Fermentative properties of starter culture during manufacture of kefir with new prebiotics derived from lactulose

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ABSTRACT

In this paper we study the fermentation properties of starter culture during kefir manufacture with one emerging prebiotic as oligosaccharides derived from lactulose (OsLu), [as well as with well-recognized prebiotics as](#) galactooligosaccharides (GOS) and lactulose at different doses (2 and 4% of prebiotic). Microbial growth, glycerol, lactic and citric acids and [short-chain fatty acids](#) (SCFAs) and carbohydrate utilization during fermentation and cold storage of control and prebiotic supplemented kefir were determined. Prebiotic levels remained unaltered during fermentation (24 hours) and storage (28 days), with the exception of a decrease (7.3%) of lactulose in kefir with 4% of prebiotic content. Consequently, the viability of lactic acid bacteria and yeasts, as well as the pH or level of fermentation metabolites was similar for all kefir. Therefore, our data highlight the suitability of kefir as a matrix for the consumption of a variety of prebiotics, including that of novel synthesis as OsLu, widening their potential food uses and applications.

Keywords: kefir, prebiotics, OsLu, lactulose, GOS, SCFAs

1. Introduction

Kefir is a fermented milk product originally from the Caucasian mountains produced by the addition of kefir grains or commercial starter cultures to milk. The making and consumption of kefir has increased in many parts of the world. Concretely, the main European countries consuming sugary kefir beverage include France, Greece, Turkey, Romaine, Russia, the United Kingdom, Netherlands, Norway, Sweden, Spain and Portugal (Sarkar, 2007; Zhou, Liu, Jiang, & Dong, 2009).

~~The microbial composition in the kefir grains is based on probiotic lactic acid bacteria species of *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Lactococcus* ssp. (Glibowski & Zielińska, 2015), fermenting yeasts (*Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*) and acetic acid bacteria (Piermaria, Mariano, & Abraham, 2008). Lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Lactococcus* ssp.), fermenting yeasts (*Kluyveromyces*, *Saccharomyces*) and acetic bacteria are the most common microorganisms present in kefir (Glibowski & Zielińska, 2015; Piermaria, Mariano, & Abraham, 2008).~~ Lactic acid bacteria include both homofermentative that produce lactic acid, and heterofermentative producing acetic acid, ethanol, carbon dioxide and formic acid, apart from lactic acid (Mayo et al., 2010). All these microorganisms have a cross-feeding relationship (Lopitz-Otsoa, Rementeria, Elguezabal, & Garaizar 2006), and they are contained in a water-soluble polysaccharide named kefiran, which produces specific sensory properties because of sugary kefir consumption (Fiorda et al., 2017). Several homofermentative species of *Lactobacillus* such as *Lactobacillus kefiranofaciens* ~~and *Lactobacillus kefir*~~ could enhance the production of kefiran (Cheirsilp, Shimizu, & Shioya, 2003). ~~In consequence, kefir is widely known as an excellent source of~~

~~probiotics, which must be able to survive under the harsh conditions of human gastrointestinal tract and colonize the colon, conferring a health benefit on the host (FAO/WHO, 2001) (Nielsen, Gürakan, & Ünlü, 2014).~~ Currently, several scientific studies have supported the health benefits of kefir in the organism such as antimicrobial, anti-inflammatory, antioxidative, anticarcinogenic, and anti-hypertensive activities (de Lima et al., 2017; Rosa et al., 2017).

The microbiological, chemical and sensorial characteristics of the final kefir product could depend on factors such as the grain or powder starter culture to milk, incubation, agitation, and storage conditions (Öner, Karahan, & Cakmakci, 2010). For standard production, industries use starter cultures that involve pure kefir microflora strains (Gul, Mortas, Atalar, Dervisoglu, & Kahyaoglu, 2015). There are several authors who have reported the evolution of starter cultures (Grønnevik, Falstad, & Narvhus, 2011) and kefir grains (Irigoyen, Arana, Castiella, Torre, & Ibanez, 2005), as well as the organic acid composition (Puerari, Magalhães, & Schwan, 2012) during fermentation and storage processes.

~~Prebiotics are selectively fermented ingredients that produces changes, both in the composition and/or activity in the gastrointestinal microflora which confers benefits to host well-being and health (Roberfroid, 2007)~~Prebiotics are selectively fermented ingredients that selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Gibson et al., 2017). Lactulose (4-O- β -D-galactopyranosyl-D-fructose) is a prebiotic disaccharide that can reach the proximal colon and stimulate the growth of *Bifidobacterium* and *Lactobacillus spp.* (Olano & Corzo, 2009). However, this disaccharide cannot reach distal regions of colon where most diseases occur such as colon cancer. Our

research group synthesized new oligosaccharides derived from lactulose (OsLu) by transgalactosylation reaction. These novel oligosaccharides are considered as emerging prebiotics due to their ability to be selectively fermented by *Bifidobacterium* and *Lactobacillus spp.* (Cardelle-Cobas et al., 2012), and to exert higher resistance to mammalian digestibility than conventional galactooligosaccharides (GOS) (Ferreira-Lazarte, Olano, Villamiel, & Moreno, 2017; Hernández-Hernández et al., 2012). This piece of evidence together with the fact that OsLu have a higher DP (≥ 3) than lactulose point out the OsLu capacity to be fermented in the distal regions of the colon (Moreno, Montilla, Villamiel, Corzo, & Olano, 2014).

The effect produced by a combination of ~~probiotics~~ the microorganisms and prebiotics of present in kefir ~~and prebiotics~~ could improve the survival of the probiotic bacteria during the passage through the upper intestinal tract and could stimulate their growth in the colon (Casiraghi, Canzi, Zanchi, Donati, & Villa, 2007). The addition of prebiotics, such as lactulose (Nacheva, Loginovska, & Valchkov, 2018), galactooligosaccharides (GOS), fructooligosaccharides (FOS) (Oh et al., 2013), oligofructose (Glibowski & Zielińska, 2015), inulin (Simsek, Sanchez-Rivera, El, Karakaya, & Recio, 2017), or isomaltooligosaccharides (Mei, Feng, & Li, 2017) to kefir has been described during last years, but this information has been mainly focused on their effect on physicochemical and sensory properties, as well as on microbiological growth and organic acid content; however ~~.. Therefore,~~ there is still a lack of knowledge on the resistance of prebiotics to kefir fermentation and cold storage ~~potential effect of prebiotics in lactic acid bacteria and yeast behavior during kefir production.~~ Therefore, the focus of this work -was to determine the biological stability during kefir fermentation and cold storage of novel galacto-oligosaccharides derived from lactulose (OS-Lu) in comparison with lactulose and commercial galacto-oligosaccharides derived from lactose (GOS). ~~in this work a study of~~

~~of fermentative properties of starter culture during fermentation and cold storage of kefir~~
~~manufactured with a new prebiotic as OsLu as well GOS and lactulose.~~ Microorganism
growth, evolution of pH and carbohydrate fraction as well as formation of glycerol, and
organic acids (lactic, citric and SCFAs) determinations were also performed in order to
provide a full picture of the fermentation properties of starter culture during kefir
manufacture. ²

2. Materials and methods

2.1. Kefir starter culture and prebiotics

Powder kefir starter culture (Vital-Fermente, A.Vogel, Bioforce, Spain) was used to
produce kefir beverages. It was composed of *Streptococcus thermophilus*, *Lactobacillus*
kefir, *Lactobacillus acidophilus*, *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp.
cremoris, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, *Leuconostoc mesenteroides*
ssp. *cremoris*, *Kluyveromyces marxianus* var. *marxianus* and *Saccharomyces unisporus*, as
constitutive microflora. Storage and maintenance of the cultures were carried out according
to the specifications of the manufacturer.

Lactulose (powder), trade name “Galactofructose[®]”, was kindly provided by Solactis
Group (Paris, France) with a composition of 74.5% lactulose, 7.5% galactose, 8% lactose
and 10% epilactose, tagatose and fructose per 100g of total carbohydrates. Vivinal-GOS[®]
(syrup) was obtained from Borculo Domo (Hanzeplein, Groningen, The Netherlands) and
the composition was 74% GOS, 16% monosaccharides and 10% lactose per 100g of total
carbohydrates. Oligosaccharides derived from lactulose (OsLu) (syrup) were previously
synthesized in our laboratory using α β -galactosidase from *Aspergillus Oryzae* and lactulose

(Cardelle-Cobas et al., 2016). The composition was 43% OsLu, 19% fructose, 12% galactose, 1% glucose and 25% lactulose per 100 g of total carbohydrates.

2.2. Kefir production

Pasteurized milk (1 L) was heated at 75°C during 45 min and subsequently cooled to 25°C. Different batches (50 mL) of kefir without (control) and with prebiotics (OsLu, GOS, or lactulose) at 2 and 4% (w/v) were prepared. The starter culture (0.7 g/L) was inoculated and these batches were incubated at 25°C during 24 hours and, then, refrigerated and stored at 4°C for 28 days according to manufacturer's protocol. Control and prebiotic supplemented kefirs were prepared in duplicate and samples for analysis were taken at 0, 2, 6, 12 and 24 hours during fermentation process, and at 7, 14, 21 and 28 days during the storage period of cold storage at 4°C. All analyses of kefir samples were carried out in triplicate.

2.3. Measurement of pH

The pH was measured in all kefir samples with an electrode pH-meter (Metler Toledo, Five Easy Plus) during fermentation (0, 2, 6, 12 and 24 hours) and refrigerated storage (7, 14, 21 and 28 days).

2.4. Enumeration of starter culture microorganisms

The enumeration of each type of bacteria in the starter culture was performed by optimizing their microbiological analysis, using different mediums, different temperatures of incubation and aerobic/anaerobic conditions. Optimized conditions, as described below, were chosen for the identification of the different microorganisms from kefir.

Samples collected during fermentation and cold storage were analyzed within 24 hours after collection. Bacterial counts were carried out following fermentation (0, 2, 6, 12 and 24 hours) and cold storage (7, 14, 21, 28 days) processes.

Phosphate buffer saline (PBS) at a concentration of 1 g/L was used to prepare the dilutions for the microbiological analysis. *Streptococcus thermophilus* and *Lactococcus ssp.* were quantified according to Simova et al. (2002), with some modifications. They were incubated aerobically into M-17 agar at 30°C for 72 h, containing 1% lactose (Sigma Aldrich, Steinheim, Germany) and 1.2% bacteriological agar (Scharlab, Barcelona, Spain).

Leuconostoc mesenteroides ssp. cremoris was identified following Abekhti, Daube, & Kihal (2014) methodology, with some modifications. They were incubated aerobically at 23°C for 5 days in MSE agar supplemented with 0.0075% of sodium azide (to inhibit *Lactobacillus ssp.* growth), 10% of sucrose, 0.5% of glucose (Sigma Aldrich, Steinheim, Germany) and 1.5% agar (Scharlab, Barcelona, Spain). Viscous cocci colonies were enumerated as *Leuconostoc mesenteroides ssp. cremoris*. For enumeration of *Lactobacillus ssp.*, they were incubated aerobically at 30°C for 5 days in MSE agar supplemented 10% of sucrose, 0.5% of glucose, 1.5% agar, without any trace of sodium azide.

Yeasts (*Kluyveromyces marxianus var. marxianus* and *Saccharomyces unisporus*) were determined by using 3M Petrifilm™ (3M, Neuss, Germany) and they were incubated at 20°C for 5 days. Small green colonies were identified as yeasts.

2.5. Microbial metabolite analyses

Organic acids (lactic and citric acids, and SCFAs) as well as glycerol production were determined by HPLC following the methods of da Costa, da Silva Frasao, da Costa Lima, Rodrigues, & Junior (2016). Before analysis, 1 mL of kefir was mixed with 10 mL

of 0.0045 M sulfuric acid. The resulting mixture was stirred in an ultrasonic bath and centrifuged at 10,000 g for 10 min for removal of proteins. The supernatant was filtered through a 0.22 µm pore membrane.

The analyses were performed in a LC Agilent Technologies 1220 Infinity LC System 1260 equipped with a dual detection system consisting of an Ultraviolet detector (UV) and a Refractive Index detector (RID). Separations of compounds were performed in a ion exchange column with sulfonated styrene-divinylbenzene spheres REZEXTM ROA, crosslinked resin (8% hydrogen; 300 x 7.8 mm and 8mm particle size) (Phenomenex, Torrance, CA, USA) thermostated at 40°C. Detection of organic acids was carried out by UV ($\lambda = 210$ nm) while glycerol was detected using RID. Elution was in isocratic mode and the mobile phase was 0.005 M sulfuric acid at a flow rate of 0.5 mL/min.

Quantification of organic acids was carried out by the external standard method using standard solutions of citric acid, pyruvic acid, lactic acid, acetic acid, formic acid, propionic acid and butyric acid prepared in the concentration range of 0.002-2.0 mg/mL. Glycerol quantification was carried out by the external standard method using standard solutions at a concentration range of 0.02-1 mg/mL. Determination coefficients obtained from these calibration curves were linear over the range studied ($R^2 > 0.99$).

2.6. Analysis of carbohydrates

2.6.1 Sample preparation

Mono-, di- and oligosaccharides from control and prebiotic supplemented kefir were determined by GC-FID. Before analysis, 1 g of kefir sample (2 and 4% w/v) were precipitated with 10 mL of methanol at 99.9% (Merck Millipore, Spain) during 1 hour at room temperature in order to precipitate proteins and fat. Later, the supernatant was

centrifuged at 10,000 x g for 5 min and 500 µL of supernatant was evaporated with 400 µL of internal standard (phenyl-β-glucoside) solution, which contained 0.5 mg/mL.

2.6.2 Analysis

Carbohydrates present in kefir samples were determined as trimethyl silylated oximes (TMSO) prepared following the method of Brobst & Lott, (1966). First, oximes were formed by adding 250 µL of hydroxylamine chloride (2.5%) in pyridine to dried samples and heating the mixture at 70°C for 30 min. Then, oximes were silylated with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) and kept at 50 °C for 30 min. Reaction mixtures were centrifuged at 10,000 g for 2 min at room temperature. Supernatants were injected or stored at 4 °C prior to analysis.

Analyses of TMSO derivatives were carried out following the method of Montilla, Van de Lagemaat, Olano, & Del Castillo, (2006) in an Agilent Technologies 7890A Gas Chromatograph (Agilent Technologies, Wilmington, DE, EEUU) equipped with a flame ionization detector (FID) using a fused silica capillary column DB-5HT, bonded, crosslinked phase (5% phenyl-methylpolysiloxane; 15 m × 0.32 mm i.d., 0.10 µm film thickness) (J&W Scientific, Folson, California, USA). The oven temperature was initially 150°C increasing at a rate of 3°C/min to 380°C and held this temperature during 76 min. The temperature of injector and detector were at 280°C and 385°C, respectively. Injections were carried out in split mode (1:20) using nitrogen as carrier gas at a flow rate of 1 mL/min.

In the case of kefirs containing lactulose, and due to coelution with lactose, analyses were performed using a fused silica capillary column DB-17, bonded, crosslinked phase (50% phenyl-50% methylpolysiloxane; 30 m x 250 µm id., 0.25 µm film thickness) (J&W Scientific, Folson, California, USA) under the following conditions: initially oven

temperature was 200°C, then increased at 4°C/min to 230°C and held this temperature during 7.5 min. Later, increase at 1°C/min to 250°C until 27.5 min and finally, increase at a rate of 2°C/min up to 255°C, and maintaining this temperature for 30 min. The temperature of injector and detector were at 280°C and 295°C, respectively. Injections were carried out in split mode (1:20) using nitrogen as carrier gas at a flow rate of 0.4 mL/min.

Quantification of each sugar was performed by internal standard calibration using phenyl- β -glucoside (0.5 mg/mL). Response factors were calculated after the analysis of standard solutions of glucose, galactose, fructose, lactose, lactulose, raffinose and nystose over the expected concentration range in samples (0.3-5 mg/mL). Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software.

2.7. Statistical Analysis.

The experimental data of pH, bacterial counts and carbohydrate, lactic acid, glycerol and SCFAs concentrations during fermentation and cold storage were presented as mean values $\pm$ standard deviations. Mean values of these parameters were submitted to analysis of variance using General Linear Model (GLM) by the Statistica Software 6.0 (SPPS) to find statistical differences among all prepared kefir. When it was needed, the experiments were also organized with factorial-repeated measures ANOVA, considering the influence of the time, the type and the amount of prebiotic, as the main variables, by using the Tukey test at significance level $P < 0.05$.

3. Results and discussion

3.1. Evolution of pH during kefir manufacture and cold storage

During the fermentation process of prebiotic supplemented kefir a significant pH decrease was observed after 6 hours of fermentation, being the decrease rate especially faster after 12 hours, with pH values going down to 4.4 in all cases at the end of fermentation (**Figure 1**). This behavior agreed with those previously described in kefirs with oligofructose or inulin at 4% where a pH value of 4.4 was observed at the end of fermentation (Glibowski & Zielińska, 2015). The acidification was likely due to the growth of lactic acid bacteria and lactic acid production as will be ~~shown~~ demonstrated below.

During the storage period (**Figure 1**), the ~~slight~~ negligible decrease of pH observed in all kefirs took place mainly during the third week of storage, with values closed to of 4.3 at the end of storage, in agreement with data reported for traditional and kefirs with inulin and ~~oligofructosa~~ oligofructose during 21 and 28 days of storage, respectively (Glibowski & Zielińska, 2015; Güzel-Seydim, Seydim, Greene, & Bodine, 2000; Irigoyen, Arana, Castiella, Torre, & Ibanez, 2005). This fact could be attributed to the low acidifying activity of the starter cultures during the refrigerated storage that was carried out at 4 °C.

Lastly, the addition of the three different prebiotics, at different doses, did not have any significant influence ($P < 0.05$) on the fermentation time nor the pH of kefirs supplemented with prebiotics (**Figure 1**).

3.2. Effects of prebiotics on microorganism counts

Figure 2 shows changes in the viability of microorganisms during fermentation and storage observed in all prepared kefirs. In general, lactic acid bacteria increased in all prebiotic supplemented kefirs during fermentation period.

In the case of *Lactococcus lactis* (*Streptococcus lactis*) and *Streptococcus thermophilus* enumerated as “viable streptococci” (**Figure 2 a**), the count at the beginning of fermentation process was of 9.5 log CFU/mL and they significantly increased ($P<0.05$) at 12 hours of fermentation up to 9.8 log CFU/mL. During storage, a sharply decrease during the first week was produced, achieving values of ≈ 9.3 log CFU/mL to, then, remain stable during the last three weeks of storage. The initial levels of these microorganisms were mainly attributed to *Lactococcus ssp.*, which are the most predominant species in kefir samples. Thus, Simova et al. (2002) reported higher levels of *Lactococcus ssp.* than *Streptococcus thermophilus* but the presence of these bacteria highly depends on the type of grain used. Additionally, other studies reported levels of 8 log CFU/mL in *Lactococcus ssp.* counts at the beginning of storage with a decrease at 14 days to values of 6.5 log CFU/mL, although *Streptococcus thermophilus* was not determined (Grønnevik, Falstad, & Narvhus, 2011; Irigoyen, Arana, Castiella, Torre, & Ibanez, 2005).

The initial counts of *Lactobacillus ssp.* were 8.3 log CFU/mL, but a significant increase ($P<0.05$) was found after 6, 12 and 24 hours by reaching maximum values of $\approx \log 9.3$ CFU/mL in all studied kefirs. Afterwards, these values remained constant during the whole storage period (**Figure 2 b**). *Lactobacillus ssp.* levels in our work are much higher than those reported by previous authors with values of 6-6.5 log CFU/mL at the end of storage (Grønnevik et al. 2011; Irigoyen et al. 2005), but similar to those described by Leite et al. (2013) who reported a population of *Lactobacillus ssp.* of 10 log CFU/mL at 24 hours of fermentation and remained constant after 28 days of storage.

The counts of *Leuconostoc mesenteroides* ssp. *cremoris* had initial values of 4.5 log CFU/mL (**Figure 2 c**), to be then significantly increased ($P<0.05$) during the whole fermentation process, mainly between 6 and 24 hours of fermentation, with final values of 6.3 log CFU/mL at the end of fermentation process. These data were in line with those reported in traditional kefir, with an increase during the first hours of fermentation, reaching final values of 7.5 log CFU/mL (Fontán, Martínez, Franco, & Carballo, 2006). During the entire refrigerated storage period, levels of this microorganism remained unaltered in all prepared kefir.

Finally, the initial yeast content in the studied kefir samples was 2.0 log CFU/mL (**Figure 2 d**) and remained constant during the first 6 hours of fermentation and then, to be increased up to values of 2.3 log CFU/mL at 12 hours of fermentation. These values were kept constant until the end of the fermentation and during the subsequent cold storage. Yeast counts were low according to the Codex standard for fermented milks (CODEX Alimentarius Commission, 2003) which reported a minimum of 4 log CFU/mL in kefir. Nevertheless, other studies on kefir production also reported low values (3.3 log CFU/mL and lower than 3 log CFU/mL) at the end of the fermentation period (Fontán, Martínez, Franco, & Carballo, 2006; Grønnevik, Falstad, & Narvhus, 2011).

Overall, ~~the~~ time is an important variable to consider in the viability on lactic acid bacteria (*Lactococcus* ssp., *Streptococcus thermophilus*, *Lactobacillus* ssp. and *Leuconostoc mesenteroides* ssp. *cremoris*) and fermenting yeasts (*Kluyveromyces marxianus* and *Saccharomyces unisporus*). However, the type and dose of added prebiotic did not influence significantly the bacterial counts.

As it will be shown below, lactose, which is the major component in the matrix, is likely metabolized by the Embden-Meyerhoff-Parnas pathway (EMP), promoting the growth

of all ~~acid~~-lactic acid bacteria. This consumption seems to take place mainly between 6 and 24 hours of fermentation, according to the significant increases observed in *Lactococcus* ssp. *Streptococcus thermophilus*, *Lactobacillus* ssp. and *Leuconostoc mesenteroides* ssp. *cremoris* (**Figure 2 a-c**), which is, in turn, concomitant with the sharp pH decrease previously described (**Figure 1**).

3.3. Formation of organic acids (lactic, citric and SCFAs) and glycerol during kefir elaboration

Lactic and citric acids and SCFAs, as well as glycerol were quantified in control and prebiotic supplemented kefirs during fermentation and cold storage (**Figure 3**). Lactic acid was the major organic acid detected in all kefirs as it could be expected attending to lactose consumption by kefir microbiota (**Figure 3 a**). In all studied kefirs, the initial content at the beginning of fermentation were ≈ 0.90 mg/g kefir. During the fermentation process, the level of this acid increased significantly ($P < 0.05$) from the sixth hour, reaching levels of 6.0-6.4 mg/g kefir at 24 hours of fermentation. These data are in accordance with decrease of pH and viable cell counts of microorganisms observed. During cold storage, the content of lactic acid significantly increased during the first week of storage and then, it remained constant, reaching final values of 6.8 mg/g kefir at the end of the studied period. Previous studies have reported similar lactic acid content after 21 days of cold storage (7.7 mg/g kefir) (Güzel-Seydim, Seydim, Greene, & Bodine, 2000).

The content of citric acid, originally present in milk, at the beginning of fermentation was ≈ 1.40 mg/g kefir and it decreased significantly after 12 hours of fermentation reaching values of ≈ 0.95 mg/g kefir at the end of the fermentation process in all kefirs (**Figure 3 b**). Then, citric acid also decreased significantly ($P < 0.05$) after 7 days of cold storage (≈ 0.36 mg/g kefir) and remained constant until the end of storage, ~~attained with~~ final values

of ≈ 0.29 mg/g kefir in all samples (a total decrease of $\approx 76\%$). The decrease in citrate content can be attributed to the metabolism of lactic acid bacteria that could convert citric acid to pyruvate and acetic acid and, then, pyruvate could be transformed into lactic acid and other volatile compounds, such as acetoin and diacetyl (Verhue & Tjan, 1991). Grønnevik, Falstad, & Narvhus, (2011) also reported a decrease in citric acid in kefir, reaching final values of ≈ 0.10 mg/g kefir after 28 days of storage.

SCFAs (acetic, formic, propionic and pyruvic acids) formation ~~were~~was also studied during elaboration of control and prebiotic supplemented kefir. With respect to acetic acid (**Figure 3 c**), the initial levels were rather moderate in all cases ≈ 0.11 mg/g kefir. During fermentation process, the content of acetic acid began to increase significantly ($P < 0.05$), mostly after 12 hours. Nevertheless, during cold storage, acetic acid remained constant, with values about ≈ 0.33 mg/g kefir after 28 days. The increase observed during the fermentation process agrees with the consumption of citric acid by lactic acid bacteria since acetic acid is an intermediate in citrate metabolism and one of the organic acids produced by heterofermentative lactic acid bacteria (Verhue & Tjan, 1991). Grønnevik, Falstad, & Narvhus, (2011) also observed an increase in acetic acid from 12 to 24 hours of fermentation to remain, then, constant until 28 days of storage, reaching final values of 0.80 mg/g kefir. These results were in agreement with those found by Gul, Mortas, Atalar, Dervisoglu, & Kahyaoglu, (2015) in kefir stored for 21 days.

Formic acid was detected in all kefir, with values about 0.17 mg/g kefir at the beginning of fermentation process, remaining, then, stable during studied fermentation and storage periods, indicating that it is not metabolized by kefir microbiota. Propionic acid and pyruvic acids were also detected but the contents were below the limit of quantification. Low quantities of pyruvate could be explained because certain microorganisms of kefir

microbiota such as *Lactococcus lactis*, *Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Kluyveromyces marxianus* could transform pyruvate directly to lactic acid by EMP pathway. The presence of propionic, pyruvic (Güzel-Seydim, Seydim, Greene, & Bodine, 2000; Puerari, Magalhães, & Schwan, 2012) and formic acids (Leite et al., 2013; Oh et al. 2013) is not always described in kefir, which could be explained by the variation in the ratio and types of microorganisms in kefir grains and starter cultures.

Glycerol was also determined during kefir elaboration, thus the initial content at the beginning of fermentation period was rather low (less than 0.10%) (**Figure 3 d**). Nevertheless, this compound increased significantly ($P < 0.05$) during the fermentation process, mostly from the sixth hour. During cold storage, the content of glycerol remained fairly stable, showing values of 0.54% at the end of studied period. These low values of glycerol in control and prebiotic supplemented kefir were consistent with the levels detected by Puerari, Magalhães, & Schwan, (2012) at 72 hours of fermentation. Glycerol is the main secondary product in alcoholic fermentations by *Saccharomyces* ssp. (mainly *Saccharomyces cerevisiae*). In kefir here elaborated, *Saccharomyces unisporus* could metabolize glucose derived from the hydrolysis of lactose and produce glycerol, ethanol and esters as final products (Fiorda et al., 2017). This process could take place mainly during fermentation process, when the hydrolysis of lactose and the consumption of glucose ~~is~~are mostly carried out.

In consonance with the data on viability of lactic acid bacteria and yeast, the addition of prebiotics as OsLu, GOS or lactulose to kefir at the two studied concentrations (2 and 4%) did not affect neither the production organic acids (lactic and citric acids and SCFAs) nor the glycerol during the fermentation and storage processes (**Figure 3**). However, the time of

fermentation and storage is also an important factor to consider in the formation of organic acids.

3.4. Changes in the carbohydrate fraction during kefir manufacture

3.4.1. Monosaccharides.

The evolution of the monosaccharides: galactose, glucose and fructose were studied during fermentation and cold storage in all prepared kefirs. The content in galactose increased at 24 hours (**Figure 4 a**). This fact could be explained by the hydrolysis of lactose carried out by lactic acid bacteria and *Kluyveromyces marxianus*, releasing galactose and glucose. Galactose is subsequently metabolized by the Leloir pathway or by tagatose pathway (PKP) to produce lactic acid, among other products (Srinivas, Mital, & Garg, 1990). During the storage, the concentration of galactose remained stable in all prebiotic supplemented kefirs. Grønnevik, Falstad, & Narvhus, (2011) also reported low values of galactose in traditional kefirs during 28 days of storage.

Glucose was also found in small quantities (<0.1%) at the beginning of fermentation process in control and kefirs containing OsLu and lactulose and no changes were observed during fermentation and storage. In kefirs supplemented with GOS at 2 and 4%, levels of 0.61 and 1.01%, respectively, were found and these values significantly decreased until 0.17 and 0.38%, respectively. This different behavior could be due to the higher initial levels of glucose in kefirs with added GOS, as compared to the rest of assayed kefirs. The glucose released from lactose hydrolysis could be metabolized by EMP pathway to produce lactic acid or via hexose monophosphate pathway (HMP) to produce lactic acid, glycerol, acetic acid, ethanol and CO₂ (Puerari, Magalhães, & Schwan, 2012).

Finally, fructose was only found in kefir with OsLu at levels of 0.44 and 0.79 % at the beginning of fermentation process. During fermentation and cold storage, fructose was slightly degraded by kefir microflora being the final content 0.16% and 0.55% in kefir with 2 and 4% of OsLu, respectively.

3.4.2. Lactose.

The major carbohydrate in the kefir matrix is lactose, which is hydrolyzed into two monosaccharides (galactose and glucose) by lactic acid bacteria and *Kluyveromyces marxianus* during fermentation and storage of kefirs. The initial concentration of lactose in all samples was 4.8 g/ 100 g kefir (**Figure 4 b**) and a significant decrease ($P<0.05$) during fermentation process was produced, mainly between 12 and 24 hours achieving a final concentration of 3.3-3.4 g/ 100 g kefir. The highest consumption of lactose during fermentation was concomitant with the maximum counts of all lactic acid bacteria revealing the efficient role of lactose as carbohydrate substrate for the used kefir starter culture. Our results were in agreement with previous studies, which reported values of 4.0 g/ 100 g of kefir at the end of the fermentation process (Fontán et al., 2006; Grønnnevik et al., 2011).

During cold storage, the concentration of lactose did not change significantly in studied kefirs after 28 days of storage, this fact agreed with the stability of the pH during storage (**Figure 1**). Grønnnevik, Falstad, & Narvhus, (2011) also reported a constant concentration of lactose during the cold storage of kefir.

Lastly, the addition of GOS, OsLu or lactulose at different concentrations did not affect the hydrolysis rate of lactose by lactic acid bacteria and *Kluyveromyces marxianus*, as compared to the control kefir.

3.4.3. Prebiotic oligosaccharides

The evolution of prebiotic carbohydrates content in kefir during fermentation and storage processes is shown in **Figures 5** and **6**. GOS (**Figure 5 a and b**) or OsLu (**Figure 5 c and d**) remained unaltered ($P>0.05$) during fermentation and storage processes in all kefir samples regardless of their degree of polymerization and of the amount added. To the best of our knowledge, Oh et al. (2013) reported the only study on the addition of GOS in kefir, but they did not provide their evolution during fermentation and storage. Nevertheless, that study demonstrated that the levels of lactic acid bacteria were not affected by the addition of this prebiotic probably because their inability to hydrolyze these complex carbohydrate structures in the presence of lactose and monosaccharides. A similar behavior has been described following the addition of GOS in yogurts (Vénica, Wolf, Bergamini, & Perotti, 2016; [Delgado-Fernandez, Corzo, Olano, Hernández-Hernández, & Moreno, 2019](#)).

The evolution of lactulose content in kefir samples was dependent on its concentration (**Figure 6**). Thus, lactulose levels remained constant ($P>0.05$) during fermentation and storage processes when adding at the lowest tested concentration, i.e. 2%. However, the addition of lactulose at 4% resulted in a moderate but significant consumption with a decrease of 7.3% at the end of storage process. The partial degradation of lactulose could be explained because this prebiotic could be hydrolyzed by certain species of *Lactobacillus*, such as *Lactobacillus acidophilus* (Oliveira, Florence, Perego, De Oliveira, & Converti, 2011).

4. Conclusions

Findings described in this work point out the feasibility to elaborate kefir using either emerging, OsLu, as well-recognized prebiotics GOS and lactulose, added at two concentrations (2 or 4%). Despite the complex composition in microbial species of kefir starter culture, the lactose assimilation prevailed over the prebiotic consumption during

fermentation period and the subsequent cold storage, since only a moderate but significant decrease of lactulose could be found following its addition at 4%. Therefore, the supplementation of kefirs with OsLu, GOS or lactulose did not affect the viability of lactic acid bacteria and fermentative yeasts nor modify the production of glycerol, lactic acid, pH and SCFAs, while the stability of prebiotics was not jeopardized.

Prebiotics have been shown to remain unaltered during manufacture and subsequent cold storage of kefir without affecting the fermentation process or the characteristics of the final product. To conclude, our data highlight the suitability of kefir as a vehicle for the delivery of ~~of probiotics microorganisms and~~ a variety of prebiotics, including that of novel synthesis as OsLu, widening their potential food applications.

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FIGURE CAPTIONS

Figure 1. Evolution of pH in control and prebiotic (oligosaccharides derived from lactulose, OsLu; galactooligosaccharides GOS or lactulose) supplemented (2 and 4%) kefirs during fermentation and cold storage. Error bars represent standard deviation ($n=6$).

Figure 2.- Viability of *Lactococcus ssp.* and *S.thermophilus* **(a)**, *Lactobacillus ssp.* **(b)**, *Leuconostoc mesenteroides ssp. cremoris* **(c)** and yeasts **(d)** in control and prebiotic (oligosaccharides derived from lactulose, OsLu; galactooligosaccharides GOS or lactulose) supplemented (2 and 4%) kefirs during fermentation and cold storage. Error bars represent standard deviation ($n=6$).

Figure3. Evolvment of lactic acid **(a)**, citric acid **(b)**, acetic acid **(c)** and glycerol **(d)** in control and prebiotic (oligosaccharides derived from lactulose, OsLu; galactooligosaccharides GOS or lactulose) supplemented (2 and 4%) kefirs during fermentation and cold storage. Error bars represent standard deviation ($n=6$).

Figure 4. Changes in the content of galactose **(a)** and lactose **(b)** in control and prebiotic supplemented kefirs during fermentation and cold storage. Error bars represent standard deviation ($n=6$).

Figure 5. Changes in the content of GOS and of oligosaccharides derived from lactulose (OsLu) following their addition at 2 % **(a)** and **(c)** and 4 % **(b)** and **(d)**, respectively, during fermentation and cold storage of kefirs. Error bars represent standard deviation ($n=6$).

Figure 6. Changes in the content of lactulose in control and prebiotic supplemented 2 % **(a)** and 4% **(b)** kefirs during fermentation and cold storage of kefirs. Error bars represent standard deviation ($n=6$).