugary kefir grains have been adapted to many names from being around for so long, and shared by so many cultures around the world. Some of the names are similar to milk kefir because of the lack of distinguishing between them two through history (just as both are called 'kefir' but only distinguish by saying milk or sugary).

The history of water kefir is not well known. Although structurally similar to milk kefir, the origin, distribution and consumption drew an own route. Figure 1 is tracing the distribution of water kefir grains over centuries based in data described by

Yemoos (2015).


**Figure 1.** Origin of water kefir, distribution and consumption.
*Red area – originated area (Caucasus); green area – high conspumtion

Kefir grains were passed from generation to generation among the tribes of Caucasus (red area in Figure 1), being considered a source of family wealth. From there, kefir grains were distributed to countries of European, African and Asian continents. When the habit of drinking kefir spread all over Europe, kefir grains won the "New World" and its use expanded throughout the American continent. Today, kefir is prepared by culturing fresh or pasteurized substrates with kefir grains in homes all over the world. The green area in Figure 1 shows the places with highest consumption of water kefir, including USA, Mexico, Canada, Thailand, Malaysia, Japan, Greece, Turkey, Romaine, United Kingdom, Netherlands, Norway, Sweden, Spain, Chile and Peru (Yemoos, 2015).

## 3. MANUFACTURING OF NON-DAIRY KEFIR BEVERAGE

Traditionally, non-dairy kefir is made from a brown sugar solution (3 to 10%), but also other alternative substrates are being developed, such as honey, fruit matrices, juices, tea, olives and other vegetables (Fiorda et al., 2016; Mousavi et al., 2011; Peres et al., 2012; Prado et al., 2015; Prado at al., 2008; Schrezenmeir & De Vrese, 2001). In Figure 2 is proposed a possible process for manufacturing water kefir at industrial level.

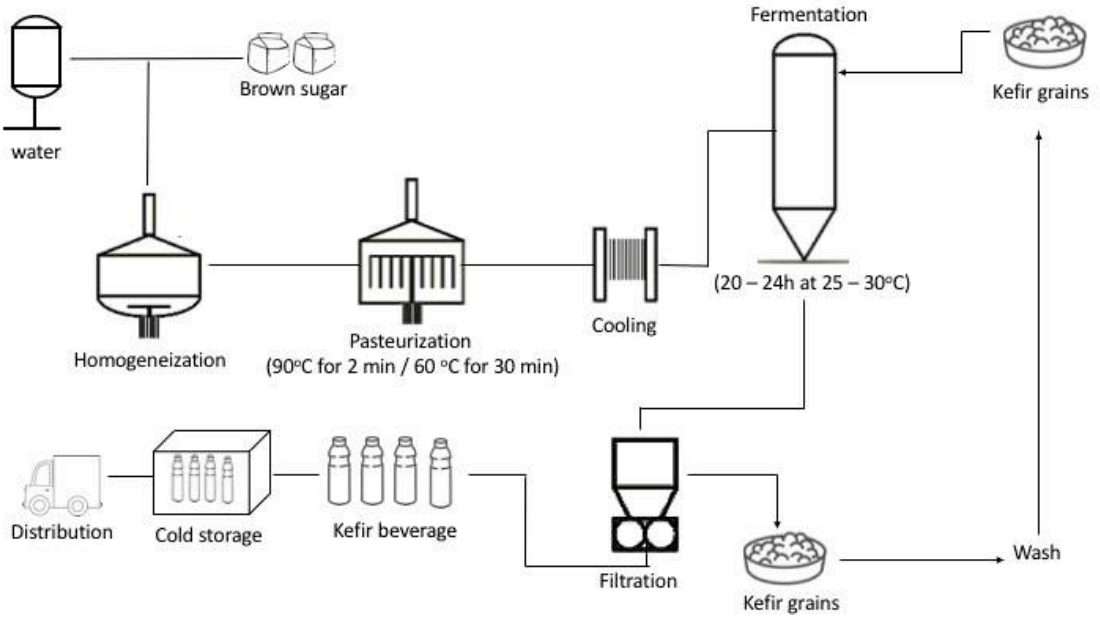


**Figure 2.** Manufacturing proposal for non-dairy kefir production

In this process, kefir grains are directly added to the pasteurized and cooled substrate and incubated for approximately 24 h at 25 to 30 °C. After fermentation, the grains are separated from the substrate by filtering with a sterile sieve and can be dried at room temperature and kept at cold storage for the next inoculation (Guzel-Seydim et al., 2010; Otles & Cagindi, 2003). Kefir beverage is stored at 4 °C and then is ready for consumption. After fermentation at 25 to 30 °C for 20–24 h, the product can be stored at refrigeration temperatures up to 20 days (Guzel-Seydim et al., 2010). Other alternative processes for production of kefir beverage are currently proposed, such as the use of

lyophilized starter cultures containing LAB and yeast isolated from kefir fermentation (Guzel-Seydim et al., 2010; Mistry, 2004).

In the next sections will be updated the microbial diversity and major alternative substrates for non-dairy kefir fermentation. The physicochemical properties, benefits and spoilage of non-dairy kefir beverages will also be reviewed as a support for future industrial applications.

## 4. SUGARY KEFIR GRAIN COMPOSITION AND MICROBIOTA

Sugary kefir grains are very similar to milk kefir grains in terms of their structure, associated microorganisms and products formed during the fermentation process. However, the distribution of strains varies according to the carbon and energy sources available for grain fermentation and these microbial changes will further affect the granulation and growth of the grains (Hsieh et al., 2012). Visual differences between water and milk kefir grains are illustrated in Figure 3.

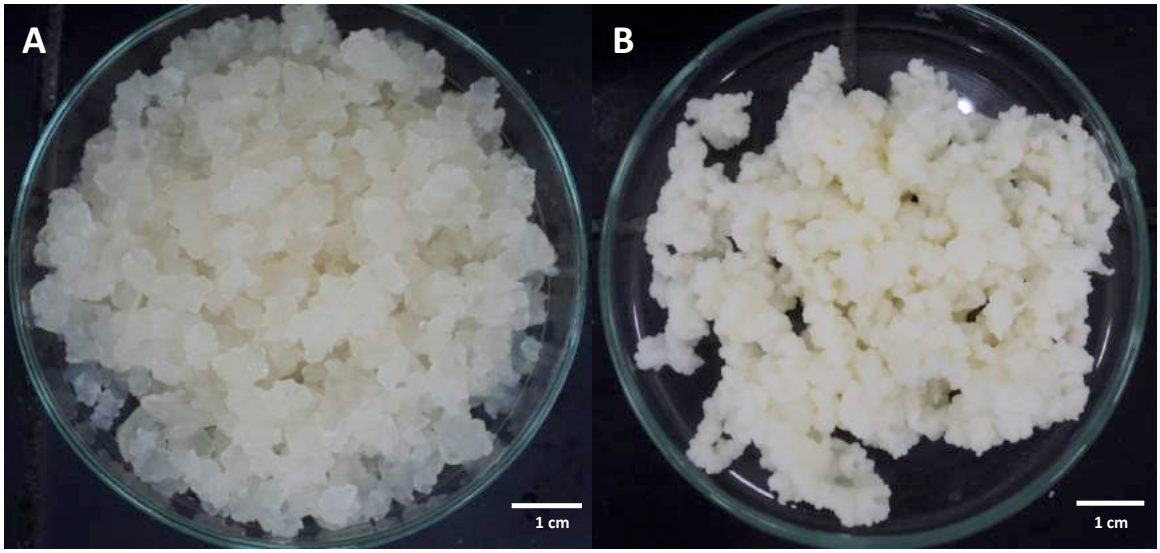


**Figure 3.** A - Non-dairy kefir grains
B - Milk kefir grains.
Source: The author

Research on microbiology chemical and structural composition of water kefir is still very limited compared to milk substrates. To date, it is known that microbial

species diversity of water kefir consists of a stable consortium of mainly LAB, acetic acid bacteria and yeasts, as evaluated by both culture-dependent and culture-independent techniques (Gulitz et al., 2013; Laureys et al., 2016; Miguel et al., 2011; Magalhães et al., 2010; Waldherr et al., 2010, Marsh et al., 2013). Different species of mainly *Lactobacilli, Streptococci, Acetobacter, Saccharomyces* and *Pichia* are found in a symbiotic relationship, meaning that they survive or propagate by sharing their bioproducts as an energy source or growth-stimulating source (Lopiz-Otsoa et al., 2006).

Some of the frequently isolated species from sugary kefir fermentation are *Lactobacillus kefiri, L. kefiranofaciens, Lactobacillus kefirgranum, Lactobacillus parakefir* and *Candida kefyr*. Also, new species have been detected in kefir, such as *Saccharomyces turicensis* (Whyder et al., 1999) and *Bifidobacterium aquikefiri* sp. (Laureys et al., 2016). The taxonomic nomenclature of the different species of yeast and bacteria that compose kefir has varied along with the advances in taxonomic classification methods. In addition, complete knowledge of yeast life cycles (telemorphic and anamorphic phases) in some of these microorganisms has resulted in the use of different nomenclature for classification (Lopiz-Otsoa et al., 2006). A complete description of the different yeast and bacteria that have been identified during fermentation of sugary kefir are shown on Table 1.

**Table 1.** Microrganisms isolated from water and milk kefir grains

| | Genus | Water kefir | Milk kefir | References |
|---|---|---|---|---|
| **Bacteria** | *Acetobacter* | *A. fabarium, A. orientalis, A. lovaniensis* | | Laureys et al. (2016), Gulitz et al. (2013), Gulitz et al. (2011), Magalhães et al. (2010) |
| | *Lactobacillus* | *L. brevis, L. buchneri, L. casei subsp. Casei, L. casei subsp. Rhamnosus, L. diolivorans, L. fermentum, L. harbinensis, L. hilgardii, L. hordeii, L. kefiranofaciens, L. kefiri, L. lactis, L. mali, L. nagelli, L. paracasei, L. parafarraginis, L. perolens, L. plantarum, L. satsumensis* | *L. acidophilus, L. brevis, L. buchneri, L. casei subsp. Pseudoplantarum, L. delbrueckii, L. fermentum, L. helveticus, L. kefiranofaciens, L. kefiri, L. otakiensis, L. paracasei, L. parabuchneri, L. plantarum, L. rhamnosus, L. sake, L. sunkii* | Fiorda et al. (2016), Laureys et al. (2016), Zanirati et al. (2015), Gulitz et al. (2013), Gulitz et al. (2011), Kesmen and Kacmaz (2011), Magalhães et al. (2010), Sabir et al. (2010), Chen et al. (2008), Witthuhn et al. (2005), Simova et al. (2002), Garrote et al. (2001), Galli et al. (1995), Pidoux (1989), Moinas et al. (1980) |
| | *Leuconostoc* | *L. citreum, L. mesenteroides* | *L. mesenteroides* | Fiorda et al. (2016), Gulitz et al. (2013), Gulitz et al (2011), Kesmen and Kacmaz (2011), Magalhães et al. (2010), Sabir et al. (2010), Waldherr (2010), Garrote et al. |

| | | | | |
|---|---|---|---|---|
| | | | | (2001) |
| | *Lactococcus* | | *L. cremoris, L. lactis, L. raffinolactis* | Magalhaes et al. (2011), Kesmen and Kacmaz (2011), Sabir et al. (2010), Yuksekdag et al. (2004) |
| | *Pediococcus* | | *P. acidilactici, P. dextrinicus, P. pentosaceus* | Sabir et al. (2010) |
| | *Streptococcus* | | *S. durans, S. thermophilus* | Kesmen and Kacmaz (2011), Chen et al. (2008), Yuksekdag et al. (2004), Simova et al. (2002) |
| | *Other species* | *Lysinibacillus sphaericus, Oenococcus kitaharae, Bifidobacterium psychraerophilum* | | Fiorda et al. (2016), Zanirati et al. (2015), Gulitz et al. (2013) |
| **Yeast** | *Candida* | | *C. iconspicua, C. kefir, C. krusei, C. lambica, C. maris* | Witthuhn et al. (2005), Simova et al. (2002) |
| | *Saccharomyces* | *S. cerevisiae* | *S. cerevisiae, S. turicensis* | Fiorda et al. (2016), Laureys et al. (2016), Gulitz et al. (2013), Puerari et al. (2012), Magalhães et al. (2010), Wang et al (2008), Simova et al. (2002) |
| | *Pichia* | *P. membranifaciens, P.* | *P. fermentans* | Fiorda et al. (2016), |

| | | | |
|---|---|---|---|
| | *kudriavzevii* | | Wang et al. (2008) |
| *Lanchancea* | *L. fermentati, L. meyercii* | *L. meyercii* | Fiorda et al. (2016), Gulitz et al. (2011), Magalhães et al. (2011), Magalhães et al. (2010) |
| *Kluvyromices* | *K. lactis, K. marxianus* | *K. lactis* | Puerari et al. (2012), Magalhaes et al. (2011), Magalhães et al. (2010), Wang et al. (2008), Garrote et al. (2001) |
| *Kazachstania* | *K. aerobia, K. unispora* | | Puerari et al. (2012), Magalhães et al. (2010) |
| *Hanseniaspora* | *H. valbyensis, H. uvarum* | | Fiorda et al. (2016), Gulitz et al. (2011) |
| *Other species* | *Zygotorulaspora florentina, Issatchenkia orientalis, Saccharomycetes* sp., *Zygosaccharomyces fermentati, Dekkera bruxellensis* | *Cryptococcus humicolus, Geotrichum candidium, Zygosaccharomyces fermentati* | Fiorda et al. (2016), Laureys et al. (2016), Gulitz et al. (2011), Witthuhn et al. (2005) |

Studies on the microbiology of water kefir fermentations have been performed over the lasts 30 years from different origins, such as Brazil, Belgium, Germany, Serbia, Taiwan, China, Ireland, Italy, Argentina, Yemen and others (Magalhães et al., 2010; Laureys & De Vuyst, 2014; Gulitz et al., 2011; Davidovic et al., 2015; Hsieh et al., 2012; Marsh et al., 2013; Blaiotta et al., 2014; Diosma et al., 2014; Alsayadi et al., 2013). Questions about this microbial diversity in sugary kefir started in 1892 in London with Dr. Ward (Ward, 1982). In more recent years, studies using molecular techniques (e.g. DGGE, ARDRA, metagenomic) have lead to major advances in understanding the diversity of yeasts and bacteria during kefir fermentation. However, the overall microbiology and biochemistry of water kefir fermentation is poorly studied as yet when compared to milk matrixes.

Gulitz et al (2011) compared the microbial consortia residing in the grains of three Germany sugary kefir of different media using RAPD PCR method and 16S rDNA for sequence analyses. The authors reported the dominance of *Lactobacillus hordei*, *L. nagelii*, *Leuconostoc mesenteroides* in the LAB group, and *Hanseniaspora valbyensis*, *Lachancea fermentati*, *Saccharomyces cerevisiae* and *Zygotorulaspora florentina* in the yeast group. Magalhães et al (2010) evaluated the microorganisms associated with sugary Brazilian kefir beverage using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and indicated that bacteria, such as *Lactobacillus paracasei, Lactobacillus kefiri, Lactobacillus parabuchneri* and *Acetobacter lovaniensis* as well as yeast, such as *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, were the predominant microorganisms present in the beverage. In this study, the PCR-DGGE technique enabled the detection of the species *Acetobacter lovaniensis* and *Kazachstania aerobia* for the first time in sugary kefir. Miguel et al (2011) also studied Brazilian kefir samples by PCR-DGGE analysis and reported the

bacteria's *Gluconobacter liquefaciens* and *Bacillus cereus* and the yeast *Pichia cecembensis*, Pichia caribbica and *Zygosaccharomyces fermentati* for the first time in water kefir grains. In Thailand, PCR-DGGE was also used to assess the diversity of microorganisms present in sugary kefir, being composed for a similar microbial diversity (*Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Gluconobacter japonicas*, *Bacillus cereus*, *Saccharomyces cerevisiae and Candida ethanolica*) compared to other grains from different parts of the world (Sarikkha et al., 2015).

The first extensive description of sugary kefir bacterial microbiota with 16S metagenetic analysis by Gulitz et al. (2013) set a new milestone with the first detection of bifidobacteria as part of the water kefir consortium. Later, Laureys et al. (2016) also proved the presence bifidobacteria group (i.e., *Bifidobacterium psychraerophilum*/*crudilactis)* in kefir grains from Belgium using culture-independent analysis. The unexpected presence of bifidobacteria in the analyzed water kefir samples and the difficulty of cultivating these species indicate that the role of bifidobacteria in other kefir matrixes may also be underestimated. Bifidobacteria species are widely known for aid in the synthesis of B-complex vitamins and vitamin K in the intestine. This synthesis protects the body from deficiencies of these vitally important nutrients, necessary to improve bone health, prevent bone fractures and reduce the risk of bleeding associated with long-term antibiotic use. Bifidobacteria also increased concentrations of organisms associated with decreased fecal concentrations of potentially pathogenic bacteria and decreased levels of carcinogenic and putrefactive compounds in the digests (Liu et al., 2006; Laureys & De Vuyst., 2014; Laureys et al., 2016). Thus, the consumption of water kefir beverage can be linked to functional features derived from *Bifidobacteria* metabolism.

## 5. COMPOSITION OF YEAST AND BACTERIA IN WATER KEFIR

Figure 4 shows a comparison of the different microbial groups present in water and milk kefir.

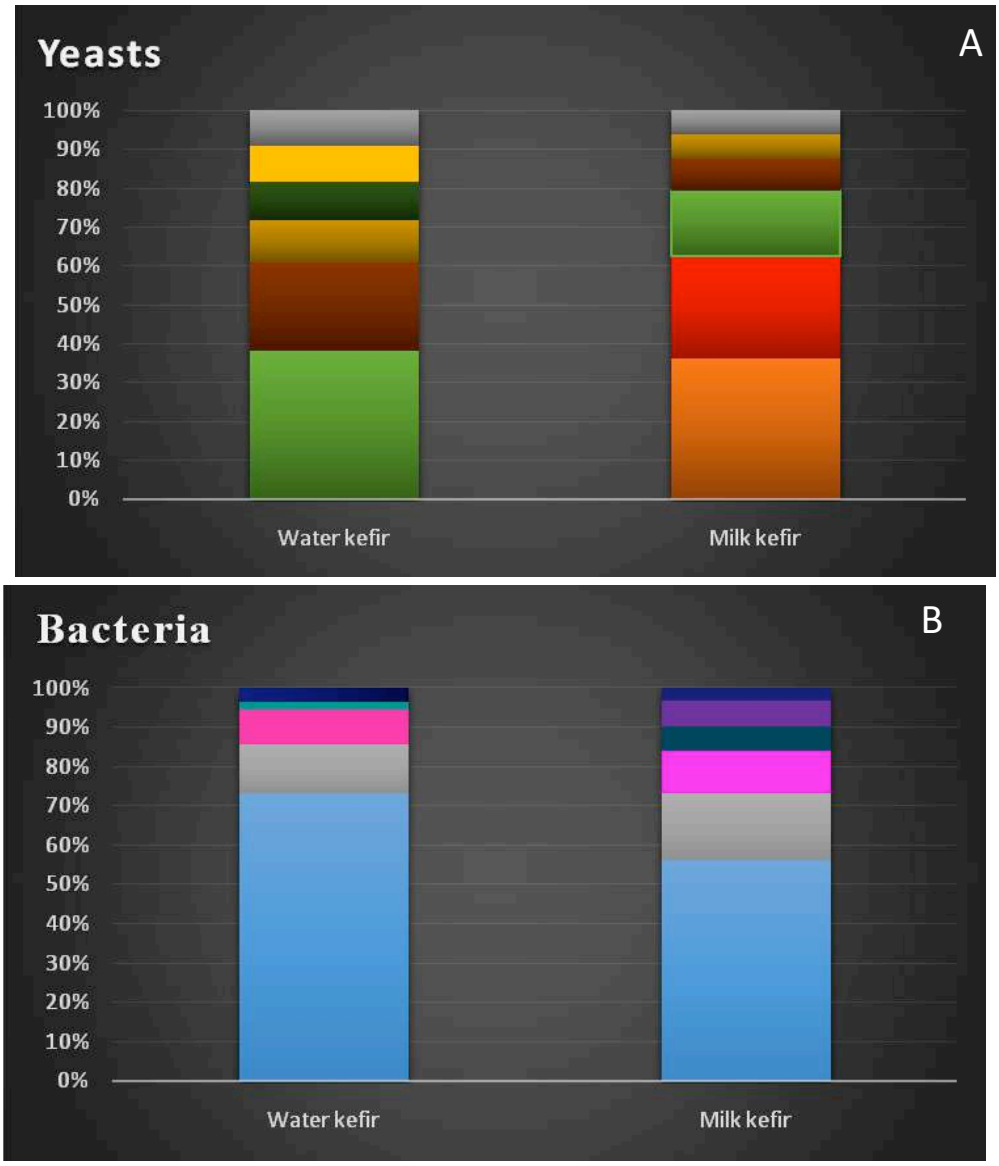


**Figure 4.** Microbial diversity of kefir on family level. Each color represents a different bacteria and yeast family. Source: Table 1
*Yeast:
*Candida* (), *Kazachstania* (), *Sacharomyces* (), *Lanchancea* (), *Pichia* (), *Hanseniaspora* (), *Kluvyromices* (), others ().
** Bateria:
*Lactobacillus* (), *Leuconostoc* (), *Acetobacter* (), *Bifidobacteria* (), *Streptococcus* (), *Pediococcus* (), *Lactococcus* (), others ()

Yeasts are extremely diverse group comparing both substrates which indicates that their metabolism is dependent on carbon and energy sources availability during grain fermentation (Figure 4A). Probably, the high sucrose content present in sugary matrixes stimulates the growth of *Saccharomyces* species which are able to convert sucrose into the monosaccharides glucose and fructose by the enzyme invertase so that the yeast cells have glucose as a free metabolite (Ikram-Ul-Haq & Ali, 2007). In addition, *Saccharomyces* species, which exhibits strong fermentative metabolism and tolerance to ethanol, is known to be superior to non-Saccharomyces yeast in the process of alcohol fermentation, regarding spontaneous fermented sugar cane (Bernardi et al., 2008). On the other hand, the disaccharide lactose present in dairy matrixes stimulates the growth of other non-*Saccharomyce*s yeasts, since *Saccharomyces* species are not able to convert lactose into monosaccharides (Schwan et al., 2001). The presence of *Saccharomyces cerevisiae* in kefir contributes to the enhancement of sensory quality in kefir beverages, promoting a strong and typically yeasty aroma, as well as its refreshing, pungent taste (Magalhaes et al., 2010). This yeast also reduces the concentration of lactic acid, removes the hydrogen peroxide and produces compounds that stimulate the growth of other bacteria, thus increasing the production of kefiran exopolysaccharides (Cheirsilp et al., 2003).

In relation to bacteria composition, a more stable diversity is observed comparing both substrates, with a strong dominance of *Lactobacillus* group (Figure 4B). However, it is possible to observe a higher dominance of acetic acid bacteria belonging to the genus *Acetobacter* in sugary kefir in relation to milk. This indicates that the metabolism of acetic acid bacteria is stimulated in sugary matrixes that utilizes the ethanol produced by the yeast for their growth and metabolism of acetic acid. This indicates a particular symbiosis in sugary kefir fermentation between yeast and acetic acid bacteria. This

ethanol conversion into acetic acid by acetic acid bacteria occurs after 12 h of fermentation. These bacteria have alcohol dehydrogenase activity, which converts ethanol to acetaldehyde (Beshkova et al., 2003), decreasing the pH value. This is of great importance, since acids inhibits the development of undesirable or pathogenic microorganisms, due to the substrate acidity increase (Magalhães et al., 2010).

Other yeasts with high fermentative capacity are mainly isolated from water kefir, such as *Hanseniaspora, Pichia and Lachancea*. These species of yeasts are generally isolated in the first stage of fermentation, before *Saccharomyces cerevisiae* takes over (Morrissey et al., 2004). These yeasts are usually used in the fermentation process to make wine and cachaça (a drink made from fermented sugar), contributing on production of aromatic compounds in the final beverage (Nova et al., 2009; Li et al., 2010; Dhaliwal et al., 2011, Bernardi et al., 2008). The presence of such yeasts in sugary kefir can contributes to the enhancement of the sensory quality of the probiotic beverage, promoting a strong and typically yeasty aroma, as well as its refreshing, pungent taste. In addition, some of these yeast species also reduces the concentration of lactic acid, removes the hydrogen peroxide and produces compounds that stimulate the growth of other bacteria, thus increasing the production of kefiran exopolysaccharides (Cheirsilp et al., 2003).

## 6. COMMUNITY DYNAMICS

The microbial flora present in kefir grains has been studied from a symbiotic community point of view by Linn Margulis since 1995 (Margulis, 1995). Accordingly, it has been stated that separated cultures of microbial kefir grains, either do not grow in sugar solution or have a decreased biochemical activity, which further complicates the study of the microbial population of kefir grains.

The mechanism of symbiogenesis of kefir grains from distinct microbial strains is unknown, although there are some data about the recover of their structure and probiotic properties from lyophilization, and even so, about the formation of an artificial consortium produced by bits of kefir grains transferred to a yeast extract-sucrose solution (Pidoux, 1989).

Kefir grains are a matrix of bacteria and yeast that work together to feed off the natural sugars (and sometimes proteins and fats too) found present in the sugar-water. The yeast and bacteria co-operate, making the nutrients that are inaccessible to one digested into accessible nutrients for the other. Yeasts break down the simple sugars like glucose and fructose, turning them into ethanol and acetic acid. Lactic and acid-producing bacteria (such as lactobacilli) convert sugars (such as sucrose) and complex carbohydrates (starches, etc) into simpler sugars and lactic acid. Lactic and acetic acids naturally preserve as well as stave off harmful foreign bacteria. The result is a drink that has had much of the sugar converted to simpler sugars, lactic and acetic acids, carbon dioxide and ethanol. It also contains millions of probiotics and is more nutritious in some regards because of the more bio-available and digestible nutrients from the sugars including an increase in vitamin C and many B vitamins (Corona et al., 2016).

During water kefir fermentation process, the community dynamics, the species diversity, and the kinetics of substrate consumption and metabolite production is still not very clear. However, according to many researches (Fiorda et al., 2016; Laureys and De Vuyst, 2014, Stadie et al., 2013) after 192 h of fermentation, a carbon recovery level of 100 % was obtained, indicating that all major substrates and metabolites were recovered from this water kefir fermentation. After 72 h of fermentation, the majority of the metabolic activity had taken place. The major end products of the fermentation were ethanol, carbon dioxide, lactic acid, acetic acid, and others (glucose, fructose, mannitol,

glycerol, organic acids, etc.) besides the synthesis of water kefir grain mass, as shown in Figure 5.

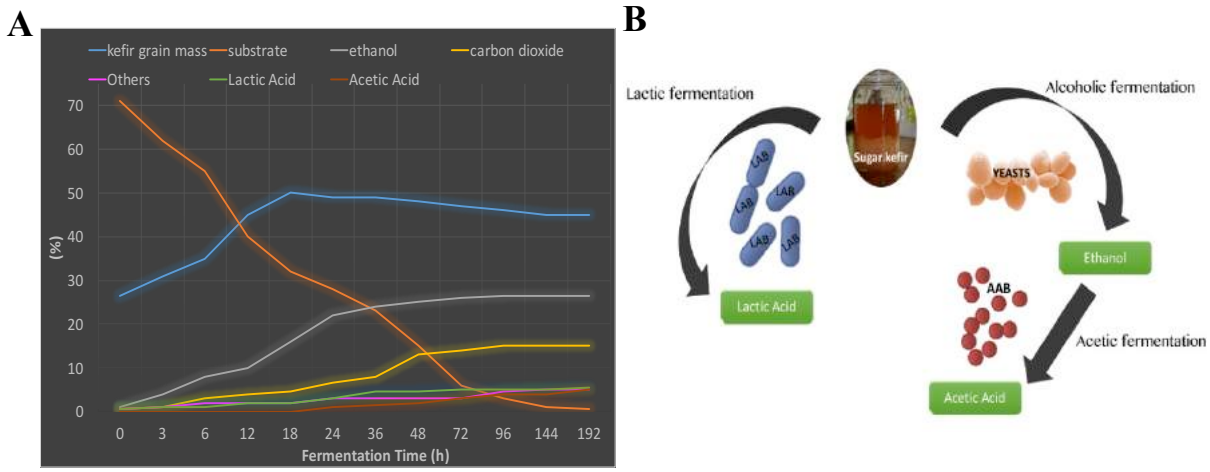


**Figure 5. A -** Presence of different carbon-containing constituents of the water kefir fermentation process, as a function of time (h), expressed as a percentage (%) of the total amount of carbon recovered. **B –** Different fermentations in kefir product

Aa aforementioned, brown sugar is the substrate used in sugary kefir, composed by sucrose (90%), reducing sugars (6%), minerals as K, Ca, P, Mg, Na, Fe, Mn, Zn e Cu, (1.5%) and moisture (2.5%) (Guerra & Mujica 2010). Sucrose is the main compound and at the start of the fermentation and the concentration of sucrose decrease quickly after 24 h of fermentation. This decrease in sucrose concentration consumed by yeasts during alcoholic fermentation gave rise to an increase in the ethanol concentration, which reaches a maximum after 24 h of fermentation. After that, part of ethanol is consumed in acetic fermentation, producing acetic acid. In this time frame, the lactic acid is produced by LAB during lactic fermentation consuming part of glucose and fructose content. After 72 h, most of the carbohydrates are consumed. The ethanol concentration increases linearly. The lactic acid and acetic acid concentrations increases and others by products as fructose, glucose, mannitol, glycerol and organic acids concentrations increases as well.

Mannitol has a sweet taste and possesses antioxidant activity (Shen et al., 1997); both properties might be desirable in water kefir. Glycerol is a slightly sweet molecule

that may slightly increase the viscosity of a fermented beverage but does not seem to have a direct influence on the taste and aroma of fermented beverages (Picinelli et al., 2000).

## 7. NON-DAIRY KEFIR BEVERAGES

Fruit juices contain water, sugar, proteins, amino acids, vitamins and minerals being a suitable and rich medium for microbial growth that can be used to prepare fermented beverages, like kefir, wine and other products (Duarte et al., 2010). Moreover, the fermentation of these substrates makes appreciated kefir beverages with acidic taste, refreshing, slightly carbonated, low alcoholic and acetic content (Gronnevik et al., 2011; Miguel et al., 2011).

Since the consumption of vegetables and fruits is strongly advised by many Governments to reduce the risk of several diseases and functional declines associated with aging (Temple, 2000; Willett, 1994, 1995), their fermentation might widen the choice for the consumption of these products. Over the years, new and diverse methods for processing fruits have been studied in an effort to minimize production losses, increasing farmers' income, and to introduce new products to the market (Duarte et al., 2010). The development of non-dairy fermented beverage with kefir may be perceived by consumers as healthy (Puerari et al., 2012).

Many researches have been done for developing new alternatives for non-dairy kefir beverage due to the numerous positive effects of kefir as well as vegetables and fruits on the human health. In Figure 6 are shown some alternative substrates used for kefir grain fermentation. The name of the resulting product is changed in case of additional fruit, tea or vegetable is used as medium of fermentation (Figure 6).

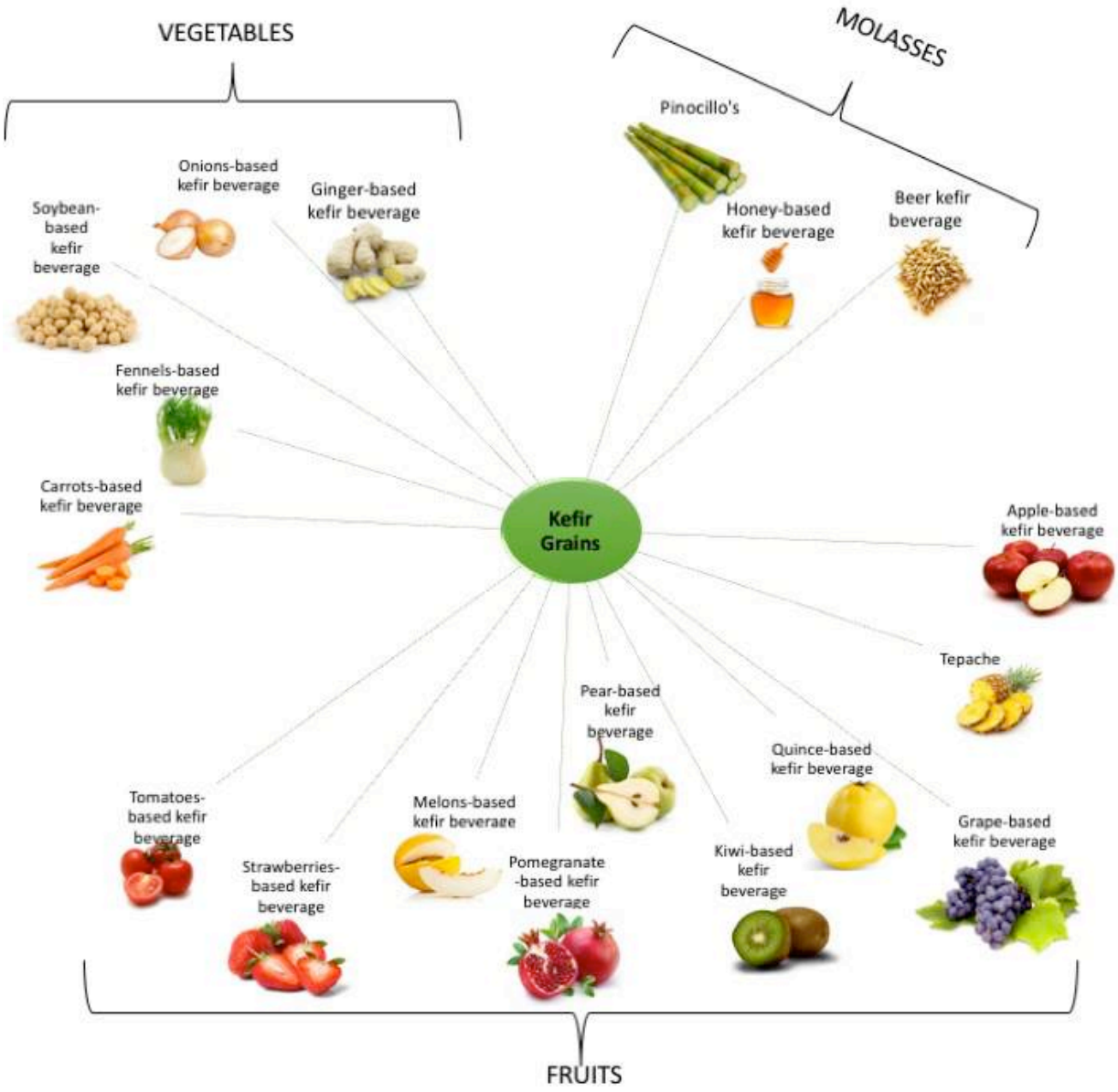


**Figure 6.** Non-dairy products produced with water kefir grains

Water kefir grains are known and widely popular among the Latin communities currently, and it is being used to make Tepache, a fermented beverage produced by kefir grains inoculation and adaptation in pineapple, brown sugar and cinnamon (Fuente-Salcido et al., 2015). After one or two days, a refreshing, pleasant, sweet beverage with low alcohol content (less than 1%) is obtained. The drink is available in small shops called *tepacherias* or from peddlers (Moreno-Terrazas et al., 2001). Water kefir grains

are often fed by Piloncillo's, a dried syrup from whole sugar cane juice shaped into cones (Rubio et al., 1993). These are quite easy to find in any Latin or Mexican Market

Ginger is also used as a substrate for sugary kefir grains, it is commonly named as 'Gingerbeer Plant' and it is very similar to sugar water kefir. Though they look alike from a distance, ginger beer crystals are known to be smoother, tinier and more opaque than water kefir crystals. The gains also tend to ferment more slowly in ginger beer. Ginger beer is widely known in many areas and is still made by the locals in the rural village of Corfu - Greek as a local specialty (Daker et al., 1938; Ward, 1892). Today in Eastern Africa (especially in Kenya and Tanzania), ginger beer is a very popular drink. It is called *Tangawizi*, which is the Swahili word for 'ginger'.

Fruit juices are the usual medium for experimentation with excess kefir grains. After a couple times of fermenting, they will typically become discolored, get white specks or a filmy coating, and may start to disintegrate or stop performing. Grape-based kefir beverage, also called as “Kefir d'uva” is simply kefir grains in grape juice - which make for a very acceptable drink, but it usually is not sustainable. It is needed to keep a separate traditional batch going in case these die. It is also fermented beverage with canned fruit (which has its own sugars and juice in the can simply add water and use a can of lychees, pineapple or peaches for example). Coconut water is one another of the most liquids to ferment with water kefir grains. And it is also possible to ferment other mediums such as coconut milk, soy milk, rice milk, or almond milk and tea (Gaware et al., 2011; Fiorda et al., 2016).

Kefir-like beverages were obtained after fermentation of juices extracted from fruits (apple, quince, grape, kiwifruit, prickly pear and pomegranate) cultivated in Sicily (southern Italy) with water kefir microorganisms, in order to develop new non-dairy fermented beverages. Beverages were produced by backslopping: the freeze-dried

microbial mixture (0.125 g) was first activated in fruit juices (50 mL) at 25 $^{o}$C for 72 h to develop the inoculants; each In was then added (4%, v/v) to 1 L of the corresponding juice and the fermentation was statically performed at 25 $^{o}$C for 48 h. Microbiological, chemical and sensory features of kefir-like beverages obtained after the fermentation were investigated. Results indicated that both lactic acid bacteria and yeasts were able to develop in the fruit juices tested, but the highest levels were registered with prickly pear fruit juice. In particular apple and grape juices fermented with kefir grains, had high added value and appreciated by testers in sensory evaluation (Randazzo et al., 2016).

Honey and soybean hydrolyzed extract were used as substrates in fermentation for production of non-dairy kefir beverages. The products had high antioxidants compounds and functional properties. In addition, honey-based kefir beverage showed protection effect on DNA damage and had a high sensory quality compared to traditional kefir beverage. Potentially probiotics were isolated from this new beverage, suggesting that kefir grains were adapted to this non-dairy substrate (Fiorda et al., 2016).

Musts prepared with 150 g/L of pilsen and vienna malt were used to produce a beer fermented by sugary kefir grains. To start the fermentation, kefir grains (30 g/L) were added to fermenter chambers and kept for 7 days at 18 °C. After this souring period, the beers were transferred to another container for maturation for 10 days. Finally, the beers were bottled with 5 g/L of sucrose to provide a second fermentation and carbonation. The plausible anti-inflammatory and anti-ulcerogenic activities were evaluated to further the development of a potential candidate alcoholic functional food for the human diet. The overall results suggest a synergistic effect of the kefir beer that

involves the polyphenol content of barley malt together with some of the probiotic and prebiotic properties inherent to the kefir itself (Rodrigues et al., 2016).

Juices extracted from carrots, fennels, melons, onions, tomatoes and strawberries were fermented with water kefir microorganisms, and the characteristics of kefir-like beverages were evaluated in order to develop new non-dairy fermented products. The extracted juices were subjected to pasteurisation at 75 ºC for 5 min and cooled at room temperature before processing. Higher volumes of vegetable juices (1 L) were then inoculated with the corresponding In (4% v/v) and the fermentation processes were performed at 25 ºC for 48 h. Physico-chemical and organoleptic properties of some vegetable-based kefir beverage were evaluated. Results indicated that lactic acid bacteria and yeasts were capable of growing in the juices tested. Melon juice registered the highest numbers of microorganisms. Almost all juices underwent a lactic fermentation. In particular, esters were present in high amounts after the fermentation, especially in strawberry, onion and melon, whereas carrot and fennel registered a significant increase of terpenes. Changes in colour attributes were registered. Strawberry, onion and tomato juices retained a high antioxidant activity after fermentation. The overall quality assessment indicated that carrot kefir-like beverage was the product mostly appreciated by the judges (Corona et al., 2016).

## 8. RESISTANCE, SHELF LIFE AND SAFETY

As a well-structured gelatinous grains with diverse microbial strains in their composition, it was hypothesize that the bacteria and yeasts present in kefir could be protected inside the polysaccharide matrix, exhibiting a different resistance under physical and chemical stresses than freely strains in solution (Schneedorf et al., 2012). The ancient culture of symbiotic kefir showed a strong resistance against ultraviolet

radiation exposure (UV), antibiotic and gas treatment (ozone), allowing a retrieval close to the normal growth after the disturbances (Pichara et al., 2001).

Kefir grains are very resilient and will strive to maintain their health at all times. They may get stressed or be responding to seasonal changes and change shape or smell a bit (more yeasty or increase/decrease in size). They are constantly adapting and working in symbioses with their environment and it's not a concern if they don't look the same in winter as in summer - this is a result of their ability to adapt (different strains do well at different temperatures, making kefir an interesting symbiotic blend that is able to survive at many temperatures). They range from clear to an opaque dark brown in color, depending on the sugar and dried fruit it is with (some fruits like figs will be pink). Even when they are not growing they can still often produce a healthy drinkable kefir, though its preferable to use optimal conditions so they can grow.

Kefir grains could become fully active after two or three propagations. It has a limited shelf life when left wet. During storage at 4 °C, kefir grains lose their activity within 28 to 30 days. However, dried grains are active for 12 to 18 months. This is an important data for manufacturing process. Excessive washing and improper utilization alter the microbiota of grains and as well as the quality of the final product. For long time storage, kefir grains can be dried at room temperature for 36–48 h and stored in a cool and dry place or be kept in a frozen state (Mistry, 2004). Garrote et al. (1997) showed that the kefir produced from grains stored at − 20 °C and − 80 °C had the same microbiota and quality characteristics with kefir produced from unstored kefir grains. Freeze-dried kefir culture has been suggested for kefir manufacture to obtain uniform quality (Mistry, 2004). Fermentation can continue in kefir beverage during storage and after some time cause extremely strong and undesirable products because of the relatively high presence of yeasts.

Spoilage of kefir beverage could rapidly occur when contaminated grains with coliforms, *Bacillus* spp., *Micrococcus* spp. and mold were used in production (Mistry, 2004). Microbiological quality of 50 kefir samples was investigated and the average count of *Lactobacillus, Lactococcus, Enterococcus, Enterobacteriaceae, S. aureus* and yeast has been reported as $3.6 \times 10^7$ cfu/mL, $1.8 \times 10^8$ cfu/mL, $4.8 \times 10^4$ cfu/mL, $7.3 \times 10^3$ cfu/mL, $2.4 \times 10^2$ cfu/mL and $7.7 \times 10^4$ cfu/mL, respectively. Twenty four and 11 of 50 kefir samples were contaminated with coliform and *E. coli*, respectively (Cetinkaya & Elal-Mus, 2012). The contaminations of kefir samples with pathogenic bacteria such as *E. coli* and *S. aureus* possess health risks for consumers. Avoid spoiled fruit or sugar that might be scooping dirty or food-covered spoons into the bag. Also, fermenting too little grains or too much sugar may encourage the pathogenic bacteria to compete and out-do the small amount of grains (and too warm of a room can encourage this further). As long as using clean utensils, keeping the temperature reasonable and maintaining reasonably clean equipment and covering the flasks properly there is little risk of contamination.

It's very difficult to have truly contaminated kefir due to the very nature of the billions of cultures in contains. If however it is contaminated, it will be an off color, thick texture to the water and/or off smell and it will be able to be recognized (it will not be subtle).

## 9. BENEFICIAL EFFECTS OF NON-DAIRY KEFIR

Non- dairy kefir has long been considered good for health and various studies suggested that kefir grains may stimulate the mucosal immunity; produce metabolites, such as short-chain fatty acids and bacteriocins, encouraging the growth of bifidobacteria in the gut; reduce plasma cholesterol; and exhibit wound healing

properties and antimicrobial, anti-carcinogenic, and anti-inflammatory activities, among others (John & Deeseenthum, 2015).

Many health benefits have been attributed to kefir, including its antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, and fungi (Garrote et al., 2000). In in vitro tests with cell-free extracts of kefir, the growth of *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Clostridium tyrobutyricum* and *Listeria monocytogenes* was inhibited (Van Wyk, 2001). In general, the antimicrobial activity of kefir is ascribed to lactic acid, volatile acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and/or bacteriocins produced by LAB (Havenaar et al., 1993; Helander et al., 1992).

Kefir has been tested for antimicrobial and cicatrizing activities against several bacterial species (Rodrigues et al., 2005). The most sensitive was *Streptococcus pyogenes* followed by *Staphylococcus aureus, Salmonella typhimurium, C. albicans and Listeria monocytogenes*. The kefir showed cicatrizing activity since a faster reduction of the wound diameter was observed compared to negative control in rats, indicating that kefir biofilms and their polysaccharide compounds may be good antimicrobial, antiinflammatory and cicatrizing agents for use in a variety of infections (Schneedorf, 2012).

Some studies refer to kefir's antimicrobial activity and suggest that the probiotic strains in kefir might influence against many pathogenic microrganisms. Kefir inhibits the growth of *Streptococcus pyogenes* and *Candida albicans* and strains of *Lactococcus cremoris, Lc. lactis, Str. thermophilus* (Rodrigues et al., 2005), and *Str. durans*, isolated from kefir inhibited the growth of *S. aureus* (Yuksekdag et al., 2004). In addition, two strains of *Lc. lactis* and a strain of *Lc. cremoris* inhibited the growth of *E. coli* and *Pseudomonas aeruginosa* and a strain of *St. thermophilus* was active against *P.*

*aeruginosa* (Yuksekdag et al., 2004). A bacteriocin produced by a strain classified as *Lactobacillus* spp. had activity against *L. innocua* F. (Atanassova et al.,1999). Also, a number of *Lactobacillus* spp. isolated from kefir displayed antimicrobial activity against enteropathogenic bacteria and affected the adhesion of *Salmonella typhimurium* to Caco-2 cells (Santos et al., 2003).

In addition, kefir might also promote competitive adhesion to the gastrointestinal epithelium surface (Chiu et al., 2007). Lactobacillus isolated from kefir showed antimicrobial activity against *Enterobacteria* and verified that ingestion of kefir specifically lowered microbial populations of *Enterbacteriaceae* and *Clostridia* (De Oliveira Leite et al., 2013).

Probiotic properties of LAB isolated from kefir such as *L. acidophilus, Lactobacillus helveticus, L. casei, Pediococcus dextrinicus, Pediococcus acidilactici, P. pentosaceus, Lactococcus cremoris*, and *Lactococcus lactis* are able to survive at low pH values, at different bile salt concentrations, and were able to autoaggregate and coaggregate with *E. coli*. (Sabir et al., 2010). In addition, *L. acidophilus* and *L. kefiranofaciens* had the best probiotic characteristics tested within the *Lactobacillus* spp. (*L. kefir, L. brevis, L. paracasei, L. plantarum, L. acidophilus* and *L. kefiranofaciens*) (Santos et al., 2003).

Anti-inflammatory responses of sugary kefir and its derivatives are poorly related in the literature. Notwithstanding, kefir may exert a beneficial effect on acute inflammatory responses, additionally improving the immune status of treated animals. Rodrigues et al (2016) evaluated plausible probiotic activities of a beer made with water kefir grains as a protective agent against damages induced in rat tissues after injection of inflammatory agents of carrageenan (rat paw edema), or acute intoxication due to ethanol administration (gastric ulcer). The kefir beer treatment resulted in greater

weight loss as compared to the control beer group and even with the group receiving the kefir suspension, which is an intriguing finding that is probably linked to the combined antioxidant and probiotic activities of kefir beer. In addition, kefir beer presented a significant reduction in carrageenan-induced paw edema as compared to the control beer ($P < 0.05$), and a pronounced inhibition with histamine-induced edema in a more effective manner than the control.

Honey-based kefir beverage showed DNA protection effect against damage caused by hydroxyl radical (Fiorda et al., 2016). It is known that some human diseases such as cancer and neurodegenerative disease involve in imbalance between oxidant and antioxidant defense system and oxidative DNA damage caused by reactive oxygen species including hydroxyl radical, superoxide anion, and hydrogen peroxide are responsible for these diseases (Lin et al., 2012). Therefore, DNA protection capacity of honey-based kefir may contribute in defense system against oxidative damage reactions, avoiding formation of free radicals and/or repairing the damage caused by them.

## 10. CONCLUSION

The microflora of kefir grains when adapted to other sources of substrates is similar to milk kefir. However, a slight pressure to specific species within the broad range of species within the grain is observed. For example, stimulation of *Saccharomyces* metabolism, which generates a higher ethanol content in these fermentations. This also seems to stimulate the growth of acetic acid bacteria that benefit from increased ethanol production to its growth and acetic acid metabolism. Inoculation of kefir grains in alternative substrates also demonstrates functional activities as antimicrobial, anti-carcinogenic and anti-inflammatory activities. A range of potential probiotic species is also isolated from this process. In addition, new non-dairy beverages are being

developing with water kefir grains, offering alternatives for consumption of fruits and vegetables and another option as a probiotic product for people with special needs (lactose intolerance).

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# CHAPTER 2

## DEVELOPMENT OF KEFIR-BASED PROBIOTIC BEVERAGES WITH DNA PROTECTION AND ANTIOXIDANT ACTIVITIES USING SOYBEAN HYDROLYZED EXTRACT, COLOSTRUM AND HONEY

Manuscript published in *LWT – Food Science and Technology*. Volume 68, 2016, Pg 690–697 – ANNEX A

**Abstract**

The aim of this study was to evaluate the use of different functional substrates (soybean hydrolyzed extract, colostrum and honey) to design novel probiotic beverages using kefir grains as starter culture. The fermentations were carried out at 30 °C for 24 h and physical-chemical composition and functional aspects were determined. It was found that fermentation processes with kefir grains increased the functional quality of all substrates evaluated. Honey-based kefir beverage had higher antioxidant activity and its microbial composition was assessed using molecular approaches (Rep-PCR and 16S rRNA gene sequencing). High levels of lactic acid bacteria and yeast populations (over $10^6$ CFU/mL) were found in the product and were mainly composed of potential probiotic strains of *Lactobacillus statsumensis, Leuconostoc mesenteroides, Bacillus megaterium, Saccharomyces cerevisiae* and *Lachancea fermentati.* In addition, the honey-based kefir beverage showed protection effect on DNA damage and had a high sensory quality compared to traditional kefir beverage. The results demonstrated that honey could be an ideal alternative substrate for the production of functional cultured beverage, especially for vegans and lactose intolerant consumers.

**Keywords:** kefir beverage, soybean hydrolyzed extract, colostrum, honey, non-dairy functional beverage

## 1. INTRODUCTION

Probiotic food products are formulations containing sufficient numbers of selected live microorganisms ($10^6$–$10^7$ CFU/mL) that can beneficially modify the intestinal microbiota of the host (Rathore et al., 2012). Kefir beverage is commonly manufactured by fermenting milk with kefir grains, which supports a complex microbial symbiotic mixture of lactic acid bacteria (e.g., *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus*) and yeasts (e.g., *Kluyveromyces* and *Saccharomyces*) (Magalhães et al., 2011). Some of these different bacteria and yeasts found in kefir have been recognized as probiotics, e.g., *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* (Leite et al., 2015).

Kefir grains can be applied to ferment different substrates besides milk. These include cheese-whey, fruit juice and molasses or sugar syrups (Cui et al., 2013; Puerari et al., 2012). The development of alternative substrates used in production of fermented kefir beverage is an ideal way for the conversion of sugars to produce organic acids and alcohol. It is considered a simple and valuable biotechnology based method for maintaining and/or improving the safety, nutritional, sensory and shelf-life properties of fermented beverages (Prado et al., 2008). Colostrum is a dairy substrate of great interest due to its positive functional properties (De Dea Lindner et al., 2011). It is a complex biological fluid and a source of immunological compounds and nutrients, many proteins, immunoglobulins, non-protein nitrogen, fat, vitamins and minerals that can be used to treat or prevent infections of the gastrointestinal tract (Uruakpa et al., 2002). Additionally, soybean hydrolyzed extract and honey are both non-dairy matrixes with attractive color, good aroma and sweet sour mouthfeel, besides being a source of natural antioxidants and other functional benefits, such as hypolipidemic, anticholesterolemic, antiatherogenic and the effects of fructooligosaccharides presented in these substrates

(Escriche et al., 2011; Pandey & Mishra, 2015). Soybean hydrolyzed extract and honey can serve as healthy alternatives for dairy probiotics to overcome problems as lactose intolerance, allergenic milk proteins and cholesterol contents (Soccol et al., 2012; Soccol & Prado, 2007).

The aim of this study was to evaluate the use of different functional products as raw material (soybean hydrolyzed extract, colostrum and honey) to design a probiotic beverage using kefir grains as starter culture. Functional characteristics and physic-chemical composition of these novel beverages were determined and compared to traditional milk kefir. In addition, the microflora, sensory quality and DNA protection effect of honey kefir beverage was evaluated due to its higher antioxidant activity. The bioprocess for the production of honey beverage fermented with kefir grains is part of a patented application process (No. BR 102014021724 0) authored by Soccol, Fiorda, Prado & Bellettini, 2014 (ANNEX B).

## 2. MATERIAL AND METHODS

### 2.1. KEFIR GRAINS AND INOCULUM PREPARATION

Kefir grains from Tibet (Province of Sigatse) and Mexico (Province of Guanajuato) were obtained from families that traditionally consumed kefir. The samples of Tibetan Kefir were preserved in sterilized milk (5%, w/v) and the samples of Mexican Kefir were preserved in brown sugary solution (10% w/v). To preserve the kefir grains the substrate was renewed daily for a period of seven days. The grains were then washed with sterile distilled water and subsequently used to inoculate different raw materials (soybean hydrolyzed extract, colostrum and honey).

## 2.2. MUST PREPARATION

### *2.2.1. Soybean hydrolyzed extract*

Mature soybean seeds were obtained from local market in Curitiba, Paraná - Brazil. The seeds were thoroughly washed and soaked overnight at 25 $^{o}$C with 10 times their weight of distilled water. Using a blender, the soybean seeds were homogenized at low speed for 1 min. Soybean hydrolyzed extract was obtained from the resulting slurry by the removal of an insoluble residue (soybean pulp fiber) by filtration. The soybean hydrolyzed extract was heated at 96$^{o}$C for 40 min and then cooled to room temperature (25$^{o}$C).

### *2.2.2. Honey media*

Honey was obtained from local market in Curitiba, Paraná - Brazil. Honey-based media was prepared by mixing honey with sterile distilled water in proportion to obtain a must of 40 $^{o}$Brix, therefore, was used the Equation 1.

$$W_{honey} \times {}^{o}Brix_{honey} = W_{must} \times 40^{o}Brix \quad \text{(Equation 1)}$$

Where $W_{honey}$ is the necessary amount of honey and $W_{must}$ is the amount of must is desired to produce. After determining the required amount of honey, the amount of water being added was estimate by Equation 2. The honey must was pasteurized at 63 °C/30 min before use.

$$W_{water} = W_{must} - W_{honey} \quad \text{(Equation 2)}$$

### *2.2.3. Bovine Colostrum*

Bovine colostrum was collected within the first 12 h after calving from three healthy cows (breed "jersey") kept under veterinary supervision at a dairy farm localized in the city of Castro (24º 47' 28" S and 50º 00' 43" W) Southern of Brazil. The colostrum was defatted by centrifugation (3,000 x g/20 min/ 2°C), pasteurized at 63 °C/30 min and divided into aliquots that were kept frozen at −20 °C until use (De Dea Lindner et al., 2011).

## 2.3. PRODUCTION OF KEFIR BEVERAGE

The Tibetan kefir grains were inoculated into soybean hydrolyzed extract, colostrum and cow milk substrates, while Mexican kefir grains were inoculated into honey must. The selection of raw material and its respective kefir inoculum (Tibetan or Mexican) was based on preliminary tests carried out with biomass growth (data not shown). Wet weight cells of 100 g were transferred into 2L of fermentation substratum. A batch aerobic fermentation was carried out in static conditions at 30 °C for 24 h. The pH kinetic of the fermented kefir beverages was determined using a pH meter and measured after 0, 12, 24, 36 and 48h. Even thought the fermentation time was 24 h, the pH was measured until 48 h in order to determine the change in pH over the period of fermentation time.

## 2.4. PHYSICAL-CHEMICAL CHARACTERIZATION OF KEFIR BEVERAGES

### *2.4.1. Volatile flavor compounds*

Aroma compounds of kefir beverages produced after 24 h of fermentation were measured by headspace analysis in a gas chromatograph (Shimadzu model 17A)

equipped with a flame ionization detector at 230°C. Aroma compounds were identified by comparing the peak retention times against those of authentic standards purchased from Sigma. The operation conditions were as follows: a 30 m × 0.32 mm HP-5 capillary column, column temperature of 40 to 150 °C at a rate of 20 °C/min, injector temperature at 230 °C. Individual volatiles were expressed as μmol/L of headspace, as ethanol equivalent (Pereira et al., 2014).

### *2.4.2. HPLC Analyses*

Sugars (glucose and lactose), ethanol and lactic acid were quantified by high-performance liquid chromatography (HPLC). The kefir beverages were separated by centrifugation at 6,000 × g and filtered through 0.22-μm pore size filter (Millipore Corp., Billerica, MA). The filtered samples were injected (50 μL) into HPLC system equipped with an HPX-87H column (300 by 7.8 mm; Bio-Rad Laboratories, California) connected to a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company). The column was eluted with a degassed mobile phase containing 5 mM $H_2SO_4$ at 60 °C at a flow rate of 0.6 mL/min (Prado et al., 2015).

## 2.5. FUNCTIONAL ASPECTS

The functional aspects (antioxidant activity and exopolysaccharides production) were performed in samples at the start of the fermentation (0 h) and after 24 h of fermentation.

### *2.5.1. Antioxidant capacity*

#### **2.5.1.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay**

The DPPH radical scavenging activity was measured in kefir beverages (0 and 24 h of fermentation) according to the procedure described by Rufino et al. (2010). A DPPH· solution (80 μM) was freshly prepared in 95% methanol. A volume of 250 μL of this solution was allowed to react with 35 μL sample and the absorbance was measured at 515 nm, for 30 min. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = (Ac\text{-}A/Ac) \times 100 \qquad \text{(Equation 3)}$$

Where *Ac* is the absorbance of the control solution and *A* is the absorbance of the samples. The results were plotted and analyzed by exponential regression to obtain the concentration of antioxidant necessary to decrease the initial DPPH concentration by 50% (IC50).

#### 2.5.1.2. ABTS (2,20 -azino-bis(3-ethylbenzothiazoline-6-sulphonic acid))radical scavenging assay

The ABTS assay was performed according to Vasconcellos et al. (2014). The ABTS solution was produced by reacting 7mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) for 12-16 h, in the dark, at room temperature. Prior to use, the ABTS working solution was prepared by diluting the stock solution with EtOH to an absorbance of 0.70 ± 0.02 at 734 nm. The samples and Trolox standards (20 μL) were combined with the ABTS working solution (170μL, absorbance 0.70 ±0.02) in 96-well microplate. After 6 min of incubation at 30°C, the absorbance at 734 nm was read with a microplate reader. The antioxidant activity was calculated

throughout the range of the response curve of Trolox (M Trolox/g of sample) and expressed as Trolox equivalent antioxidant capacity (TEAC).

### *2.5.2. Quantification of Exopolysaccharides (EPS)*

For EPS quantification, the samples were centrifuged at 8,000 x g at 5$^{o}$C for 20 min. EPS in the supernatant fluid was precipitated by adding three times volume of chilled 95% ethanol (−20$^{o}$C) and put at 4 $^{o}$C for 24 h. The sample was then centrifuged at above given conditions and the pellet was retained. The sample was re-dissolved in distilled water. The quantification was followed by Phenol–sulfuric acid method (Cuesta et al., 2003).

## 2.6. ENUMERATION OF POTENTIAL PROBIOTIC BACTERIA AND YEASTS OF HONEY-BASED KEFIR BEVERAGE (HKB)

The HKB was chosen to be analyzed microbiologically due its higher antioxidant capacity. Ten milliliters of HKB sample was added to 90 mL sterile saline-peptone water, followed by serial dilution. Enumeration of microorganisms was carried out using MRS agar (lactic acid bacteria population), M17 agar (*Lactococcus* population) and YM agar (yeast population). Plating was performed, in triplicate, with 100 µL of each diluted sample. Plates were incubated aerobically at 37°C for 48 h for bacteria and 30 $^{o}$C for 72 h for yeast. Following incubation, the colony forming units ($\log_{10}$ c.f.u./mL) were quantified. For each type of medium a square root of the number of isolated colonies (numbers of microorganisms identified of each species $|n| = \sqrt{n}$) was taken at random for identification (Holt et al., 1994). Isolates were purified by streaking

and the yeast and bacteria species were separated after microscopic examinations. The purity of bacteria isolates was monitored by catalase activity and Gram staining.

## 2.7. rRNA GENE SEQUENCING AND Rep-PCR

Bacterial and yeasts isolates were re-suspended in 40 μL of PCR buffer and the DNA from pure cultures was extracted using a QIAamp DNA Mini kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

The isolates were identified by sequence analysis of the partial 16S rRNA gene or the ITS region for bacteria and yeast, respectively. The primers 27F and 1492R were used to amplify 16S rRNA gene of bacteria isolates (Lane et al., 1985) while the primers ITS4 and ITS5 were used to amplify ITS region of yeast (Bertini et al., 1999). The PCR products were sequenced using an ABI3730 XL automatic DNA sequencer. The sequences were then compared to the GenBank database and the searches were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained, using the BLAST algorithm (National Center for Biotechnology Information, MA, USA).

The isolates were characterized at strain level using repetitive extragenic palindromic (Rep)-PCR technique with $GTG_5$ primer (Pereira et al., 2012). The amplified products were separated by electrophoresis in a 1% (w/v) agarose gel at 80 V for 40 min and stained with ethidium bromide (0.5 μg/mL, Sigma). The size of the products was estimated using a 100-bp DNA ladder. The gels were visualized via UV trans-illumination LTB20x20 HE (Loccus, Brazil) and images were captured using a camera.

## 2.8. PROTECTION AGAINST DNA BREAKAGE

The assay was conducted to determine the protective ability of the HKB against supercoiled DNA by the method of Kang et al. (2008) with some modifications. *Escherichia coli* DH5a cells were transformed with pPICZalpha C plasmid DNA and then grown overnight in the LB medium containing ampicillin (50 μg/mL) at 37°C. Plasmid DNA was purified using the QIAGEN Plasmid Kit (Macherey-Nagel, Düren, Germany). The "damage" solution (1 mM $^{\cdot}OH$) was prepared by adding 3.1 μL of 30% $H_2O_2$ into 100 mL of 1 mM $FeSO_4$. The reaction solution consisted of 5 μL of plasmid DNA, 5 μL of "damage" solution, and 5 μL of the HKB sample. As a negative control, HKB sample was replace for sterile water. Three microlitres of loading buffer [30 mM EDTA, 36% (v/v) glycerol, 0.05% (w/v) xylene cyanol FF and 0.05% (w/v) bromophenol blue] were added after 1 hour of incubation in dark, and the reaction products were then electrophoresized in 1% of agarose gel for 60 min under 120 V condition. Agarose gel was stained with 0.05% (w/v) ethidium bromide and then analyzed with image analyzer (LTB20x20 HE, Loccus, Brazil).

## 2.9. SENSORY EVALUATION

The sensory characteristics of HKB were compared with traditional kefir beverage (TKB), fermented in brown sugar solution (10% w/v) for 24 h at 30$^{o}$C. The sensory evaluation was conducted by a panel of 100 untrained panelists. Color, aroma, appearance, thickness, taste and overall acceptability were evaluated using in a hedonic rating scale with 9-point, where 1 was the lowest value (disliked extremely) and 9 the highest (liked extremely) (Stone & Sidel, 1993) (Appendage 1, 2 and 3). In addition, purshase intent was evaluated using a 5-point scale (5 = would certainly buy, 1 = would

certainly not buy). Samples were refrigerated at 5°C and 20 mL were served immediately after their opening under white light. The beverages tested were numerically coded and tap water was provided to the panelist for cleansing their palate between sampling. The sensorial test was previously approved by Ethic Committee, process n. 1.171.202 (ANEXX D). Data were expressed as the mean of all the scores.

### 2.10. STATISTIC ANALYSES

The results were expressed as mean ± standard deviation from 3 replicate determinations. Differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey´s post-hoc test. P-values $< 0.05$ were considered to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. pH KINETIC

The pH value decreased mainly during 24 h and reached similar values (~4.0) at the end fermentation processes (Figure 1). Typical pH of kefir beverages made in dairy factory is between 4.0 and 4.4 (Irigoyen et al., 2005) and the measured values in this study were in this range. These results demonstrate that the kefir grains were well adapted to the raw materials tested and the bacteria and yeast metabolism resulted in pH reduction along with the production of organic acids, ethanol, carbon dioxide and other volatile compounds (Athanasiadis et al., 2004).

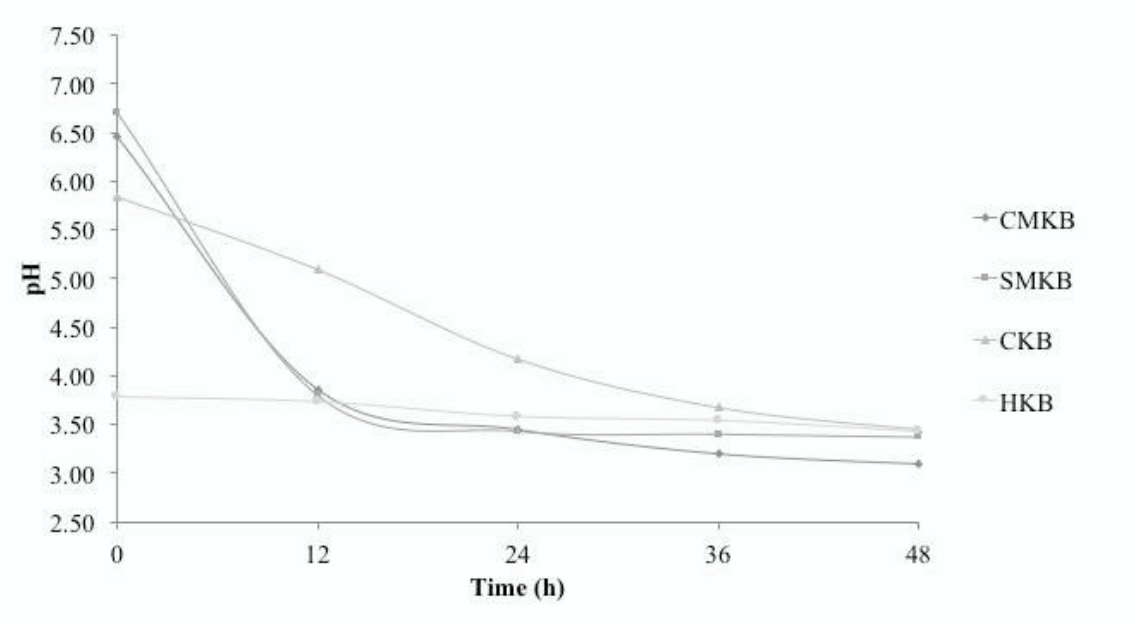


**Figure 1.** Time evolution of pH on fermented beverages using kefir grains.
CMKB: Cow Milk-based Kefir Beverage; SMKB: Soybean-Milk Kefir Beverage; CKB: Colostrum-based Kefir Beverage; HKB: Honey-based Kefir Beverage

## 3.2. PHYSICAL-CHEMICAL CHARACTERIZATION OF KEFIR BEVERAGES

The analytical parameters measured in the kefir beverages produced in this study are shown in Table 1.

**Table 1.** Chemical characteristics of fermented beverages obtained after 24h incubation with kefir grains

| | Compounds | Kefir Beverage | | | |
|---|---|---|---|---|---|
| | | **CMKB** | **SMKB** | **CKB** | **HKB** |
| GC Analyzes* | Ethyl acetate | ND | $2.5 \pm 0.1^{b}$ | ND | ND |
| | 2,3-butanedione | $11.3 \pm 0.3^{b}$ | ND | ND | ND |
| | Ethyl propionate | ND | $0.8 \pm 0.1^{b}$ | ND | ND |
| | Acetaldehyde | ND | ND | ND | $74.8 \pm 7.8^{a}$ |
| HPLC Analyzes** | Lactose | $25.15 \pm 1.07^{a}$ | $1.45 \pm 0.20^{c}$ | $24.34 \pm 1.35^{a}$ | ND |
| | Glucose | $0.44 \pm 0.03^{c}$ | $1.14 \pm 0.10^{b}$ | $0.75 \pm 0.12^{c}$ | $106.41 \pm 8.40^{a}$ |
| | Lactic acid | $30.45 \pm 1.63^{a}$ | $5.65 \pm 0.47^{c}$ | $13.67 \pm 1.55^{b}$ | $3.51 \pm 0.19^{b}$ |
| | Ethanol | $3.54 \pm 0.09^{bc}$ | $4.50 \pm 1.19^{b}$ | $1.80 \pm 0.08^{c}$ | $9.34 \pm 0.74^{a}$ |

*Values expressed in µmol/l of ethanol equivalent as means of triplicate (mean ± standard deviation).
** Values expressed in g/L (mean±standard deviation).
ND: not detected. Means in each row bearing the same letters are not significantly different ($p > 0.05$) from one another, using Tukey's test.
CMKB: Cow Milk-based Kefir Beverage; SMKB: Soybean-based Kefir Beverage; CKB: Colostrum-based Kefir Beverage; HKB: Honey-based Kefir Beverage

Colostrum-based kefir beverage (CKB) was very similar to traditional milk kefir, with high lactose and lactic acid content and low ethanol concentration. This

higher lactic acid content in dairy matrixes could probably be due to the characteristics of colostrum and milk, that are richer in nutrients (primarily proteins) than honey or soybean hydrolyzed extract, and stimulates the development of lactic acid bacteria. This result is important since lactic acid provides pleasant taste and inhibits the development of undesirable or pathogenic microorganisms (Magalhães et al., 2010).

Honey-based kefir beverage (HKB) was characterized by highest content of glucose and ethanol and lower levels of lactic acid. This demonstrates that the microbial metabolism during honey fermentation was more selective, increasing the conversion of glucose into ethanol, and could indicate alterations in carbohydrate metabolism of the kefir microorganisms in relation to milk fermentation. Although yeasts such as *Saccharomyces, Hanseniaspora* and *Pichia* (Table 2) are primarily responsible for the conversion of sugar into ethanol during kefir fermentation, some heterofermentative bacteria (e.g. *Lactobacillus kefir*) are also capable of producing ethanol. Acetaldehyde - which imparts floral and fruity flavor to the final beverage (Sanz et al., 2002) - was also found in high concentration in HKB (Table 1). It is possible that acetaldehyde is derived from compounds present in the floral plants where of bees collect pollen to produce the honey used in this study. Acetaldehyde has been identified in previous studies on honey aromatics compounds (Escriche et al., 2011). In addition, it may also have been formed by streptococci or yeast groups during fermentation process (Pereira et al., 2014).

In the case of soybean-based kefir beverage (SMKB), the main positive characteristic was related to the high content of volatile esters, namely ethyl propionate and ethyl acetate (Table 1). These compounds were not detected in soybean hydrolyzed extract prior to inoculation with the kefir grains (data not shown). Volatile compounds are important contributors to the flavors of beverages, as they determine different desirable sensory characteristics (Rossi et al., 2009). The above esters are known for

their fruity aroma contribution and may have been derived from soybean hydrolyzed extract and/or as a secondary metabolism of kefir yeasts (Kourkoutas et al., 2002; Pereira et al., 2014). This attribute makes SMKB an attractive beverage with enhanced aromatic value.

## 3.3. FUNCTIONAL ASPECTS

### *3.3.1. Antioxidant activity*

It was found that fermentation of kefir grains increased the functional quality of all substrates tested, in terms of increased levels of DDPH (reduction of $IC_{50}$ values; Figure 2A) and Trolox equivalent antioxidant capacity (Figure 2B). This indicates that some antioxidants components present in the kefir grains were transferred to the product during fermentation (Liu et al., 2005). Interestingly, HKB and SMKB had higher antioxidant capacities compared to dairy matrixes (i.e., colostrum and cow milk), as determined by both tests employed in this study (Figure 2).

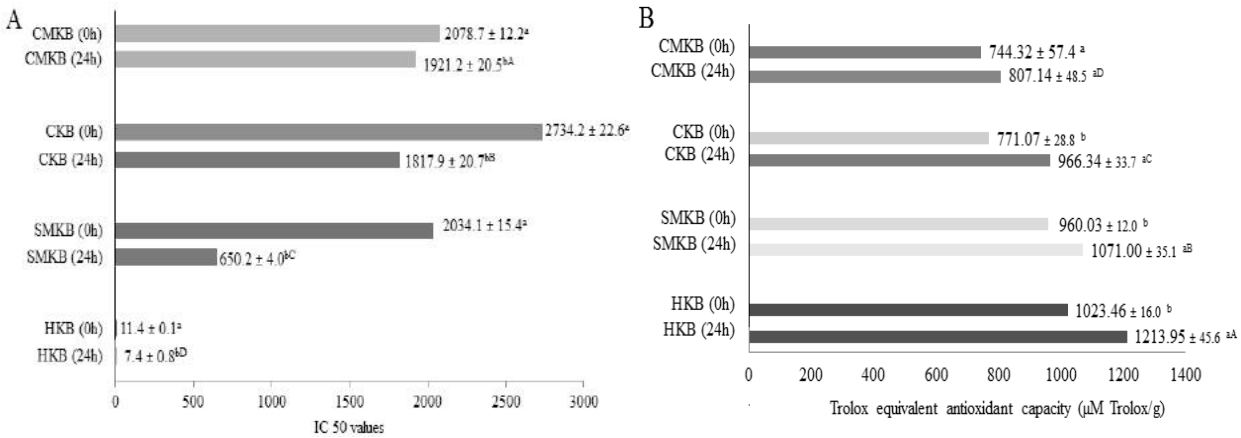


**Figure 2 .** A - $IC_{50}$ values of kefir beverages in antioxidant assays
B - Trolox equivalent antioxidant capacity (µM Trolox/g)

* Mean ± standard deviation of 3 replicates.
**Upper-case letters show significant differences between different beverages, and lower-case letters show significant differences between the beverage 0h and 24h, as determined by Tukey´s test ($p < 0.05$).
CMKB: Cow Milk-based Kefir Beverage; SMKB: Soybean-based Kefir Beverage; CKB: Colostrum-based Kefir Beverage; HKB: Honey-based Kefir Beverage

The antioxidant properties of soy and honey have been attributed to high levels of specific flavonoids, i.e., genistein and daidzein in soybeans (Pratt & Birac, 1979) and rutin in honey (Oomah & Mazza, 1996). In addition, it is important to highlight that kefir fermentation improved the antioxidant activity of both these substrates. Some studies have demonstrated that lactic acid bacteria (e.g., *Lactobacillus acidophilus, L. bulgaricus, Streptococcus thermophilus,* and *Bifidobacterium longum*) scavenge reactive oxygen species and some of this species are found in kefir microbiota (Nishino et al., 2000).

### *3.3.2. Quantification of Exopolysaccharides (EPS)*

The amount of EPS in kefir beverages did not exceed 1.527 g/L (Figure 3). The higher EPS content in dairy beverages, i.e., CKB and CMKB, can be due to bacterial cells interaction with milk protein, which may remain attached to the cells and/or interact with proteins (Vlahopoulou et al., 2001).

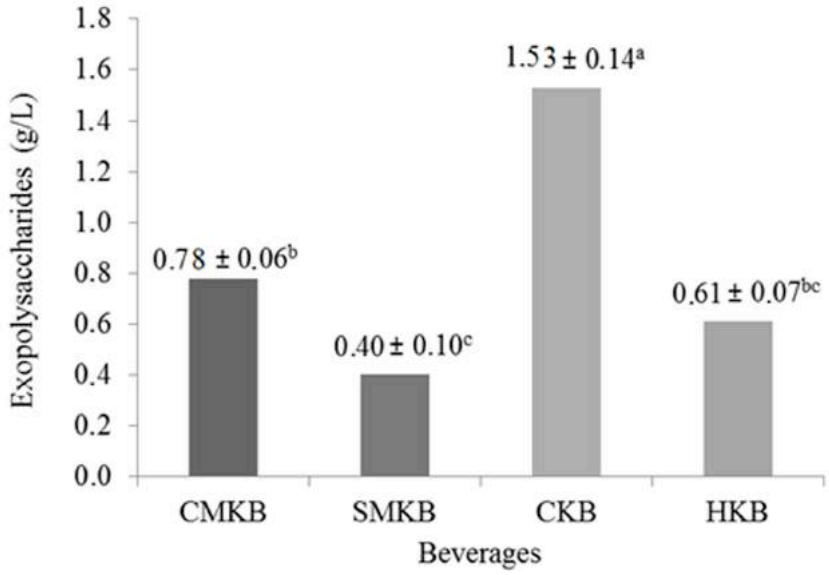


**Figure 3.** Exopolysaccharides (EPS) amounts in different kefir beverages
* Mean ± standard deviation of 3 replicates.
** Means followed by a different letter are significantly different ($p < 0.05$) as determined by Tukey's test.
CMKB: Cow Milk-based Kefir Beverage; SMKB: Soybean-based Kefir Beverage; CKB: Colostrum-based Kefir Beverage; HKB: Honey-based Kefir Beverage

The EPS content of SMKB (0.401 g/L) and HKB (0.61 g/L) probable came from glucose and other carbon source present in these substrates (Table 1), which is converted into EPS by the microbial growth. Generally, limiting concentrations of some nutrients and excess carbohydrate assists the production of polysaccharides (Ernandes & Garcia-Cruz, 2011; Sutherland, 2001).

## 3.4. IDENTIFICATION OF POTENTIAL PROBIOTIC BACTERIA AND YEAST IN HKB FERMENTATION

The microbial composition of HKB was assessed in subsequent experiments due its higher antioxidant activity. It has been suggested that fermented products required probiotic bacteria at $10^7$ cfu/mL in order to give health benefits to the gastrointestinal tract when consumed (Mirdula & Sharma 2015; Ouwehand & Salminen, 1998). In this study, the microbial density immediately after inoculation was lower $10^3$ cfu/mL (data not shown). After fermentation, high levels of *Lactococcus* population ($10^7$; M17 medium), total lactic acid bacteria ($10^6$ cfu/mL; MRS medium) and total yeast ($10^7$ cfu/mL; YM medium) in the manufactured HKB. These results indicated that honey offers a good potential as vehicle for the production of probiotic beverages.

Seventy-five isolates (39 bacteria and 36 yeasts) were identified by partial rRNA gene sequencing. A number of yeast species (*Hanseniaspora uvarum*, *Issatchenkia orientalis, Lachancea fermentati, Pichia membranifaciens*, *P.* kudriavzevii, *Saccharomyces cerevisiae* and *Zygosaccharomyces fermentati*), LAB (e.g., *Leuconostoc mesentereoides*, *Lactobacillus satsumensis* and *Lysinibacillus sphaericus*) and *Bacillus megaterium* were identified. Previous studies showed that a variety of different species

of *Lactobacillus* and *Leuconostoc* have been isolated and identified in kefir grains from around the world (Güzel-Seydim et al., 2005; Magalhães et al., 2010). These LAB species commonly produce antimicrobial substances with effect against gastric and intestinal pathogens and/or compete for cell surface and mucin binding sites (Berry, 2012).

Very few yeast strains have been studied as possible biotherapeutics agents and most reported effects of yeasts as probiotic organisms in clinical trials for alleviation of antibiotic-associated diarrhea, infectious diarrhea, irritable bowel syndrome and inflammatory bowel diseases (Foligné et al., 2010). This study demonstrated that HBK was composed of a wide variety of yeast species (Table 2). The presence of yeast contributes to the enhancement of the sensory quality of the probiotic beverage, promoting a strong and typically yeasty aroma, as well as its refreshing, pungent taste (Magalhães et al., 2010). In addition, some of these yeast species also reduces the concentration of lactic acid, removes the hydrogen peroxide and produces compounds that stimulate the growth of other bacteria, thus increasing the production of kefiran exopolysaccharides (Cheirsilp et al., 2003).

The variation of strain composition of yeast and bacteria isolates was analyzed by repetitive element PCR (Rep-PCR). By using the $(GTG)^5$-primer pair, rep-PCR produced strain-specific DNA fingerprints (Table 2), including those of *Lactobacillus satsumensis* (5 strains), *Leuconostoc mesenteroides* (3 strains), *Lactobacillus sp.* (3 strains), *Saccharomyces cerevisiae* (2 strains), *Hanseniaspora uvarum* (2 strains) *and Pichia membranifaciens* (2 strains).

**Table 2.** Identification of representative bacteria and yeasts isolated from honey-based kefir beverage

| | Isolates species | Number of isolates identified | Number of rep-PCR Genotypes | Identity | GenBank accession n° |
|---|---|---|---|---|---|
| **Bacteria** | | | | | |
| | *Lactobacillus satsumensis* | 6 | 5 | 98% | NR_028658.1 |
| | *Lactobacillus* sp. | 4 | 3 | 99% | AY681129.1 |
| | *Bacillus* sp. | 7 | 1 | 99% | HM566766.1 |
| | *Bacillus megaterium* | 4 | 1 | 99% | KF933665.1 |
| | *Leuconostoc mesenteroides* | 17 | 3 | 99% | KF697619.1 |
| | *Lysinibacillus sphaericus* | 1 | 1 | 99% | GQ279292.1 |
| **Yeast** | | | | | |
| | *Hanseniaspora uvarum* | 4 | 2 | 97% | KF953898.1 |
| | *Issatchenkia orientalis* | 3 | 1 | 96% | EF198000.1 |
| | *Issatchenkia* sp. | 1 | 1 | 98% | DQ667976.1 |
| | *Lachancea* sp. | 2 | 1 | 99% | KJ451620.1 |
| | *Lachancea fermentati* | 3 | 1 | 99% | GQ340439.1 |
| | *Pichia* sp. | 2 | 1 | 99% | KM252959.1 |
| | *Pichia membranifaciens* | 2 | 2 | 99% | DQ223427.1 |
| | *Pichia kudriavzevii* | 4 | 1 | 97% | AB369918.1 |
| | *Saccharomyces cerevisiae* | 12 | 2 | 99% | KC515373.1 |
| | *Saccharomycetes* sp. | 1 | 1 | 92% | HM224412.1 |
| | *Zygosaccharomyces fermentati* | 2 | 1 | 99% | AY046206.1 |

Besides identification, the potential of rep-PCR to help determine strain level variation would facilitate selection of bacterial and yeast strains with desirable attributes for controlled fermentation or for their utilization in newer functional foods. In addition, particular yeast and bacteria strains may positively influence development of high levels of secondary compounds, such as volatile, flavoring compounds in beverage production process (Oliveira et al., 2005).

### 3.5. DNA PROTECTION EFFECT OF HKB

In this study, DNA protection capacity was used to further investigate the effect of HKB. Plasmid DNA has three forms on agarose gel electrophoresis, namely supercoiled circular DNA, open circular form and linear form (Figure 4).

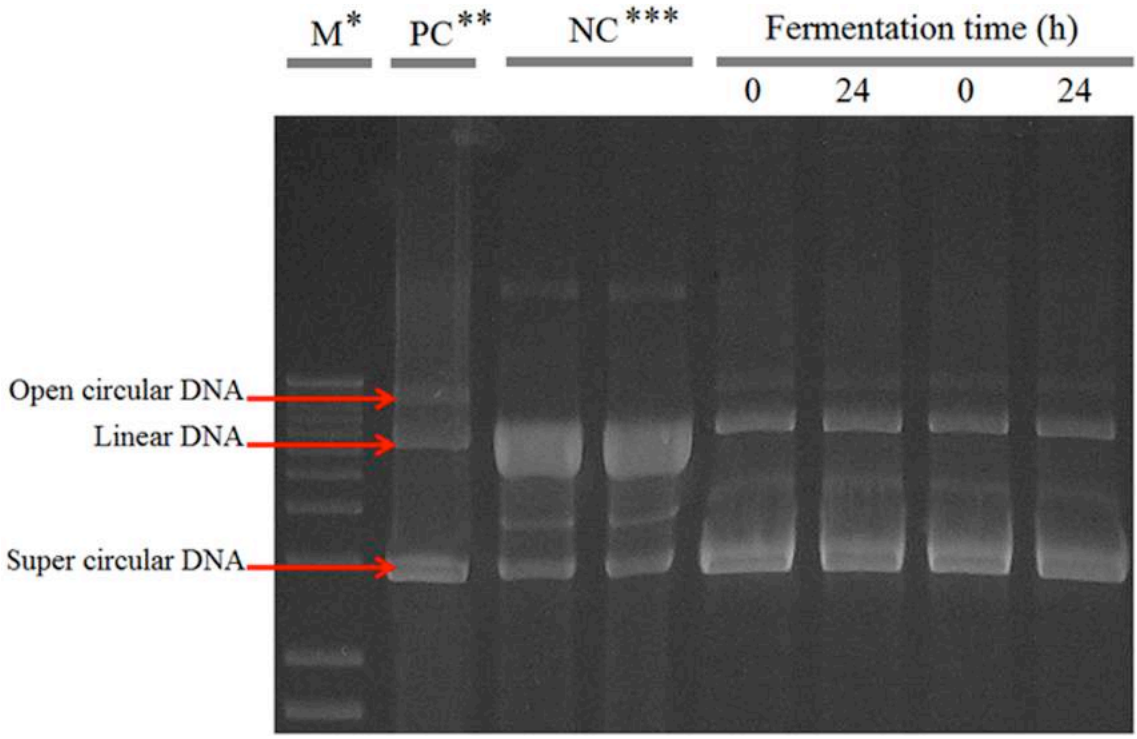


**Figure 4.** DNA damage protection potential of honey-based kefir beverage based on movement of bands with differents DNA structures.
*Molecular Marquer (1Kb); ** Positive Control (plasmid DNA without the addition of damage solution) ***Negative Control (water + plasmid DNA + damage solution)

The hydroxyl (·OH) radicals (negative control) were able to cleave DNA strand resulting in the cleavage of supercoiled circular DNA to open circular and linear forms. As shown in Figure 4, the HKB (0 and 24h) showed DNA protection effect against damage caused by hydroxyl radical. It is known that some human diseases such as cancer and neurodegenerative disease involve in imbalance between oxidant and antioxidant defense system and oxidative DNA damage caused by reactive oxygen species including hydroxyl radical, superoxide anion, and hydrogen peroxide are responsible for these diseases (Lin et al., 2012). Therefore, DNA protection capacity of HKB may contribute in defense system against oxidative damage reactions, avoiding formation of free radicals and/or repairing the damage caused by them.

## 3.6. SENSORIAL EVALUATION

The sensory assessment of HKB and traditional kefir beverage (TKB) produced at the end of the 24 h fermentation is shown in Figure 5.

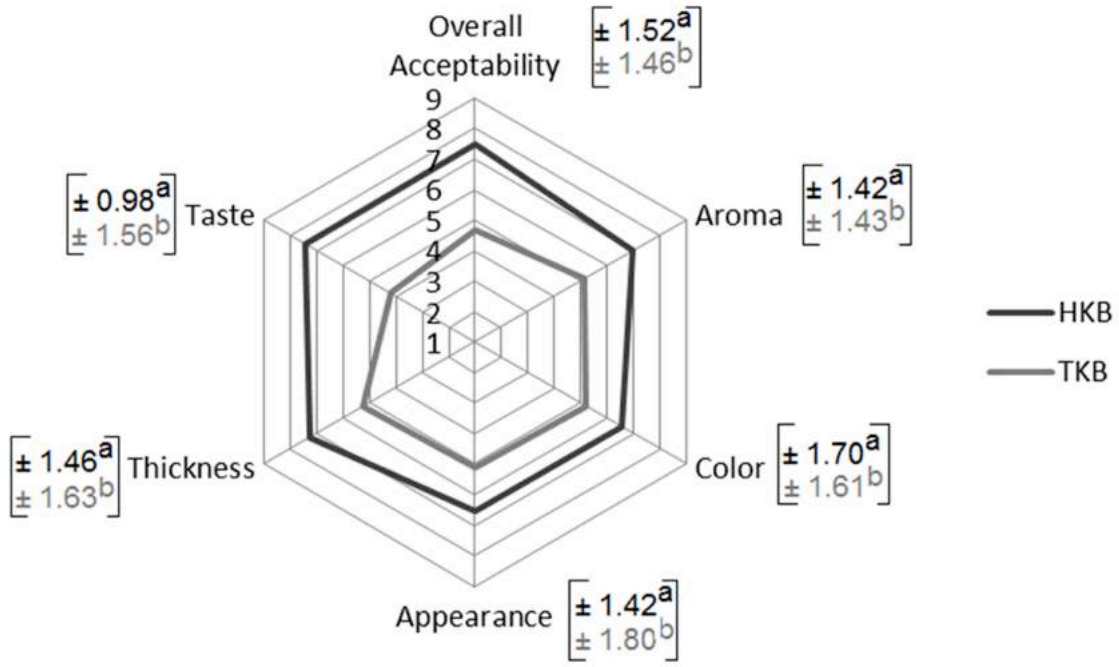


**Figure 5.** Sensory assessment of honey-based kefir beverage and traditional kefir beverage produced at the end of the 24 h fermentation

* Attributes ± standard deviation followed by a different letter are significantly different ($p < 0.05$) as determined by Tukey's test.

For all attributes assessed, HKB received significantly ($p<0.05$) higher approval scores and had an average score of 7.5 on a 9 point scale. This corresponded to the "liked moderately" and "liked very much" level in the score sheet. In the purshase intention test, the HKB received an average score of 4.01 on a 5 point scale, corresponding to a classification between "would certainly buy" and "possibly would buy". In addition, HKB received a score two times higher than TKB for purshase intention in the panel feedback (Figure 5). The results demonstrate the high sensory quality of the HKB produced in this study.

## 4. CONCLUSION

The results of the present study provided evidence indicating that soybean hydrolyzed extract, colostrum and honey could serve as raw materials/substrates for the production of kefir-like beverages with functional and flavoring properties. The results demonstrated that honey could be an ideal alternative substrate for production of fermented beverage with high antioxidant activity and potential probiotic composition. Additionally, the beverage had protective effect to DNA damage caused by hydroxyl radical and had very good sensory qualities. The study showed that non-dairy probiotic beverage using honey as base substrate could lead to a product which has enhanced health benefits and sensory qualities.

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# CHAPTER 3

# EVALUATION OF A POTENTIALLY PROBIOTIC NON-DAIRY BEVERAGE DEVELOPED WITH HONEY AND KEFIR GRAINS: FERMENTATION KINETICS AND STORAGE STUDY

Manuscript published in *Food Science and Technology International*. – ANNEX C

## Abstract

The aim of this work was to study the fermentation process of honey with kefir grains through a comprehensive understanding of its rheological properties, probiotic cell viability, instrumental color parameters and kinetic aspects in a batch bioreactor and during storage. The results showed that kefir grains were well adapted to bioreactor conditions, reaching high levels of cell viability (over $10^6$ CFU.mL$^{-1}$ for total yeast and bacteria), phenolic compounds content (190 GAE/100g) and acidification after 24 h of fermentation at 30ºC. Colorimetric analyses showed that lightness (L*) and redness (a*) remained constant, while yellowness intensities (b*) decreased during fermentation time. After 35 days of storage, honey kefir beverage (HKB) maintained its chemical characteristics and microbial viability as required to be classified as a probiotic product. The Ostwald-de Waele ($R^2 \geq 0.98$) and Herschel-Bulkley ($R^2 \geq 0.99$) models can be used to predict the behavior of HKB. The parameters analyzed in this study should be taken into account for production of this novel non-dairy beverage and scale up of this bioprocess.

**Keywords:** kefir beverage, fermentation, non-dairy functional beverage, kinetic, bioreactor, viscosity

## 1. INTRODUCTION

For centuries lactic acid fermentation has been used to preserve, improve or modify the flavor of milk, meats, cereals and vegetables. Lactic acid bacteria, such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, are the main agents of milk fermentation that convert sugars into lactic acid (Garcia-Fontan et al., 2006; Lourens-Hattingh & Viljoen, 2001; Penna et al., 2007). An alternative method for milk fermentation is through the use of kefir grains as starter cultures. Kefir grain consists of a polysaccharide composed by a complex microbial association among bacteria and yeasts, which works as a starter culture for milk fermentation (García-Fontán et al., 2006). The result is a naturally carbonated beverage (associated with yeast metabolism), with acid taste and creamy consistency due to lactic acid bacteria metabolism. The consumption of kefir beverage has been associated with beneficial effects on human health and several bacteria and yeasts found in kefir are recognized as probiotics (Zanirati et al., 2015; Diosma et al., 2014; Puerari et al., 2012).

The use of kefir beverage is limited for vegan and lactose intolerant consumers (De Dea Lindner et al., 2013; Rivera-Espinoza & Gallardo-Navarro, 2010). Kefir grains have been adapted to different non-dairy substrates — such as honey, vegetables, tea and juices — to produce functional, probiotic beverages with distinct sensory characteristics (Garcia-Fontan et al., 2006; Lourens-Hattingh & Viljoen, 2001; Penna et al., 2007). Honey is a natural sweet substance produced by honey bees from the nectar of plants. It is a very healthy and nutritious food with good aroma, taste, with antioxidant and functional properties (Codex Alimentarius, 2001). Hence, honey has been used by food industry either as a main raw material or as a secondary ingredient for flavor improvement.

Recently, we have evaluated the use of honey as an alternative substrate to design a novel probiotic beverage using kefir grains as starter culture at laboratory scale (Soccol et al., 2014; Fiorda et al., 2016). These studies provided evidence indicating that honey can serve as raw substrate for production of kefir-like beverages with functional properties (high antioxidant capacity, exopolysaccharides content and DNA protection effect) and with a high sensory quality compared to traditional kefir beverage. Additionally, some known probiotic species, e.g., *Lactobacillus statsumensis*, *Leuconostoc mesenteroides*, *Bacillus megaterium* and *Saccharomyces cerevisiae*, were identified by molecular approaches. However, further studies had to be performed before development of this novel product into a food industry. The aim of this work was to explore the fermentation process of honey kefir beverage through a comprehensive study of its rheological properties, probiotic cell viability, instrumental color parameters and kinetic aspects in a batch bioreactor and during storage.

## 2. MATERIAL AND METHODS

### 2.1. KEFIR GRAINS AND INOCULUM PREPARATION

Kefir grains isolated from sugary Mexican kefir beverage were used in this study. The kefir grains were first washed with distilled water and then used to inoculate (5% w/v) a brown sugar solution (10% w/v). The mixture was then incubated for 24 h at 30$^{o}$C (Laureys & De Vuyst, 2014; Magallhães et al., 2010). The kefir grains were renewed daily for a period of seven days into honey must for adaptation. After this the grains were washed with sterile distilled water and subsequently used as a starter culture for batch bioreactor studies.

## 2.2. HONEY MUST AND HONEY KEFIR BEVERAGE (HKB) PREPARATION

Honey was mixed with sterile distilled water, in specific proportions, to obtain a must with the desired amount of soluble solids as described by Fiorda et al. (2016). The honey must was then pasteurized at 63 °C.30 $min^{-1}$ using a water-bath. The pasteurized must was cooled down to 25 °C and then inoculated with the kefir working-culture (5% w/v). The fermentation conditions (nitrogen sources, temperature and honey concentration) were chosen by the experimental design program using the *Plackett-Burman* and Response Surface Methodology.

## 2.3. EXPERIMENTAL DESIGN

### *2.3.1. Optimization of nitrogen sources using Plackett–Burman design*

The Plackett–Burman design (Plackett & Burman, 1944) was used to determine the optimal nitrogen source levels required for maximize biomass production using honey must as the substrate. The biomass production was measured from the increase in the weight of grains, using an analytical balance (BEL Mark 210A), at the end of each fermentation batch. The organic nitrogen sources tested were yeast extract, sodium nitrate, ammonium acetate, peptone bacteriological, ammonium sulfate and ammonium nitrate. Each factor was tested at two extreme levels: 20 $g.L^{-1}$ (coded value +1) and 0 $g.L^{-1}$ (coded value -1); and central points 10 $g.L^{-1}$ (coded value 0). The range of these parameters were decided based on preliminary experimentation. They were screened by running 19 experiments, as shown in Table 1. The significant factors at the 5% level ($P < 0.05$) by regression analysis were considered to have a high impact on biomass production. The experiments were performed in 200 mL Erlenmeyer flasks

containing 100 mL of honey must under static conditions at points suggested by the design matrix (Table 1). The biomass production was measured by the increase in the weight of grains at the end of each fermentation batch.

**Table 1.** Nitrogen sources studied in the Plackett-Burman desing*

| Experiment | Yeast Extract | Sodium Nitrate | Ammonium acetate | Peptone | Ammonium sulfate |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | 1 | 1 | 1 | -1 | -1 |
| 3 | 1 | 1 | -1 | 1 | -1 |
| 4 | 1 | 1 | -1 | -1 | 1 |
| 5 | 1 | -1 | 1 | 1 | -1 |
| 6 | 1 | -1 | 1 | -1 | 1 |
| 7 | 1 | -1 | -1 | 1 | 1 |
| 8 | 1 | -1 | -1 | -1 | -1 |
| 9 | -1 | 1 | 1 | 1 | -1 |
| 10 | -1 | 1 | 1 | -1 | 1 |
| 11 | -1 | 1 | -1 | 1 | 1 |
| 12 | -1 | 1 | -1 | -1 | -1 |
| 13 | -1 | -1 | 1 | 1 | 1 |
| 14 | -1 | -1 | 1 | -1 | -1 |
| 15 | -1 | -1 | -1 | 1 | -1 |
| 16 | -1 | -1 | -1 | -1 | 1 |
| 17 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 |

* 0 g/L (-1)
10 g/L (0)
20 g/L (+1)

### *2.3.2. Optimization of fermentation conditions using response surface*

Experiments were carried out to optimize fermentation conditions of temperature and honey must concentration in honey kefir beverage production process. The Central Composite Rotational Design (CCRD) was used with 11 experiments and 3 replicates at the central point (Box et al., 2005). The coded values of the independent variables were 1.41; -1; 0; 1 and 1.41, while the real temperature fermentation values ranged between 22.95 and 37.05°C and honey concentration between 28 and 42% (Table 2). The

temperature and honey concentration ranges were chosen from preliminary test results in relation to biomass accumulation (data not shown).

**Table 2.** Real and coded values of temperature and inoculum size using experimental design for optimization of kefir fermentation*

| Experiment | Independent variables | |
|---|---|---|
| | Temperature ($^{o}$C) | Honey concentration (%) |
| 1 | 25 (-1) | 30 (-1) |
| 2 | 35 (+1) | 30 (-1) |
| 3 | 25 (-1) | 40 (+1) |
| 4 | 35 (+1) | 40 (+1) |
| 5 | 22.95 (-1.41) | 35 (0) |
| 6 | 37 (+1.41) | 35 (0) |
| 7 | 30 (0) | 28 (-1.41) |
| 8 | 30 (0) | 42 (+1.41) |
| 9 | 30 (0) | 35 (0) |
| 10 | 30 (0) | 35 (0) |
| 11 | 30 (0) | 35 (0) |

*Real values (coded values)

Biomass increase data were evaluated by analysis of variance, with the construction of multiple regression models. Graphs of response surface for the visualization of the effect of independent variables on the responses were constructed using the software Statistica (Statsoft, 2007).

## 2.4. PRODUCTION OF HONEY KEFIR BEVERAGE (HKB) IN BIOREACTOR AND STORAGE STUDY

Fermentations to determine kinetic parameters were conducted in a bioreactor STR (6 L, MDL B.E. Marubishi), equipped with a heater and a control unit and filled with 3 L of honey medium (40% w/v). Honey must was pasteurized inside the bioreactor ($63^{o}$C.30 $min^{-1}$) and a disc turbine propeller was used for homogenization. 150 g of kefir biomass (5% - w/v) were transferred into 3 L of fermentation medium (Alsayad et al., 2013), corresponding to approximately $10^{3}$ CFU.$mL^{-1}$ of yeast and bacteria, respectively. A batch fermentation was carried out under static conditions. Temperature was maintained at 30°C for 24 h.

The fermentation parameters of kefir beverages were determined at 0, 4, 8, 12, 16, 20 and 24 h of fermentation. At the end of the fermentation process, the grains were separated from the fermented beverage by filtration and washed prior to use in the next culture. Samples were taken into propylene flasks and were analyzed for 24 h following inoculation and also after storage at 5°C for 1, 7, 14, 21, 28 and 35 days. Microbial growth, phenolic compounds, color (L*, a* and b*), pH, viscosity, organic acids, carbohydrates and ethanol were analyzed during the fermentation process and during storage process.

### *2.4.1. Microbial growth*

Tryptone (Difco) at a concentration of 1 $g.L^{-1}$ was used to prepare the dilutions for the microbiological analyses. Lactic acid bacteria (LAB) were enumerated by pour plate inoculation in MRS agar (Merck) containing miconazol nitrate (200 mg. $L^{-1}$) to inhibit yeast growth. Yeast were enumerated by surface inoculation on YM medium (pH 7.0 ± 0.2) containing 100 $mg.L^{-1}$ chloramphenicol (Sigma) and 50 $mg.L^{-1}$ chlortetracycline (Sigma) to inhibit bacterial growth. Plates were incubated at 37°C for 48 h for bacteria and 30°C for 72 h for yeast. Following incubation, the number of colony-forming units ($\log_{10}$ $CFU.mL^{-1}$) was recorded.

### *2.4.2. Instrumental color parameters*

Color measurements were recorded using Hunter L*, a* and b* scale. In order to determine the instrumental color parameters, digital photos were taken of the beverages as described in Fiorda et al (2013). The D65 two-source illumination system was used with an angle of incidence of 45º on the product, which was placed on a white background. The digital images of the samples were processed using the Digital Color Meter 5.10 program (APPLE, CA, USA), selecting 15 regions of approximately 5x5 cm

in each photo. The images were converted into Cielab system using the pixel to pixel color reading application obtaining the values L* (luminosity), a* (red-green component) and b* (yellow-blue component).

### *2.4.3. HPLC analyses*

The samples were filtered through 0.22 μm filters. Filtered samples were injected (25 μL) into HPLC system (1260 Agilent Technologies) equipped with an HPX-87H Aminex fermentation monitoring column (300 × 7.8 mm) maintained at 50°C. Organic acids (lactic, acetic, citric, malic, galic, fumaric, succinic and oxalic acids), fructose, glucose and ethanol were quantified by using a refractive index detector model 1260 RID monitoring the absorbance at 215 nm. The mobile phase used (isocratic flow rate at 0.6 $mL.min^{-1}$) was 5 mM $H_2SO_4$. Standard curves based on peak area were calculated for the individual organic acids, carbohydrates and ethanol covering a broad range of concentrations, by comparison with standard solutions. Standards were prepared in deionized water (Milli-Q) filtered through 0.22 μm filters (Millex GV).

### *2.4.4. Total phenolic compounds*

Total phenolics in the supernatant were determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). The samples (0.1 mL) were mixed with 0.5 mL of Folin–Ciocalteu reagent, 1.5 mL of 20% sodium carbonate solution and 7 mL of distilled water. After 2 h, absorbance at 765 nm was read in the spectrophotometer. Results were expressed as g gallic acid equivalents (GAE)/100 g.

## 2.5. RHEOLOGICAL PROPERTIES

The rheological analyses were performed in HKB (0 and 24 h fermentation times) and in TKB (24h fermentation time) at two temperatures (5°C and 25°C). Rheological measurements were carried out using a Brookfield rheometer, model DVII-

Pro (Brookfield Engineering Laboratories, Massachussets, EUA), spindle SC4-18 connected with a bath Tecnal T-184 (Tecnal, Piracicaba, SP, Brazil). The apparent viscosity (ηap) and shear stress (τ) were obtained using software RHEOCALC (v3.1-1, Brookfield Engineering Laboratories, EUA). Each analysis was done at 20 points, at different shear rate in the range of 10–86 $s^{-1}$ and both upward and downward tests were performed in triplicate for each temperature for each sample. Fitted rheological models for the dependence of shear rate on shear stress were obtained by non-linear estimation procedure using the ORIGIN software (Version 8.6, OriginLab Corporation, Massachussets, USA). This was done by minimizing the sum of squared errors. The reliability of the equations was evaluated by the number of parameters, coefficient of determination ($R^2$) and analysis of residuals.

### *2.5.1. Theoretical models*

Non-Newtonians fluids do not present a direct proportionality between shear stress and shear rate. To describe their rheological behavior, different flow models are commonly used. The most frequently used are the Ostwald-de-Waele model, better known as the Power-Law model (Rao, 1999) given by Equation 1; and Herschel-Bulkley model, given by Equation 2.

$$\tau = k\gamma^{\eta} \qquad \text{(Equation 1)}$$

In Equation 1, τ is the shear stress (Pa), γ is the shear rate ($s^{-1}$), k is the consistency index ($Pa.s^{n}$) and η is the flow behavior index (dimensionless). In cases in which η = 1, k changes to η.

$$\tau = \tau OH + k\gamma^{\eta} \qquad \text{(Equation 2)}$$

In Equation 2, τ is the shear stress (Pa), τOH is the initial shear stress (Pa), K is the consistency index ($Pa.s^{n}$), γ is the shear rate ($s^{-1}$) and η is the flow behavior index.

### 2.6. STATISTIC ANALYSES

The results obtained in the study were expressed as mean ± standard deviation from 3 replicate data points. Differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey´s post-hoc test. P-values < 0.05 were considered to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. NITROGEN SUPPLEMENTATION OF HONEY MUST

In order to verify the significance of nitrogen sources in biomass production during HKB fermentation process, a *Plackett-Burmann* design was chosen and the results are presented in Pareto chart (Figure 1).

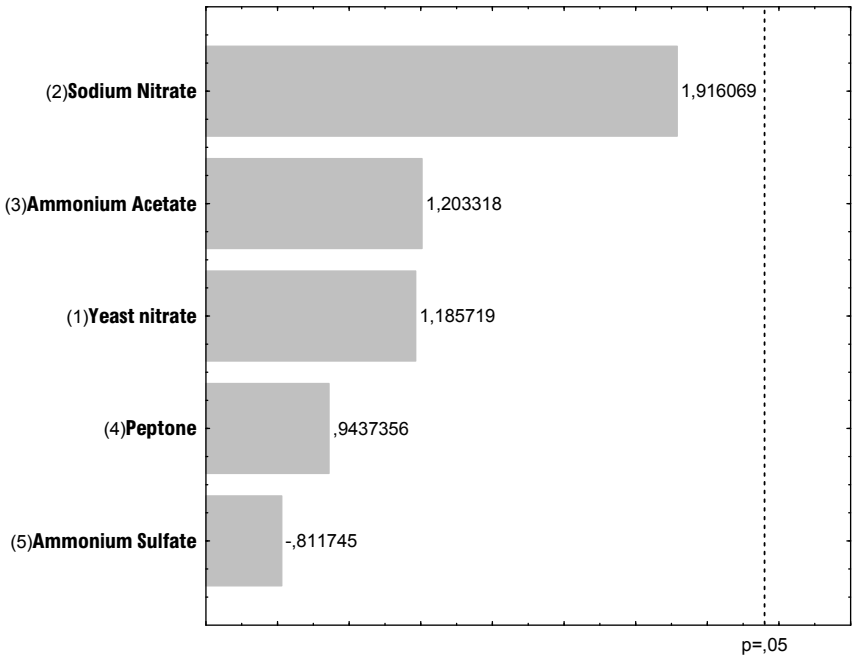


**Figure 1.** Pareto chart for biomass increase (%) in Honey Kefir Beverage (HKB) production using different nitrogen sources ($p < 0.05$).
*****Fcal=18.42; Ftab = 3.63. The maximum explained variance was 99.99 % indicating that the model was appropriate

The results showed that neither of the independent variables was statistically significant ($p<0.05$), *i.e.* nitrogen supplementation is not necessary to increase the kefir

biomass. This makes honey an interesting vehicle for kefir grains fermentation, since additional costs for nitrogen supplementation is not necessary when it is used.

### 3.2. RESPONSE SURFACE DESIGN (RSD)

The results of RSD experiments are presented in Figure 2A.

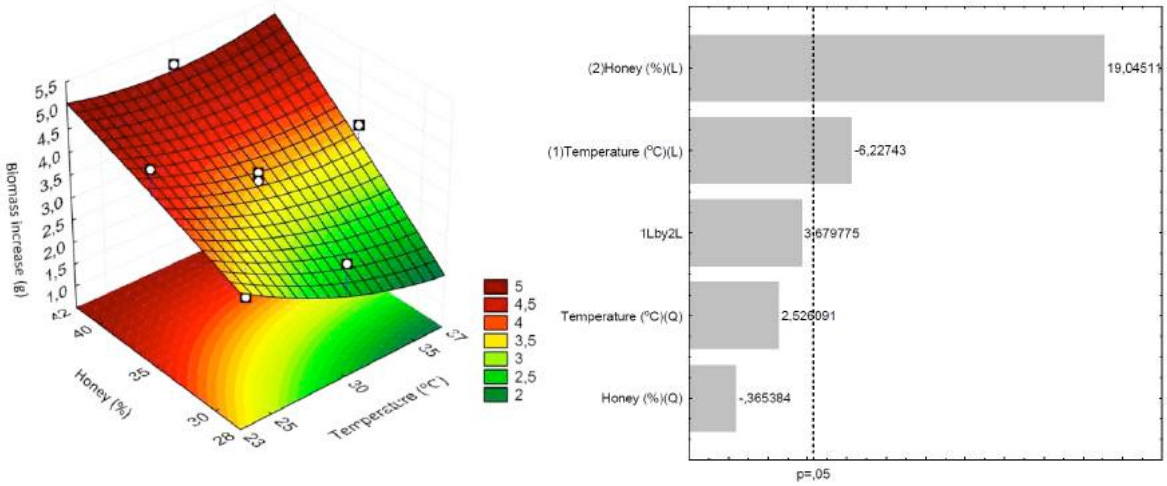


**Figure 2.** A - Response surface plot for biomass increase (g) in honey kefir beverage production ($p < 0.05$).
B - Pareto chart for biomass increase (g) in Honey Kefir Beverage (HKB) production ($p < 0.05$).

The determination coefficient ($R^2$) for biomass increase was 0.8024 and the maximum explained variance was 99.62%, indicating that the model was appropriate. The variation of 0.38% was due to other factors not included in this model. The linear regression equation (Equation 3) was obtained from the regression results of the factorial experiment with the selected at a significant level of $p < 0.05$ parameters.

$$z = 3.61\beta_0 - 0.589\beta_1 + \textit{0.285}\beta_1^2 + 1.79\beta_2 - \textit{0.04}\beta_2^2 + \textit{0.49}\beta_1\beta_2 \qquad \text{(Equation 3)}$$

Where z is the biomass increase, $\beta_1$ is temperature (°C) and $\beta_2$ is honey concentration (%). Linear effects were significant and the effects in italics were not significant ($p < 0.05$), but were held to improve the model fit. The statistical analyses and the analyses of variance (ANOVA) indicated that the proposed model suggest a

good fit. The maximum value of biomass increase (5.0 g) was obtained when the fermentation process was carried out in central temperature conditions (30 $^{o}$C) and high honey concentration (up to 40%), while the lowest biomass increase (below 2.0 g) was obtained under conditions of higher temperature (above 37 $^{o}$C) and lower honey concentration (28%) (Figure 2A).

From the response surface data, it can be observed that the temperature variable is less significant, as determined by low or no inclination of its axis. To verify the significance of this behavior, the statistical effects are presented in the Pareto chart (Figure 2B). The linear terms of temperature and honey concentration were statistically significant ($p < 0.05$), i.e., the increase of temperature decreases biomass production by 6.22% and the increase of honey concentration increases biomass growth by 19.04%. The remaining non-significant terms were kept for improved fit. These results are in agreement with the findings of Kristo et al (2003) who reported that a biomass increase occurs with a combination of low incubation temperature and high substrate concentration. According to these results, the best conditions for production of HKB are 30$^{o}$C fermentation temperature and 40% of honey concentration in must.

### 3.3. KINETIC ANALYSES AND STORAGE STUDY

Figure 3 shows the kinetic parameters for kefir grains fermentation in honey must under bioreactor conditions, as well as during beverage storage process at 5°C for 35 days. Fructose was the sugar with highest concentration in honey must (24.9 g.L$^{-1}$) and was consumed mainly after the initial microbial adaptation (lag phase) period of 4h of fermentation (Figure 3A).

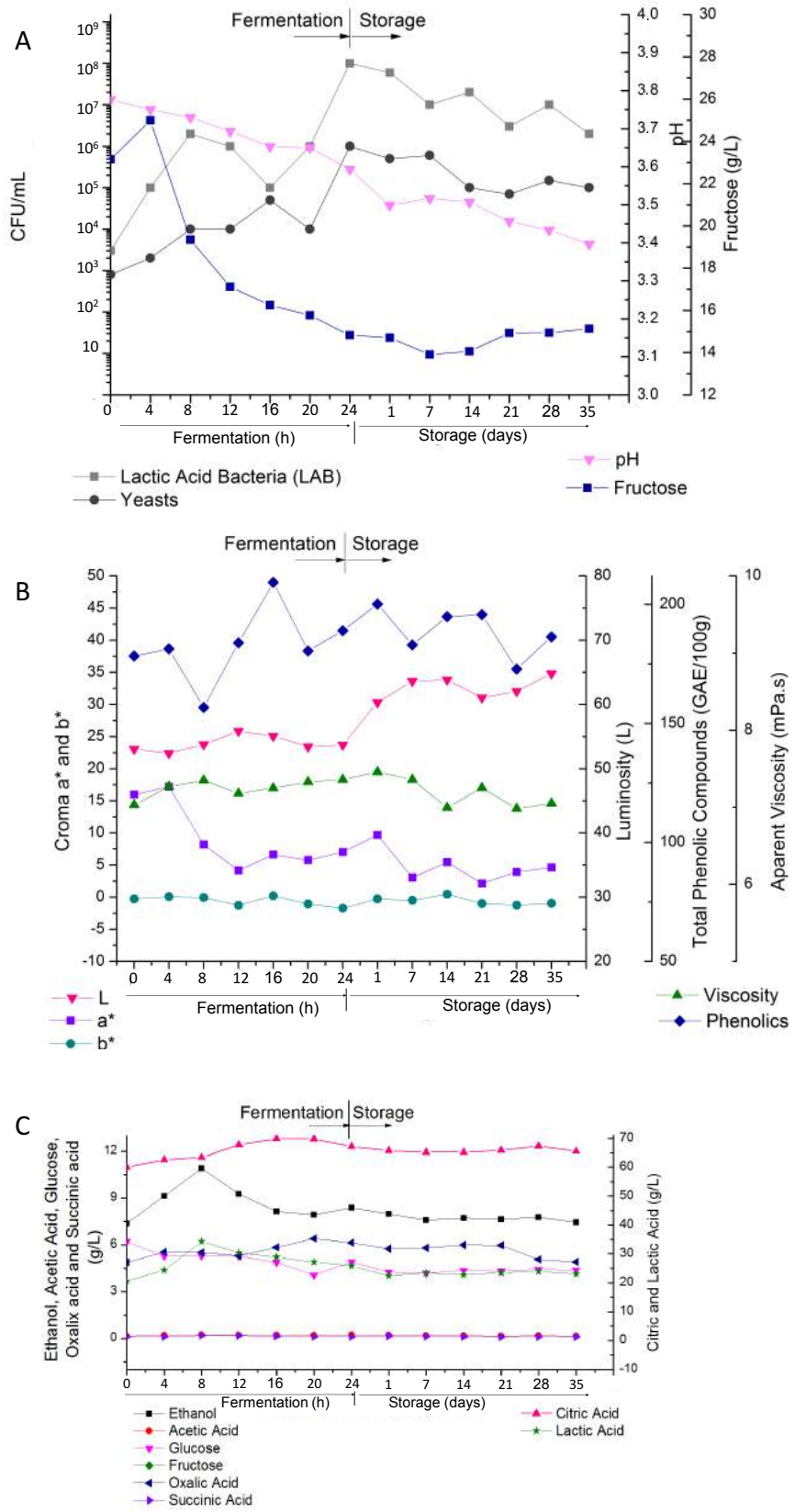


**Figure 3.** Analyses of honey kefir beverage during fermentation (0 to 24h) and storage (1 to 35 days). (A) Microorganisms growth, pH and fructose;
(B) Color parameters, phenolic compounds production and viscosity;
(C) HPLC analyses

The increase in fructose uptake rate led to a decrease in pH value (from 3.8 to 3.4) due to an increase in microorganism growth to $10^4$ CFU.mL$^{-1}$. After 16h, bacteria and yeast counts increased to $10^5$ CFU.mL$^{-1}$, and reached $10^8$ CFU.mL$^{-1}$and $10^6$ CFU.mL$^{-1}$ at the end of fermentation time (24h) for bacteria and yeast, respectively. At the same time, fructose progressively decreased to 14.82 g.L$^{-1}$. These results shows that kefir grains were well adapted to honey substrate and were able to ferment this substrate (consume sugar) and reduce pH values.

Color analysis is a process used to monitor foods and beverages in order to develop the ideal taste, texture and appearance (Chung et al., 2016). Thus, it is important to maintain the honey color during fermentation and storage in order to facilitate the consumers' perception of honey characteristics. The color parameters during fermentation and storage of HKB are illustrated in Figure 3B. It was observed that *L* and a** values did not change significantly during fermentation time. On the other hand, the luminosity (L) values indicated it is to be more clear than dark, and croma a* was 0. Croma b* decreased during fermentation, probable due its relation with sugar contents becoming less yellow and more brown (Alves et al., 2008).

The phenolic compounds production increased as microbial growth occurred, which indicated that kefir fermentation improved the antioxidant activity of honey must (Figure 3B). Some studies have demonstrated that lactic acid bacteria (e.g., *Lactobacillus acidophilus*, *L. bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium longum*) scavenge reactive oxygen species and some of this species are found in kefir microbiota (Nishino et al., 2000). Antioxidant property in food and beverages might influence positively one or more biological function in the human body, improving the state of health and wellness, and reducing the risk of developing diseases (Randazzo et al., 2016). Additionally, in industrial beverage process,

antioxidants are desirable for preserve the shelf life of beverages and prevent off-flavors developing (Preedy et al., 2014).

Figure 3C shows that citric acid (69.86 $g.L^{-1}$) was a major end-metabolite of carbohydrate metabolism during the kefir fermentation quantified by HPLC. Ethanol, lactic, acetic, oxalic and succinic acids were also produced and other selected acids such as gallic, malic and formic acids were not detected. Sugars and organic acids are widely used as food additives in many kinds of beverages, soft drinks and wines due to its mild and refreshing sourness. In addition, these compounds contribute to a wide range of functionalities, as sweetness, texture and microbiological stability increasing the sensorial acceptance (Huang et al., 2009). The ethanol content in the final beverage was approximately 8 $g.L^{-1}$ which is within the range of values (0.01–2.0%) observed by other authors for kefir from different origins (García Fontán et al., 2006; Güzel-Seydim et al., 2000).

After fermentation was completed, some product characteristics (microbial viability, total phenolic compounds, sugars, ethanol, organic acids, color parameters and viscosity) were measured during the storage process for 35 days. Yeast (approximately $10^6$ $CFU.mL^{-1}$) and LAB (approximately $10^7$ $CFU.mL^{-1}$) counts remained constant until the end of the storage period. The microorganisms enumerated in the studied kefir beverage meet with specifications suggested by FAO/WHO (2006), which recommends that probiotic beverages should contain at least $10^7$ and $10^4$ $CFU.mL^{-1}$of bacteria and yeast counts, respectively, at the end of 30 days of storage period. Total phenolic compounds, sugars, ethanol, organic acids, color parameters and viscosity were also constant during storage process (Figure 3B and 3C).

## 3.4. RHEOLOGICAL PROPERTIES

The rheological behavior of HKB (0 and 24h of fermentation) was carried out at 5°C and 25°C. The results were compared with traditional kefir beverage (TKB). In the studied ranges of temperature and fermentation time, the viscosity varied from 0.99 mPa.s to 8.14 mPa.s (Figure 4) and, as expected, an increase in the temperature induces the reduction of the beverage viscosities, as occurs with some fruit juices (Belibagli and Dalgic, 2007; Shamsudin et al., 2009; Singh and Eipeson, 2000). The viscosity values are comparable to literature in relation to sugar solutions, sugarcane juices and fruit juices with similar soluble solids contents (Zuritz et al., 2005).

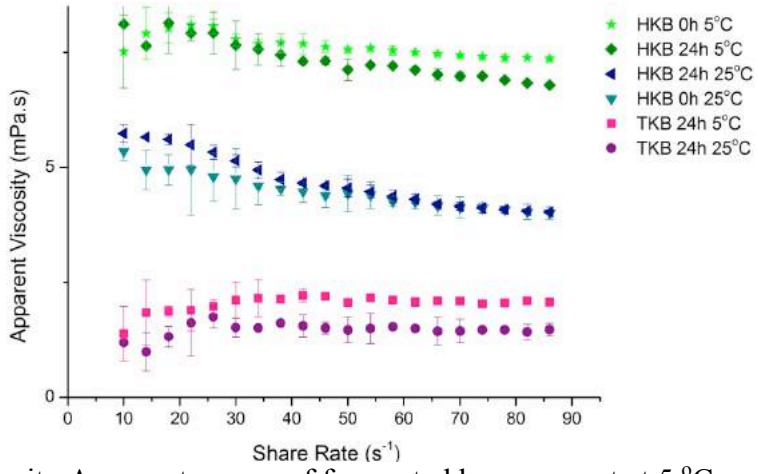


**Figure 4.** Viscosity Apparent curves of fermented beverages at at 5 °C and 25 °C
TKB: Traditional Kefir Beverage
HKB: Honey Kefir Beverage

HKB showed higher viscosity compared with TKB. The viscosity of HKB was not affected by fermentation time, however, was significantly higher at 5° compared to 25°C. According to Pelegrine et al. (2002) temperature is one factor that most affects the viscosity of various foods, as most of these products are present in the form of dispersed solids in liquids. An increase in temperature results in the decrease in viscosity of the liquid phase, increasing the movement of particles in suspension, as

noted in the behavior of HKB. The knowledge about viscosity of kefir beverages is very important from the storage and handling point of view and has many effects on food acceptability and food processing. The relationship between consumer preferences and viscosity of foods is a key part of the science of rheology (Kayacier & Dogan, 2006).

In order to obtain an evaluation of the rheological characteristics, flow curves (Figure 5), relativity shear stress (τ) versus shear rate (γ) were observed. This allows analyzes of fluid behaviors as Newtonian or non-Newtonian in the strain rate range studied.

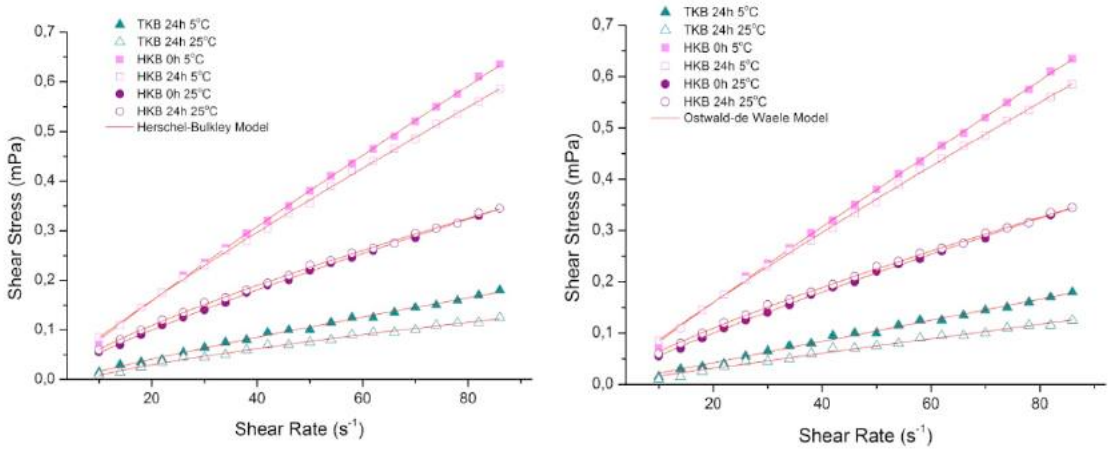


**Figure 5.** Flow curves adjusted by Ostwald-de Waele (Power Law) and Herschel-Bulkley models for fermented beverages at 5 °C and 25 °C
TKB: Traditional Kefir Beverage
HKB: Honey Kefir Beverage

According to the Ostwald-De Waele (Power Law) model, the beverages showed nearly Newtonian behavior, as indicated by the linear dependence of the shear stress on the shear rate shown in Figure 5. The Newtonian behavior of the studied beverages may be attributed to the low molar mass of the solutes. Usually, beverages and fruit juices are typically Newtonian, but at high shear rate the graph may curve (not linear) due to the pseudoplasticity nature achieved (Lannes et al., 2004; Müller, 1973). Under those conditions the behavior of the flow curves indicates that HKB and TKB can be classified as nearly a pseudoplastic fluid.

Table 3 shows the parameters according to the rheological models evaluated. On analyzing the rheological data obtained experimentally and adequately described by tested models, it was observed that all samples showed higher consistency in Herschel-Bulkley model (0.004 <$K_H$> 0.012) than Ostwald-de Waele model (0.002 <K> 0.011), and HKB (24h and 5$^{o}$C) was the most consistent beverage.

**Table 3.** Rheological ajusted parameters for Ostwald-de Waele (Power Law) and Herschel-Bulkley models for fermented beverages at 5 $^{o}$C and 25 $^{o}$C

| Model | Sample | Parameters | | | | |
|---|---|---|---|---|---|---|
| | | **K (mPa.s$^{n}$)** | **n** | **RSS** | **$R^2$** | **$\chi^2$** |
| Ostwald-de Waele (Power Law) | TKB 24h 5$^{o}$C | 0.0022 0 ± 0.0002 | 0.98656 ± 0.0231 | 2.57853 x $10^{-4}$ | 0.99413 | 1.43251 x $10^{-5}$ |
| | TKB 24h 25$^{o}$C | 0.00182 ± 0.0002 | 0.95054 ± 0.0312 | 2.46679 x $10^{-4}$ | 0.98865 | 1.37044 x $10^{-5}$ |
| | HKB 0h 5$^{o}$C | 0.00933 ± 0.0001 | 0.94719 ± 0.0050 | 1.66393 x $10^{-4}$ | 0.99969 | 9.24403 x $10^{-6}$ |
| | HKB 24h 5$^{o}$C | 0.01101 ± 0.0002 | 0.89234 ± 0.0061 | 2.31547 x $10^{-4}$ | 0.99947 | 1.28637 x $10^{-5}$ |
| | HKB 0h 25$^{o}$C | 0.00786 ± 0.0002 | 0.84844 ± 0.0061 | 8.77338 x $10^{-5}$ | 0.99939 | 4.8741 x $10^{-6}$ |
| | HKB 24h 25$^{o}$C | 0.01037 ± 0.0003 | 0.78644 ± 0.0072 | 1.3697 x $10^{-4}$ | 0.99899 | 7.60979 x $10^{-6}$ |

| Model | Sample | Parameters | | | | | |
|---|---|---|---|---|---|---|---|
| | | **$\tau_{oH}$ (mPa)** | **$K_H$ (mPa.s$^{n}$)** | **$n_H$** | **RSS** | **$R^2$** | **$\chi^2$** |
| Herschel-Bulkley | TKB 24h 5$^{o}$C | -0.014 ± 0.0070 | 0.00428 ± 0.0013 | 0.85181 ± 0.0622 | 1.99933 x $10^{-4}$ | 0.99518 | 1.17608 x $10^{-5}$ |
| | TKB 24h 25$^{o}$C | -0.02429 ± 0.0083 | 0.00686 ± 0.0024 | 0.68766 ± 0.0688 | 1.3207 x $10^{-4}$ | 0.99357 | 7.76881 x $10^{-6}$ |
| | HKB 0h 5$^{o}$C | -0.00991 ± 0.0051 | 0.01073 ± 0.0007 | 0.91877 ± 0.0150 | 1.35587 x $10^{-4}$ | 0.99973 | 7.97571 x $10^{-6}$ |
| | HKB 24h 5$^{o}$C | -0.00739 ± 0.0070 | 0.01223 ± 0.0012 | 0.87121 ± 0.0207 | 2.17116 x $10^{-4}$ | 0.99947 | 1.27715 x $10^{-5}$ |
| | HKB 0h 25$^{o}$C | -0.00626 ± 0.0040 | 0.00905 ± 0.0009 | 0.82034 ± 0.0214 | 7.91321 x $10^{-5}$ | 0.99942 | 4.65483 x $10^{-6}$ |
| | HKB 24h 25$^{o}$C | -0.00964 ± 0.0068 | 0.0126 ± 0.0017 | 0.74827 ± 0.0268 | 1.21831 x $10^{-5}$ | 0.99905 | 7.16654 x $10^{-6}$ |

*$\tau_{oH}$ is initial shear stress; **K** and **Kh** are consistency index; **n** and **nH** are flow behavior index; **RSS**: Residual Some of Squares; $R^2$ : Determination Coefficient; $\chi^2$ : qui-square.
TKB: Traditional Kefir Beverage
HKB: Honey Kefir Beverage

The models used in the present study had values of $\chi^2 \leq 9.24x10^{-6}$ and $R^2 = 0.999$. That is, fitting the data to the rheological models, provided values of coefficient of determination ($R^2$) near 1 and low $\chi^2$ values and SSR. The values also indicated Newtonian behavior ($n_H \geq 0.9$) according to the model of Ostwald-de Waele. Hence, the models Ostwald-de Waele and Herschel-Bulkley can be used to predict the behavior of HKB, providing important data for beverages industry, such as resistance to flow and sensory characteristics. They can also assist in the equipment design, adequacy of tubing systems, heat transfers, filters and pumps required for industrial process (Castro, 2003). It is clear that such data can help in design unit operations involved in beverage production using rheological characterization of the products (Pal, 2011; Steffe, 1996; Tabilo-Munizaga & Barbosa-Cánovas, 2005).

## 4. CONCLUSION

Large scale production of Honey Kefir Beverage is certainly possible, no additional cost is involved for nitrogen supplementation and low fermentation temperature is required. Furthermore, kefir grains are well adapted to honey as a substrate, producing phenolic compounds, high microorganism growth and improved desirable color aspects. The Ostwald-de Waele and Herschel-Bulkley models can be used to predict the behavior of this new non-dairy kefir beverage. The parameters analyses in honey kefir beverage production can be considered for production of a novel beverage product and scale up of this bioprocess.

## 5. ACKNOWLEDGMENT

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# CHAPTER 4

# *IN VITRO* PROBIOTIC PROPERTIES AND ANTIMICROBIAL ACTIVITY OF STRAINS ISOLATED FROM NON-DAIRY HONEY KEFIR BEVERAGE

Manuscript to be submitted for publication in *International Journal of Food Science and Technology.*

**Abstract**

Probiotics has been demonstrated to positively modulate the intestinal microflora and could promote host health. The probiotic potential and antimicrobial properties of *Lactobacillus satsumensis*, *Leuconostoc mesenteroides* and *Sacharomyces cerevisiae,* isolated from honey kefir beverage, was investigated. The isolates showed resistance to acid conditions (pH 2.0, 3.0, 4.0 and 7.0) and bile salts (0.3% and 0.6%), showing ability to survive in the presence of simulated gastric juice. There strains also survived in the presence of simulated intestinal juice and did not show hemolytic activity. The antimicrobial activity of the isolates and of honey kefir beverage was tested against *Escherichia coli* and *Staphylococcus aureus*. All the isolates exhibited antagonistic activity against *E. coli* and *S. aureus* (up to 7.0 mm). The isolate *L. satsumensis* showed resistance against the studied pathogens and was the most powerful antagonistic isolates. Honey kefir beverage had high antagonistic activity (19.5 to 27.5 mm). *L. satsumensis, L. mesenteroides* and *S. cerevisiae* isolated from honey kefir beverage could be classified as potential probiotics. The investigation of the potential probiotic features of these kefir strains should be useful for the development of novel functional beverage.

**Keywords:** functional beverage, antagonistic activity, lactic acid bacteria, probiotic properties

## 1. INTRODUCTION

Kefir is a beverage slightly carbonated with low alcohol content obtained through the use of kefir grains. These grains are clusters of lactic and acetic acid bacteria along with yeasts in a structural matrix of polysaccharides and proteins. The microorganisms present are responsible for the lactic, acetic, and alcoholic fermentation of substrate that yields a product with characteristic sensorial properties (Garrote et al., 2010). Some of these different bacteria and yeasts found in kefir have been recognized as probiotics (Leite et al., 2015).

Probiotics are defined as "living microorganisms, which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition" (Guarner, & Schaafsma, 1998). Promising probiotic strains include members of the genera *Lactobacillus, Bifidobacterium, Leuconostoc* and *Sacharomyces* (Shori, 2015; Liu, 2016; Castro-Rodríguez et al., 2015; Buntin et al., 2008). Many bacteria and yeasts are proved with probiotic functions, which are beneficial to the host when ingested in sufficient quantities. The colonization of the gut by probiotic bacteria prevents growth of harmful bacteria by competition exclusion and by the production of organic acids and antimicrobial compounds. The acid and bile tolerance are two fundamental properties that indicates the ability of a probiotic microorganism to survive through the upper gastrointestinal tract (Erkkila & Petaja, 2000; Hyronimus et al., 2000). The viability and activity of probiotic bacteria are important for survival in food during shelf life and transition through the acidic conditions of the stomach. To be potentially probiotics, bacteria must also be resistant to degradation by hydrolytic enzymes and bile salts in the small intestine (Belma & Gulcin, 2009). However, the selection of potential probiotic strains that would be capable of performing effectively in the gastrointestinal tract is a significant challenge, specially if these strains are isolated from a non-dairy matrix.

Most bacteria and yeasts are capable of producing a wide range of substances in vitro, which may be inhibitory for both these cultures, and for other bacteria. Such substances include toxins, bacteriolytic enzymes of metabolic pathway products (organic acids and hydrogen peroxide) and bacteriocins (Tagg et al., 1976). For certain microorganisms, such as probiotic such antagonism becomes a desirable property, either by production of antimicrobial substances or by competitive exclusion during its growth (Lee & Salminen, 1995).

Thus, the aim of this study was to characterize the probiotic potential of *Lactobacillus satsumensis*, *Leuconostoc mesenteroides* and *Sacharomyces cerevisiae,* isolated from honey kefir beverage, through acid and bile salts resistance, hemolytic acitivy, survival in simulated gastrointestinal tract conditions, and also to evaluate its *in vitro* antimicrobial properties against growth of two strains of pathogenic microorganisms conveyed by foods.

## 2. MATERIALS AND METHODS

### 2.1. KEFIR GRAINS AND INOCULUM PREPARATION

Kefir grains isolated from Mexican kefir beverages ("Water kefir") were used in this study. The samples were preserved in brown sugar solution (10% w/v), as this is the commonly substrate water kefir is traditionally preserved. The mixture was then incubated for 24 h at 30$^{o}$C (Laureys & De Vuyst, 2014; Magallhães et al., 2010). The kefir grains (5% w/v), were renewed daily for a period of seven days into honey must for adaptation. After this the grains were washed with sterile distilled water and subsequently used as a starter culture for batch bioreactor process.

## 2.2. HONEY KEFIR BEVERAGE PRODUCTION

Honey was obtained from local market in Curitiba, Paraná - Brazil. Honey-based media was prepared by mixing honey with sterile distilled water in proportion to obtain a must of 40 $^{o}$Brix, therefore, was used the Equation 1.

$$W_{honey} \times {}^{o}Brix_{honey} = W_{must} \times 40^{o}\,Brix \quad \text{(Equation 1)}$$

Where Whoney is the necessary amount of honey and Wmust is the amount of must is desired to produce. After determining the required amount of honey, the amount of water being added was estimate by Equation 2.

$$W\ water = W\ must - W\ honey \quad \text{(Equation 2)}$$

Honey must was pasteurized at 63 °C 30 $min^{-1}$ using a water-bath. The pasteurized must was first cooled down to about 25 °C and then inoculated with the working-culture (5% w/v).

## 2.3. FERMENTATION IN BIOREACTOR CONDITION

Fermentations was conducted in bioreactor (6 L, MDL B.E. Marubishi), equipped with a heater and a control unit and filled with 3 L of honey medium (40% w/v). Honey must was pasteurized inside the bioreactor (63 $^{o}$C 30 $min^{-1}$) and a disc turbine propeller was used for homogenization. Than, kefir grains were used to inoculate the honey must (aproximatly $10^{3}$ CFU $mL^{-1}$ for bacteria and yeast in pre-culture respectivally). Wet weight cells of 150 g (5% - w/v) were transferred into 3 L of fermentation substrate (Alsayad et al., 2013). A batch fermentation was carried out in static conditions. Temperature was maintained at 30 °C for 24 h.

After 24 h of fermentation, the grains were separated from the fermented beverage by filtration and washed prior to the next culture incubation.

## 2.4. MICRORGANISM AND GROWTH CONDITIONS

*Lactobacillus satsumensis*, *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* strains previously isolated from Honey Kefir Beverage (Fiorda et al., 2016), were used in this study. The strains were maintained as frozen (-80 $^{o}$C) stock cultures in MRS broth (for bacteria) and YM broth (for yeast) containing 20% (v/v) glycerol. *Escherichia coli* JM109 and *Staphylococcus aureus* ATCC® 6538 belonging to the collection of Biorefining Research Institute (Lakehead University, Thunder Bay, Canada), were used in antimicrobial analyzes.

## 2.5. ACID TOLERANCE

The resistance under acid conditions was carried out according to Pieniz et al. (2014) with some modifications. Cells were grown in MRS broth at 37 $^{o}$C (for bacteria) and YM broth at 30 $^{o}$C (for yeast) without shaking for 24 h. Then, the cultures were standardized at an optical density (OD600) = 1.0 ± 0.05. One milliliter of standardized culture was added into tubes containing 9 mL of respective sterile broth with the following pH values: 2.0, 3.0, 4.0 and 7.0 (adjusted with HCl), in which pH 7.0 was used as a control. Viable cell counts were determined after exposure to acidic condition for 0, 1, 2, 3 and 4 h. The experiment was performed in triplicate. Survival cell counts were expressed as log values of colony-forming units per ml (CFU/mL) by pour plate method after serial dilutions. The survival percentage was calculated as follows: % survival = final (CFU/mL)/intial (CFU/mL) x 100.

## 2.6. RESISTANCE TO BILE SALTS

After strains were grown in MRS broth (for bacteria) and YM broth (for yeast), cells were harvested by centrifugation (10,000 x g for 10 min at 4 $^{o}$C) washed three times with 0.1 M phosphate buffered saline (PBS) (pH 7.2) and suspended in 0.5% NaCl solution. The cultures were standardized at an optical density (OD600) = 1.0 ± 0.05. Then, a 0.2 ml aliquot of

suspensions were inoculated into 1.0 ml of YM broth (for *S. cerevisiae*) and MRS broth (for *L. satsumensis* and *L. mesenteroudes*) with 0% (control - pH 7.0), 0.3 % and 0.6% (w/v) of bile salts (Sigma-Aldrich®), at pH 7.4. Total viable counts were determined after exposure to bile salts solution at 0, 1, 2, 3 and 4 h of incubation, by pour plate method after serial dilutions and incubated at 37 $^{o}$C (for bacteria) or 30$^{o}$C (for yeast) for 24 h. Values were expressed as log CFU/mL (Perelmuter et al., 2008).

### 2.7. HEMOLYTIC ACTIVITY

The strains were tested for hemolytic activity using blood agar (7% v/v sheep blood) for 48 h incubation at 37 $^{o}$C (Foulquié Moreno et al., 2003). Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) were considered non hemolytic. Strains displaying blood lyses zones around the colonies were classified as hemolytic (β-hemolysis).

### 2.8. SURVIVAL IN SIMULATED GASTROINTESTINAL TRACT

Survival in simulated gastrointestinal tract was performed according to Pieniz et al. (2014). After 24 h of incubation in MRS broth at 37 $^{o}$C (for bacteria) or YM broth at 30 $^{o}$C (for yeast), cells were harvested by centrifugation (10,000 x g for 10 min at 4 $^{o}$C), washed three times with 0.1 M phosphate buffered saline (PBS) (pH 7.2) and suspended in 0.5% NaCl solution. The cultures were standardized at an optical density (OD600) = 1.0 ± 0.05. Then, a 0.2 mL aliquot of suspensions were inoculated into 1.0 mL of simulated gastric or intestinal juices and incubated at 37 $^{o}$C for 4 h. Survival cell counts were determined at initial time (0 h) and 1, 2, 3 and 4 h for the gastric tolerance and intestinal tolerance. Values were expressed as log CFU/mL.

Simulated gastric juice was prepared fresh daily containing 3 mg of pepsin (Sigma), 1 mL of NaCl solution (0.5%) and acidified with HCl to pH 3.0. Simulated intestinal juice was consisted of 1 mg of pancreatin (Merck), 1 mL of NaCl solution (0.5%) and adjusted to pH 8.0. Both solutions were sterilized by filtration through 0.22 mm membranes (Millipore, Bedford, USA).

### 2.9. ANTIMICROBIAL ACTIVITY

Antimicrobial capacity of selected strains and of honey kefir beverage were evaluated. *Escherichia coli* JM109 and *Staphylococcus aureus* ATCC® 6538 were used as photogenic microorganisms. They were grown in nutrient broth at 37 $^{o}$C for 24h and suspended in 0.85% NaCl solution standardized to OD600 of 0.150 in spectrophotometer, which corresponded to a 0.5 McFarland turbidity standard solution. One aliquot of 50 µl of culture containing grown *L. satsumensis*, *L.mesenteroides* and *S.cerevisiae* and 50 µl of honey kefir beverage was applied onto Mueller Hinton plates previously inoculated with a swab soaked in a culture of each indicator bacteria. The plates were incubated at 37 $^{o}$C and inhibition zones were measured after 24 h. Ampicillin (50 mg $mL^{-1}$) was used as standard. The diameter of inhibition zones was measured using a caliper rule and halos $\geq$ 7 mm were considered inhibitory (Bromberg et al., 2006). The experiment was performed in triplicate.

### 2.10. STATISTIC ANALYSES

The results obtained in the study were expressed as mean ± standard deviation from 3 replicate determinations. Differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey´s post-hoc test. P-values $< 0.05$ were considered to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. ACID TOLERANCE

Potential probiotic strains need to tolerate acidic environments in order to successfully pass through the stomach and small intestine. The stains were further analysed *in vitro* for their ability to survive under acidic conditions and the results are shown in Figure 1.

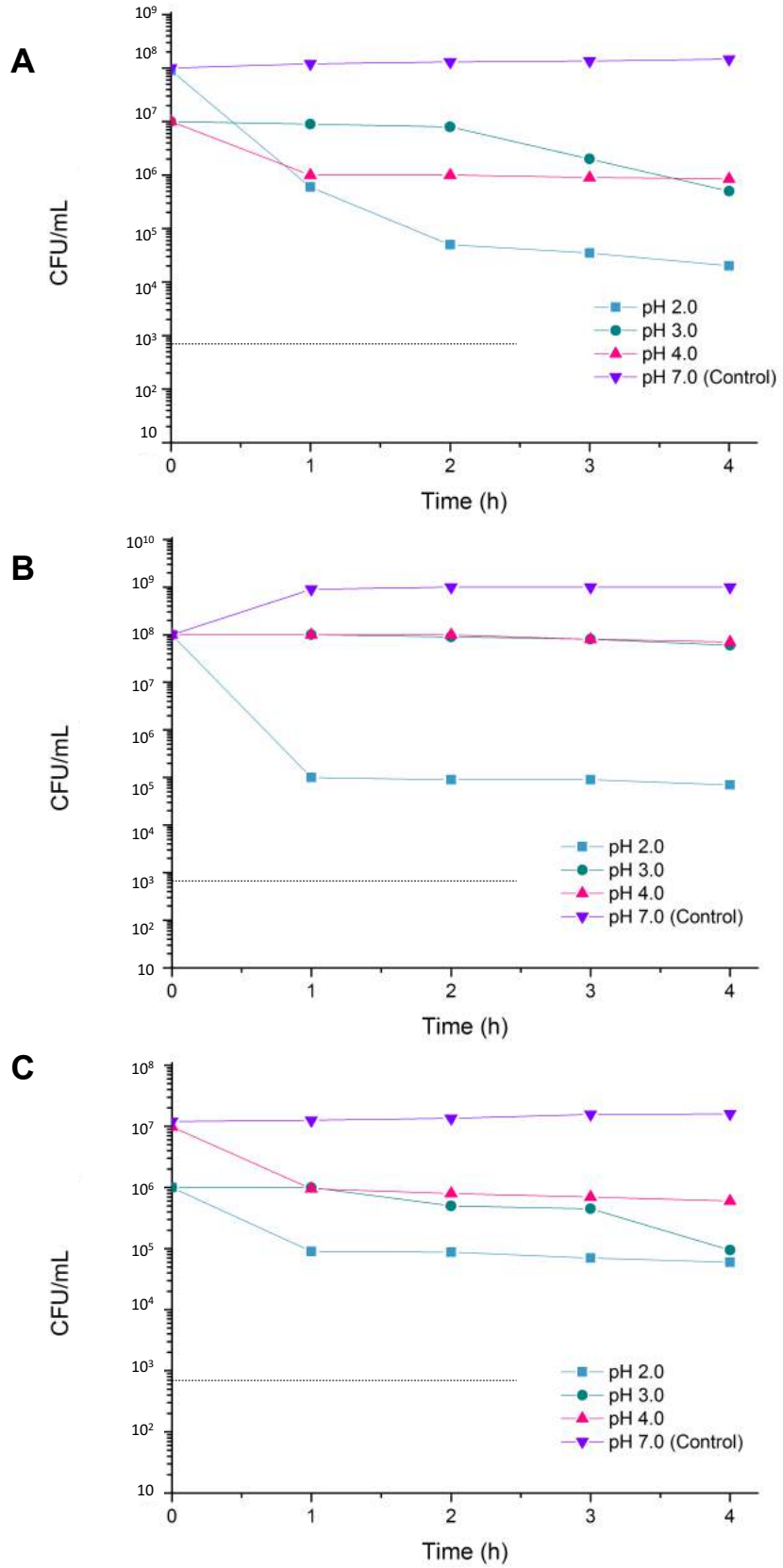


**Figure 1.** Acid tolerance test of *Lactobacillus satsumensis* (A), *Leuconostoc mesenteroides* (B) and *Sacharomyces cerevisiae* (C) showing ability to survive at the physiological pH 7.0 (control), 4.0, 3.0 and 2.0. Dotted line is detection limit.

In this study, the isolates survived in all times tested (1, 2, 3 and 4h) at pH 2, pH 3, pH 4 and pH 7 maintaining high counts at pH 3 for 2 h, which are considered to be the standard values of acid tolerance of probiotic cultures (Usman et al., 1999). The viability of isolates was satisfactory when exposed to pH 3 and 4, although it was observed a decrease in viable cell counts in pH 2 in the first hour (until 4 log CFU $mL^{-1}$). However, the viable count of all isolates remained up to the limit of $10^3$ CFU $mL^{-1}$ (dotted line) after 4h even at pH 2, and acording to Likotrafiti et al. (2013), this is the limit of detection for acid-tolerance of probiotic strains.

The pH of the stomach is between 2.5 and 3.5, although it may be lower during prolonged fasting (pH 1.5), or higher after a meal (pH 4.5) (Huang & Adams, 2004). Thus, the fact that the strain survived for a short time at pH 2 should not interfere with the probiotic ability, because it is intended to apply the strain concomitantly with the beverage, and thus the pH of the stomach is likely to be greater than 2. Hence, the ability to survive at pH 3.0 over approximately 3 h is an essential criterion for micro-organism has probiotic action (Usman et al., 1999). The highest percentage of survival was observed for *L. mesenteroides* ($10^5$ CFU $mL^{-1}$ at pH 2 after 4h). The survival residual cells were between 50% and 90% of the initial cells even after 2 h of incubation at the pH 3.

If probiotic bacteria survive through the acidic environment, the next major challenge is to withstand the presence of bile acids, a major hurdle to bacterial survival and growth in the small intestine.

### 3.2. RESISTANCE TO BILE SALTS

Another key characteristic of probiotic bacteria is their resistance and ability to grow in the presence of bile salts in order to survive in the digestive system. In this study, *L. satsumensis, L. mesenteroides* and *S. cerevisiae*, which were resistant to highly acidic

conditions, were evaluated for their ability to grow in the presence of 0.3% and 0.6% bile salts. The results are presented in Figure 2.

The results showed that all strains isolated from honey kefir bevearge were able to survive at all bile salt concentrations tested (0.3% and 0.6%) to give an exponential growth from the inoculation (0 h) until 4 h of incubation.

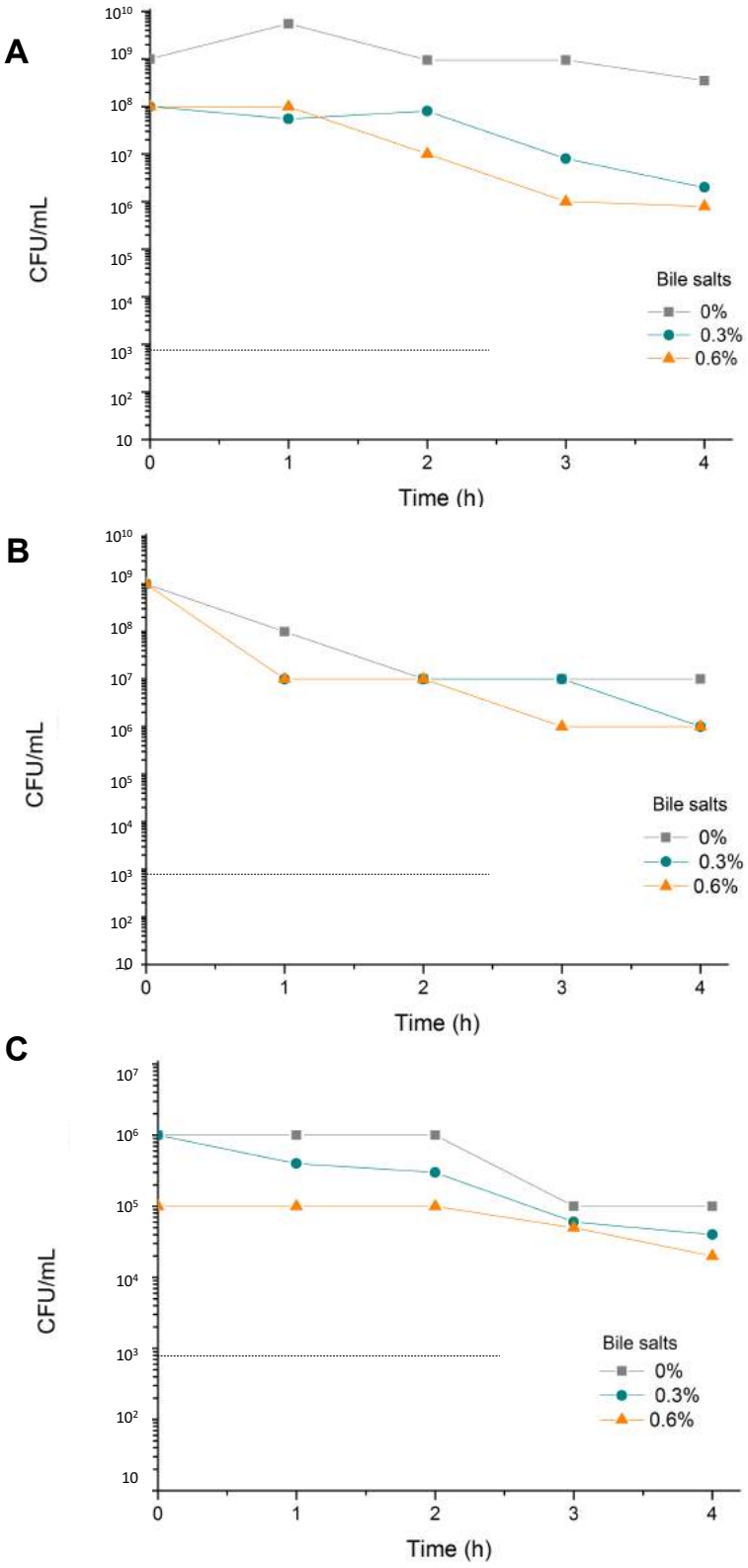


**Figure 2.** Tolerance of *Lactobacillus satsumensis* (A), *Leuconostoc mesenteroides* (B) and *Sacharomyces cerevisiae* (C) to bile salts concentration, containing 0%, 0.3% and 0.6% of bile salts. Dotted line is detection limit.

Bile tolerance by probiotics has been revealed to be dependent on bile type and the strain, with resistance levels ranging from bile concentrations of 0.125 - 2.0 % (Lian et al., 2003). It has been hypothesized that deconjugation of bile salts is a detoxification mechanism and bile salt hydrolases enzymes play a role in bile tolerance of probiotic organisms in the gastrointestinal tract. Hence, the resistence of probiotics to bile salts is due to the ability of certain species of microorganisms have to reduce the effect of the detergent for producing enzymes capable of hydrolyzing bile salts. However, *Saccharomyces cereviseae* isolated in the present study was more sensitive to bile salts than bacterias isolated from kefir. Probably owing to the capsule present in prokaryotic cells (such as bacterias) that causes protection effect in probiotic bacteria and not in probiotic yeasts. Nevertheless, *S. cerevisiae* reached up to $10^4$ CFU $mL^{-1}$ after 4h of incubation even at 0.6% of bile salts.

All the isolates were able to survive at 0.3% bile concentration for 2h, which is essential for survival of the physiological conditions of the gastrointestinal tract (Sahadeva et al., 2011). In addition, the viable count of all isolates remained up to the limit of $10^3$ CFU $mL^{-1}$ (dotted line) after 2h, and acording to Likotrafiti et al (2013), this is the limit of detection for bile salts resistence of probiotic strains.

### 3.3. HEMOLYTIC ACTIVITY

The determination of hemolytic activity is considered a safety aspect for the selection of probiotic strains (FAO/WHO, 2002), and this activity was also investigated in this study. The isolates did not exhibited any effect (γ-hemolysis); green area (α-hemolysis), and/or inhibition zone (β-hemolysis) after 48 h incubation in blood agar plates. Thus, our results showed that none of the isolates exhibited hemolytic activity.

## 3.4. TOLERANCE TO GASTROINTESTINAL JUICES

Exposure to gastric and intestinal fluids along the digestive tract is the main stress that could decrease the viability of ingested probiotics (Liong & Shah, 2005). Hence survival to pass through the gastrointestinal tract is a desirable characteristic in the choice of probiotic microorganisms since viability plays a significant role in certain of their beneficial properties (Romanin et al., 2010; Saad et al., 2013). The potential ability of the identified isolates to survive under the conditions of transit through the gastrointestinal tract as assayed indirectly in vitro is demonstrated by the results presented in Figure 3.

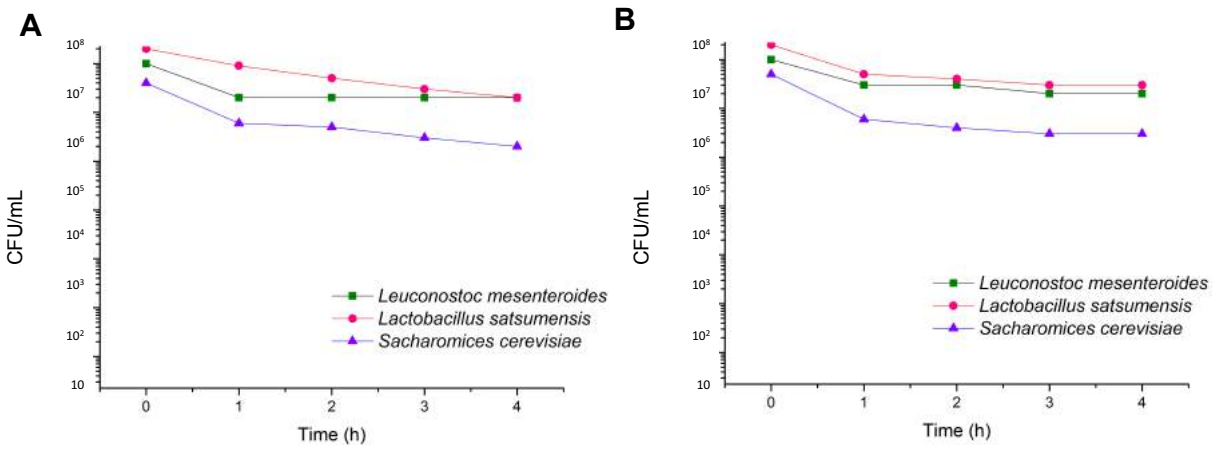


**Figure 3.** Resistance to simulated Gastric Juice containing pepsin (A) and Intestinal Juice containing pancreatin (B) of strains isolated from honey kefir beverage.

When exposed to both simulated gastric and intestinal conditions for 4 hours, the strains analyzed exhibited cell count near by $10^7$ CFU.mL$^{-1}$, that would allow it to pass through the stomach. *S. cerevisiae* was the least sensitive - but not low resistance - among the strains, while the two others had better resistance properties in both gastric and intestinal conditions.

It was observed that until 2 hours of inoculation, the cell viability of isolates did not change hardly and no difference were observed in survival of the strains when exposed to both simulated gastric and intestinal juices.

This indicate that *L. satsumensis, L. mesenteroides* and *S. cerevisiae* demonstrated high ability to survive in the presence of simulated gastric juice containing pepsin and simulated intestinal juice containing pancreatin. Therefore, they can be classified as tolerant to the gastrointestinal secretions and can be used as potentially probiotic micorganisms.

### 3.5. ANTIMICROBIAL ACTIVITY

The demonstration of antimicrobial activity towards pathogenic species *in vitro* may be considered a desirable attribute of some probiotic bacteria. The pathogens studied in the present work commonly cause different diseases, so they are used as standards in antimicrobial activity tests of potentially probiotic microorganisms (Ramirez-Chavarin et al., 2013; Yamazakia et al., 2012; Ramos et al., 2012; Tsai et al., 2008; Valdéz et al., 2005). The strains isolated from honey kefir bevegare exhibited antimicrobial activity against different indicator microorganisms (Table 1).

**Table 1**. Antimicrobial activity of strains isolated from honey kefir beverage against indicator microrganisms.

| Microrganism | Inhibition zone (mm)* | |
|---|---|---|
| | *Escherichia coli* | *Staphylococcus aureus* |
| *Lactobacillus satstumensis* | $12.5 \pm 0.50^{Ca}$ | $10.5 \pm 0.50^{Ba}$ |
| *Leuconostoc mesenteroides* | $10.5 \pm 0.50^{Ca}$ | $12.0 \pm 1.00^{Ba}$ |
| *Sacharomyces cerevisiae* | $8.0 \pm 0.10^{Ca}$ | $8.5 \pm 0.50^{Ba}$ |
| Honey kefir beverage | $27.5 \pm 1.50^{Aa}$ | $19.5 \pm 1.50^{Ab}$ |
| Control (Ampicilin 50 mg/mL) | $42.5 \pm 1.50^{Ba}$ | $23.5 \pm 0.50^{Aa}$ |

*values represent the mean ± standard deviation of three independent experiments
**Upper-case letters show significant differences between column, and lower-case letters show significant differences between lines, as determined by Tukey´s test ($p < 0.05$).

The highest inhibitory activity among isolated strains was observed against *E. coli,* followed by *S. aureus*. The smaller inhibition halos observed was in *E. coli* by *S. cerevisiae* (8.0 mm) and *L. satsumensis* shoed the most effective antimicrobial properties against *E. coli*. However, against *S. aureus, L. mesenteroides* showed the biggest halo zones of growth inhibition.

Regarding the honey kefir beverage, its observed high antimicrobial capacity both against *E. coli* and *S. cerevisiae.* In this case, honey might have increased the inhibition activity due to its physicochemical properties. Significant contributing properties are osmolarity, pH, sugar content, water content and hydrogen peroxide production. The high osmolarity and the hydrogen peroxide content assist in tissue repairing and contribute to the antimicrobial activity, as the carbohydrate concentration has a vital effect on the antimicrobial activity (Basson & Grobler, 2008). Furthermore, favorable pH levels increase the quantity of oxygen off-loaded from hemoglobin in the capillaries (Simon et al., 2009), resulting in an environment where pathogens are unable to thrive.

As *Escherichia coli* and *Staphylococcus aureus* have high pathogenic activity and is of clinical concern globally, these in vitro antimicrobial efficacy results from this study highlight the high potential of honey beverage developed with kefir grains containing strains such as *Lactobacillus satsumensis, Leuconostoc mesenteroides* and *Sacharomyces cerevisiae*.

## 1. CONCLUSION

The results obtained in this study suggest that *Lactobacillus satsumensis, Leuconostoc mesenteroides* and *Sacharomyces cerevisiae* isolated from honey kefir beverage are resistant strains to pass through the gastrointestinal tract and did not show hemolytic activity. The viability of these strains through the exposure to bile salts and acid tolerance were also observed. Also, honey kefir beverage have strong antagonistic effects against pathogenic bacteria.

In conclusion, all isolated strains exhibited some desirable probiotic properties in vitro. These strains are good probiotic candidates. However, other in vitro and in vivo assays must be performed to elucidate the potential of these new isolates, such as assays for

autoaggregation and coaggregation, the production of organic acids and other antimicrobial substances, adhesion to intestinal cells, protection against experimental pathogenic challenges, and immunomodulatory capacities in animal models.

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## CONCLUSION

The results of the present study provided evidence indicating that soybean hydrolyzed extract, colostrum and honey could serve as raw materials/substrates for the production of kefir-like beverages with functional and flavoring properties. The results demonstrated that honey could be an ideal alternative substrate for production of fermented beverage with high antioxidant activity and potential probiotic composition. Additionally, the beverage had protective effect to DNA damage caused by hydroxyl radical and had very good sensory qualities. The study showed that non-dairy probiotic beverage using honey as base substrate could lead to a product which has enhanced health benefits and sensory qualities.

Large scale production of Honey Kefir Beverage is accomplishable, no costs are involved for the nitrogen source and low fermentation temperature is required. Furthermore, kefir grains are well adapted to honey as a substrate, producing phenolic compounds, high microorganism growth and improved color aspects. The models Ostwald-de Waele and Herschel-Bulkley can be used to predict the behavior of this new non-dairy kefir beverage. The parameters analyzed in honey kefir beverage production can be considered for production of a novel beverage product and scale up of this bioprocess.

The results obtained in this study suggest that *Lactobacillus satsumensis, Leuconostoc mesenteroides* and *Sacharomyces cerevisiae* isolated from honey kefir beverage are resistant strains to pass through the gastrointestinal tract and did not show hemolytic activity. The viability of these strains through the exposure to bile salts and acid tolerance were also observed. Also, honey kefir beverage have strong antagonistic effects against pathogenic bacteria. All isolated strains exhibited some desirable probiotic properties in vitro. These strains are good probiotic candidates. However, other in vitro and in vivo assays must be

performed to elucidate the potential of these new isolates, such as assays for autoaggregation and coaggregation, the production of organic acids and other antimicrobial substances, adhesion to intestinal cells, protection against experimental pathogenic challenges, and immunomodulatory capacities in animal models.

In conclusion, kefir-based beverages have shown an alternative way to produce functional beverages with probiotic activities, especially for people with special needs (lactose intolerance) and vegan consumers. Honey could be an ideal alternative substrate for the production of functional cultured beverage, especially for vegans and lactose intolerant consumers.

## APPENDAGE 1 - Termo de Consentimento Livre e Esclarecido

**UNIVERSIDADE FEDERAL DO PARANÁ**
**PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE ALIMENTOS**

### TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está convidado (a) para participar, como voluntário (a), em uma pesquisa. Após ser esclarecido (a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa, você não será penalizado (a) de forma alguma.

### INFORMAÇÕES SOBRE A PESQUISA:

Título do Projeto: Caracterização, isolamento e identificação de linhagens de grãos de kefir e desenvolvimento de bebida fermentada probiótica.

Pesquisadora Responsável: Fernanda Assumpção Fiorda (Engenheira de Alimentos)

Orientador: Prof.° Dr.° Carlos Ricardo Soccol

Telefones para contato: 97029535 (pesquisadora)

A pesquisa tem por objetivo desenvolvimento de processo tecnológico de produção de bebida fermentada desidratada com propriedades probióticas.

A análise sensorial será realizada por meio de teste de aceitabilidade com pessoas adultas de ambos os sexos, pelo interesse e disponibilidade em participar das análises. Serão excluídos da pesquisa fumantes, analfabetos, idosos, celíacos e portadores de patologias que interferem na absorção intestinal, sensibilidade gustativa, olfativa e/ou apresentarem redução da capacidade visual.

A aceitação global será avaliada em cabines individuais com luz branca. As amostras serão servidas à temperatura ambiente, codificadas com três dígitos. Cada provador avaliará o quanto gosta ou desgosta da amostra usando uma escala de 9 pontos.

A degustação da bebida não implica em qualquer risco para os participantes da pesquisa. Além disso, os provadores não são obrigados a ingerir a amostra. O resultado da avaliação dos provadores será sigiloso.

Caso sejam comprovadas alterações na saúde dos provadores por causa da degustação, a pesquisadora Fernanda Assumpção Fiorda se responsabilizará pelo encaminhamento aos serviços médicos hospitalares.

Pesquisadores:

Fernanda Assumpção Fiorda Carlos Ricardo Soccol

Data: _____

Assinatura do participante: _____

RG: _____

## **APPENDAGE 2** - Ficha de avaliação da análise sensorial

Nome:_____ Data:_____

Prove as amostras codificadas e avalie o quanto você gostou ou desgostou da mesma em relação à aparência, cor, odor, sabor, textura e nota global utilizando escala abaixo:

1 – Desgostei muitíssimo
2 – Desgostei muito
3 – Desgostei regularmente
4 – Desgostei ligeiramente
5 – Indiferente
6 – Gostei ligeiramente
7 – Gostei regularmente
8 – Gostei muito
9 – Gostei muitíssimo

| Número da Amostra | Aparência | Cor | Odor | Sabor | Textura | Nota global |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |

Avalie a intensidade dos seguintes atributos, assinando com um traço vertical, conforme exemplo.

**Exemplo:**

Fraco ——|—— Forte

número da amostra

**Sabor**

Fraco —————— Forte

**Aroma**

Ausente —————— Intenso

**Consistência**

Muito líquido —————— Muito viscoso

número da amostra

**Sabor**

Fraco —————— Forte

**Aroma**

Ausente —————— Intenso

**Consistência**

Muito líquido —————— Muito viscoso

TESTE DE INTENÇÃO DE COMPRA

Em relação às amostras, qual seria a sua atitude de compra caso o produto possua algum efeito benéfico ao organismo?
1 – Certamente eu não compraria
2 – Provavelmente eu não compraria
3 – Talvez sim / Talvez não
4 – Provavelmente eu compraria
5 – Certamente eu compraria

Amostra_____ Resposta _____

Amostra_____ Resposta _____

Comentários:

_____

_____

**Obrigada!!**

## APPENDAGE 3

QUESTIONÁRIO PARA RECRUTAMENTO DE PROVADORES

Desejamos provadores para avaliar a aceitação de bebida probiótica, que está sendo desenvolvido em nosso laboratório. Ser um provador não exigirá de você nenhuma habilidade excepcional e não envolverá nenhuma tarefa difícil, além disso você não é obrigado a ingerir a amostra. Por favor, preencha este formulário. Se tiver qualquer dúvida ou necessitar de informações adicionais, por favor, entre em contato (Fernanda Assumpção Fiorda, fernandafiorda@hotmail.com).

Dados Pessoais

Nome _____

E-mail _____

1-Faixa etária

( ) 15-25

( ) 25-35

( ) 35-50

( ) acima de 50 anos

2-Sexo

( ) masculino

( ) feminino

3-Ocupação

( ) aluno

( ) funcionário

( ) professor

( ) outro _____

4-Escolaridade

( )1º grau

( ) 2º grau

( ) 3º grau

( ) outro _____

5) Experiência como provador:

Já participou de algum teste sensorial?

( )Não ( ) Sim

6) Consome alguma bebida fermentada não alcoólica?

( )Não ( ) Sim

7) Com qual frequência?

( ) Diariamente

( ) 3 x por semana

( ) Semanalmente

( ) Outros. Qual? _____

# ANNEX A

## Published Paper in LWT – Food Science and Technology

LWT - Food Science and Technology 68 (2016) 690–697

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journal homepage: www.elsevier.com/locate/lwt

# Development of kefir-based probiotic beverages with DNA protection and antioxidant activities using soybean hydrolyzed extract, colostrum and honey

Fernanda Assumpção Fiorda [a], Gilberto Vinícius de Melo Pereira [b], Vanete Thomaz-Soccol [b], Adriane Pedroni Medeiros [b], Sudip Kumar Rakshit [c], Carlos Ricardo Soccol [a, b, *]

[a] Food Engineering Department, Federal University of Paraná (UFPR), Curitiba, PR, Brazil
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[c] Chemical Engineering Department, Lakehead University, Thunder Bay, ON, Canada

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Soybean hydrolyzed extract
Colostrum
Honey
Non-dairy functional beverage

ABSTRACT

The aim of this study was to evaluate the use of different functional substrates (soybean hydrolyzed extract, colostrum and honey) to design novel probiotic beverages using kefir grains as starter culture. The fermentations were carried out at 30 °C for 24 h and physical-chemical composition and functional aspects were determined. It was found that fermentation processes with kefir grains increased the functional quality of all substrates evaluated. Honey-based kefir beverage had higher antioxidant activity and its microbial composition was assessed using molecular approaches (Rep-PCR and 16S rRNA gene sequencing). High levels of lactic acid bacteria and yeast populations (over $10^6$ CFU/mL) were found in the product and were mainly composed of potential probiotic strains of *Lactobacillus statsumensis*, *Leuconostoc mesenteroides*, *Bacillus megaterium*, *Saccharomyces cerevisiae* and *Lachancea fermentati*. In addition, the honey-based kefir beverage showed protection effect on DNA damage and had a high sensory quality compared to traditional kefir beverage. The results demonstrated that honey could be an ideal alternative substrate for the production of functional cultured beverage, especially for vegans and lactose intolerant consumers.



## 1. Introduction

Probiotic food products are formulations containing sufficient numbers of selected live microorganisms ($10^6$–$10^7$ CFU/mL) that can beneficially modify the intestinal microbiota of the host (Rathore, Salmerón, & Pandiella, 2012). Kefir beverage is commonly manufactured by fermenting milk with kefir grains, which supports a complex microbial symbiotic mixture of lactic acid bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*) and yeasts (e.g., *Kluyveromyces* and *Saccharomyces*) (Magalhães, de Melo Pereira, Campos, Dragone, & Schwan, 2011). Some of these different bacteria and yeasts found in kefir have been recognized as probiotics, e.g., *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* (Leite et al., 2015).

Kefir grains can be applied to ferment different substrates besides milk. These include cheese-whey, fruit juice and molasses or sugar syrups (Cui, Chen, Wang, & Han, 2013; Puerari, Magalhães, & Schwan, 2012). The development of alternative substrates used in production of fermented kefir beverage is an ideal way for the conversion of sugars to produce organic acids and alcohol. It is considered a simple and valuable biotechnology based method for maintaining and/or improving the safety, nutritional, sensory and shelf-life properties of fermented beverages (Prado, Parada, Pandey, & Soccol, 2008). Colostrum is a dairy substrate of great interest due to its positive functional properties (De Dea Lindner, Neviani, Santarelli, Soccol, & Yamaguishi, 2011). It is a complex biological fluid and a source of immunological compounds and nutrients, many proteins, immunoglobulins, non-protein nitrogen, fat, vitamins and minerals that can be used to treat or prevent infections of

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0023-6438/

# ANNEX B

## Patent N$^{o}$. BR 102014021724 0

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
PROTOCOLO GERAL
02/09/2014 015140001637
15:24 DEPR
ENR 10 2014 021724 0

< Uso exclusivo do INPI >

Espaço reservado para o protocolo | Espaço reservado para a etiqueta | Espaço reservado para o código QR

**INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL**
**Sistema de Gestão da Qualidade**
**Diretoria de Patentes**

| DIRPA | Tipo de Documento: Formulário | DIRPA | Página: 1/3 |
|---|---|---|---|
| Título do Documento: Depósito de Pedido de Patente | | Código: FQ001 | Versão: 2 |
| | | Procedimento: DIRPA-PQ006 | |

**Ao Instituto Nacional da Propriedade Industrial:**
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

**1. Depositante (71):**

1.1 Nome: Universidade Federal do Paraná

1.2 Qualificação: Autarquia Federal

1.3 CNPJ/CPF: 75095679/0001-49

1.4 Endereço Completo: Rua João Negrão, 280 2°andar Curitiba/PR

1.5 CEP: 80010-200

1.6 Telefone: 41-33607441 1.7 Fax: 41-33607416

1.8 E-mail: inovacao@ufpr.br

☐ continua em folha anexa

**2. Natureza:** ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

**3. Título da Invenção ou Modelo de Utilidade (54):**

BIOPROCESSO PARA A PRODUÇÃO DE UMA BEBIDA FERMENTADA A BASE DE MEL COM PROPRIEDADES PROBIÓTICAS

☐ continua em folha anexa

**4. Pedido de Divisão: do pedido Nº** **Data de Depósito:**

**5. Prioridade:** ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

| País ou Organização do depósito | Número do depósito (se disponível) | Data de depósito |
|---|---|---|
| | | |
| | | |
| | | |

☐ continua em folha anexa

# ANNEX C

## Published Paper in Food Science and Technology International

Article

FOOD SCIENCE AND TECHNOLOGY INTERNATIONAL

# Evaluation of a potentially probiotic non-dairy beverage developed with honey and kefir grains: Fermentation kinetics and storage study

**Fernanda A Fiorda[1], Gilberto V de Melo Pereira[2], Vanete Thomaz-Soccol[2], Sudip K Rakshit[3] and Carlos R Soccol[1,2]**

**Abstract**

The aim of this work was to study the fermentation process of honey with kefir grains through a comprehensive understanding of its rheological properties, probiotic cell viability, instrumental color parameters and kinetic aspects in a batch bioreactor and during storage. The results showed that kefir grains were well adapted to bioreactor conditions, reaching high levels of cell viability (over $10^6$ CFU $mL^{-1}$ for total yeast and bacteria), phenolic compounds content (190 GAE/100 g) and acidification after 24 h of fermentation at 30 °C. Colorimetric analysis showed that lightness (L*) and redness (a*) remained constant, while yellowness intensities (b*) decreased during fermentation time. After 35 days of storage, honey kefir beverage maintained its chemical characteristics and microbial viability as required to be classified as a probiotic product. The Ostwald-de-Waele ($R^2 \geq 0.98$) and Herschel-Bulkley ($R^2 \geq 0.99$) models can be used to predict the behavior of honey kefir beverage. The parameters analyzed in this study should be taken into account for industrial production of this novel non-dairy beverage.

**Keywords**

Kefir beverage, fermentation, non-dairy functional beverage, kinetic, bioreactor, viscosity

Date received: 15 January 2016; accepted: 3 April 2016

## INTRODUCTION

For centuries, lactic acid fermentation has been used to preserve, improve or modify the flavor of milk, meats, cereals and vegetables. Lactic acid bacteria (LAB), such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* are the main agents of milk fermentation that convert sugars into lactic acid (García Fontán et al., 2006; Lourens-Hattingh and Viljoen, 2001; Penna et al., 2007). An alternative method for milk fermentation is through the use of kefir grains as starter cultures. Kefir grain consists of a polysaccharide composed by a complex microbial association among bacteria and yeasts, which works as a starter culture for milk fermentation (García Fontán et al., 2006). The result is a naturally carbonated beverage (associated with yeast metabolism) with acid taste and creamy consistency due to LAB metabolism. The consumption of kefir beverage has been associated with beneficial effects on human health, and several bacteria and yeasts found in kefir are recognized as probiotics (Diosma et al., 2014; Puerari et al., 2012; Zanirati et al., 2015).

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[3]Chemical Engineering Department, Lakehead University, Thunder Bay-ON, Canada

**Corresponding author:**
Carlos R Soccol, Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, 81531-970 Curitiba PR, Brazil.
Email: soccol@ufpr.br

# ANNEX D

## Ethic Committee Approvment for Sensorial Tests

## TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Eu, Fernanda Assumpção Fiorda pesquisadores da Universidade Federal do Paraná, estou convidando você da comunidade da UFPR a participar de um estudo intitulado "Bioprocesso de produção de bebida probiótica a base de mel fermentada com grãos de kefir" realizando um teste de visualização e degustação de bebida fermentada a base de mel para avaliar suas características. Esta pesquisa está sendo realizada visando à busca de alternativas na produção de bebidas probióticas.

O objetivo desta pesquisa é desenvolver uma bebida fermentada a base de mel e investigar a aceitação de um produto novo no mercado através de consumidores voluntários.

a) Caso você participe da pesquisa, será necessário que compareça a uma sessão no Laboratório de Análise Sensorial, Usina Piloto B, do PPGEAL (Pós-Graduação em Engenharia de Alimentos) no Campus Centro Politécnico da Universidade Federal do Paraná (Rua Francisco H. dos Santos, S/Nº, bairro Jardim das Américas) para participar da avaliação da aceitabilidade da bebida fermentada a base de mel. A sessão será realizada em agosto de 2015, nos períodos das 9:30 às 11:00 e das 14:00 às 17:00.

b) Para tanto você deverá comparecer no Laboratório de Análise Sensorial, Usina Piloto B, do PPGEAL da UFPR para participar da avaliação da aceitabilidade desses produtos novos à base de mel. Nesta sessão serão avaliadas amostras de bebida a base de mel e a sessão terá duração de aproximadamente quinze minutos.

c) Não estão previstos riscos relacionados ao produto exceto no caso de você apresentar alergia a algum tipo de mel e, desconhecendo fato, participar do estudo. É possível ainda que sinta leve desconforto apenas por provar mais de uma amostra do produto.

d) Os benefícios esperados com essa pesquisa são: identificar um produto probiótico novo no mercado à base de mel, quanto sua aceitação pelo mercado consumidor, relacionando suas características físicas com sensoriais. No entanto, nem sempre você será diretamente beneficiado com o resultado da pesquisa, mas poderá contribuir para o avanço científico.

e) Os pesquisadores, Fernanda Assumpção Fiorda, aluna do curso de Pós-Graduação em Engenharia de Alimentos da Universidade Federal do Paraná, telefone (41)9927-0678, e-mail: fernandafiorda@gmail.com e a Prof. Dr. Carlos Ricardo Soccol, tel: (41) 3361-3965, e-mail: soccol@ufpr.br, como responsáveis por este estudo poderão ser contatados na Usina Piloto B do Programa de Pós-Graduação em Engenharia de Alimentos da UFPR, das 07:30 às 11:30 e das 13:30 às 17:30 de segunda à sexta para esclarecer eventuais dúvidas que você possa ter e fornecer-lhe as informações que queira, antes, durante ou depois de encerrado o estudo.

f) A sua participação neste estudo é voluntária e se você não quiser mais fazer parte da pesquisa poderá desistir a qualquer momento e solicitar que lhe devolvam o termo de consentimento livre e esclarecido assinado.

g) As informações relacionadas ao estudo poderão ser conhecidas pelos pesquisadores. No entanto, se qualquer informação for divulgada em relatório ou publicação, isto será feito sob forma codificada, para que a sua identidade seja preservada e seja mantida a confidencialidade.

h) As despesas necessárias para a realização da pesquisa não são de sua responsabilidade e pela sua participação no estudo você não receberá qualquer valor em dinheiro. Como membro da Comunidade da UFPR (estudante, professor, colaborador), você terá a garantia de que problemas como: alergia decorrente do estudo será assistida pela Plus Santé (tel: (41)3342-2525) atendimento de emergência contratado pela UFPR para o atendimento em qualquer local dos Campi. Após havendo necessidade de atendimento médico posterior você poderá agendar consulta na Casa III (Centro Politécnico – tel: (41)3361-3066 ou (41)3361-3643 no horário de atendimento das 07:00 às 18:00.

i) Quando os resultados forem publicados, não aparecerá seu nome, e sim um código.

Eu,_____ li esse termo de consentimento e compreendi a natureza e objetivo do estudo do qual concordei em participar. A explicação que recebi menciona os riscos e benefícios do estudo. Eu entendi que sou livre para interromper minha participação a qualquer momento sem justificar minha decisão.

Eu concordo voluntariamente em participar deste estudo.

_____
(Assinatura do participante da pesquisa ou responsável legal)

Curitiba, ____/____/____ Assinatura do Pesquisador

Comitê de ética em Pesquisa do Setor de Ciências da Saúde da FUFPR
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