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

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ABSTRACT

The fermentation properties of a starter culture during kefir manufacture was studied with the inclusion of one emerging lactulose-derived oligosaccharide prebiotic and with the well-recognised galactooligosaccharide and lactulose prebiotics at different doses (2 and 4%). Microbial growth, glycerol, lactic and citric acids and short-chain fatty acids and carbohydrate utilisation during fermentation and cold storage of control and prebiotic supplemented kefirs were determined. Prebiotic levels remained unaltered during fermentation (24 h) and storage (28 days), with the exception of a decrease (7.3%) of lactulose in kefir with 4% prebiotic. Consequently, the viability of lactic acid bacteria and yeasts, as well as the pH or level of fermentation metabolites was similar for all kefirs. 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 lactulose-derived oligosaccharides, widening their potential food uses and applications.

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. Currently, the main European countries consuming sugary kefir beverage include France, Greece, Turkey, Romania, Russia, the United Kingdom, Netherlands, Norway, Sweden, Spain and Portugal (Sarkar, 2007; Zhou, Liu, Jiang, & Dong, 2009).

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* could enhance the production of kefiran (Cheirsilp, Shimizu, & Shioya, 2003). 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 kefir grains (Irigoyen, Arana, Castiella, Torre, & Ibanez, 2005) and starter cultures (Grønnevik, Falstad, & Narvhus, 2011), as well as the organic acid composition (Puerari, Magalhães, & Schwan, 2012), during fermentation and storage processes.

Prebiotics are substrates that are selectively utilised by host microorganisms conferring a health benefit (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

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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 synthesised new oligosaccharides derived from lactulose (OsLu) utilising the 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 degree of polymerisation ($\geq$3) than lactulose explain 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 microorganisms and prebiotics present in kefir 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), GOS, fructooligosaccharides (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 the last few 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 formation; however, there is still a lack of knowledge on the resistance of prebiotics to kefir fermentation and cold storage.

Therefore, the focus of this work was to determine the biological stability during kefir fermentation and cold storage of novel galactooligosaccharides derived from lactulose (OsLu) in comparison with lactulose and commercial galactooligosaccharides derived from lactose (GOS). Determinations of microorganism growth, evolvement of pH and carbohydrate fraction, as well as the formation of glycerol, and organic acids [lactic acid, citric acid and short chain fatty acids (SCFAs)], were also performed to provide a full picture of the fermentation properties of the starter culture during kefir manufacture.

2. Materials and methods

2.1. Kefir starter culture and prebiotics

Powdered 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 culture was 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 100 g of total carbohydrates. Vivinal-GOS® (syrup) was obtained from Borculo Domo (Hanzeplein, Groningen, The Netherlands) and the composition was 74, 16 and 10% (total carbohydrates) of GOS, monosaccharides and lactose, respectively. OsLu (syrup) were previously synthesised in our laboratory using a β-galactosidase from *Aspergillus oryzae* and lactulose (Cardelle-Cobas et al., 2016). The composition was 43, 19, 12, 1 and 25% (total carbohydrates) of OsLu, fructose, galactose, glucose and lactulose, respectively.

2.2. Kefir production

Pasteurised milk (1 L) was heated at 75 °C for 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^{-1}) was inoculated and these batches were incubated at 25 °C for 24 h and, then, refrigerated and stored at 4 °C for 28 days according to the 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 h during fermentation process, and at 7, 14, 21 and 28 days 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 h) 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 optimising their microbiological analysis, using different media, different temperatures of incubation and aerobic/anaerobic conditions. Optimised conditions, as described below, were chosen for the identification of the different microorganisms from kefir.

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

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

L. mesenteroides ssp. *cremoris* was identified following the method of Abekhti, Daube, and Kihal (2014), with some modifications, being incubated aerobically at 23 °C for 5 days in MSE agar supplemented with 0.0075% sodium azide (to inhibit *Lactobacillus* ssp. growth), 10% sucrose, 0.5% glucose (Sigma Aldrich) and 1.5% agar (Scharlab). Viscous cocci colonies were enumerated as *L. mesenteroides* ssp. *cremoris*. Enumeration of *Lactobacillus* ssp., was by aerobic incubation at 30 °C for 5 days in MSE agar supplemented 10% sucrose, 0.5% glucose, 1.5% agar (no sodium azide).

Yeasts (*K. marxianus* var. *marxianus* and *S. 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 method of da Costa, da Silva Frasao, da Costa Lima, Rodrigues, and Junior (2016). Before analysis, 1 mL of kefir was mixed with 10 mL of 0.0045 M sulphuric 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). Separation of compounds was performed on an ion exchange column with sulfonated styrene-divinylbenzene spheres REZEX™ ROA, crosslinked resin (8% hydrogen; 300 × 7.8 mm and 8 mm particle size) (Phenomenex, Torrance, CA, USA) thermostatised at 40 °C. Detection of organic acids was with UV (λ = 210 nm); glycerol was detected using RID. Elution was in isocratic mode; the mobile phase was 0.005 M sulphuric acid at a flow rate of 0.5 mL min^{-1}.

Quantification of organic acids was carried out by the external standard method using standard solutions of citric, pyruvic, lactic, acetic, formic, propionic and butyric acids prepared in the concentration range of 0.002–2.0 mg mL^{-1}. Glycerol quantification was carried out by the external standard method using standard solutions at a concentration range of 0.02–1 mg mL^{-1}. 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 kefirs 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) for 1 h at room temperature to precipitate proteins and fat. Later, the supernatant was centrifuged at 10,000× *g* for 5 min and 500 μL supernatant was evaporated with 400 μL 0.5 mg mL^{-1} internal standard (phenyl-β-glucoside) solution.

2.6.2. Analysis

Carbohydrates present in kefir samples were determined as trimethyl silylated oximes (TMSO) prepared following the method of Brobst and 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, and Del Castillo (2006) in an Agilent Technologies 7890A Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionisation 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^{-1} 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^{-1}.

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 × 250 μm id., 0.25 μm film thickness) (J&W Scientific, Folson, CA, USA) under the following conditions: initially oven temperature was 200 °C, then increased at 4 °C min^{-1} to 230 °C and held this temperature for 7.5 min. Later, increase at 1 °C min^{-1} to 250 °C until 27.5 min and finally, increase at a rate of 2 °C min^{-1} 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^{-1}.

Quantification of each sugar was performed by internal standard calibration using phenyl-β-glucoside (0.5 mg mL^{-1}). 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^{-1}). 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 ± standard deviations. Mean values of these parameters were submitted to analysis of variance (ANOVA) using General Linear Model (GLM) by the Statistica Software 6.0 (SPPS) to find statistical differences among all prepared kefirs. When it was needed, the experiments were also organised 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 h of fermentation, this decrease being faster thereafter, with pH values going down to 4.4 in all cases at the end of fermentation (Fig. 1). This behaviour 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 demonstrated below.

During the storage period (Fig. 1), the 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 oligofructose during 21 and 28 days of storage, respectively (Glibowski & Zielińska, 2015; Güzel-Seydim, Seydim, Greene, & Bodine, 2000; Irigoyen et al., 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.

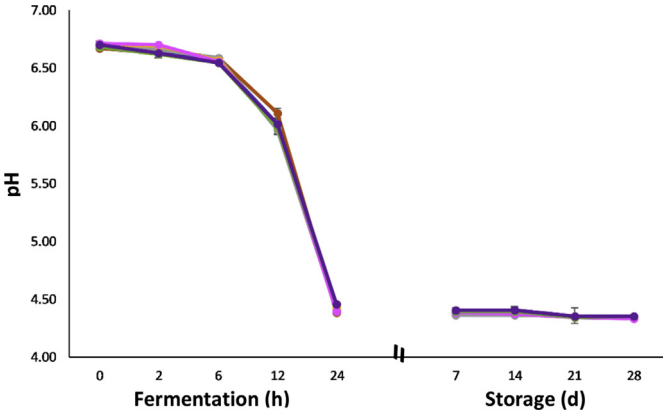

Fig. 1. Evolution of pH in control (●) and prebiotic supplemented kefirs (●, 2% OsLu; ●, 4% OsLu; ●, 2% lactulose; ●, 4% lactulose; ●, 2% GOS; ●, 4% GOS) during fermentation and cold storage. Error bars represent standard deviation ($n = 6$).

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 (Fig. 1).

3.2. Effects of prebiotics on microorganism counts

Fig. 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 *L. lactis* and *S. thermophilus* enumerated as "viable streptococci" (Fig. 2a), the count at the beginning of fermentation process was of 9.5 log cfu mL^{-1} and they significantly increased ($P < 0.05$) at 12 h of fermentation up to 9.8 log cfu mL^{-1}. During storage, a sharply decrease during the first week was produced, achieving values of approximately 9.3 log cfu mL^{-1} 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 *S. 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^{-1} in *Lactococcus* ssp. counts at the beginning of storage with a decrease at 14 days to values of 6.5 log cfu mL^{-1}, although *S. thermophilus* was not determined (Grønnevik et al., 2011; Irigoyen et al., 2005).

The initial counts of *Lactobacillus* ssp. were 8.3 log cfu mL^{-1}, but a significant increase ($P < 0.05$) was found after 6, 12 and 24 h, reaching maximum values of approximately log 9.3 cfu mL^{-1} in all studied kefirs. Afterwards, these values remained constant during the whole storage period (Fig. 2b). *Lactobacillus* ssp. levels in our work are much higher than in those reported by some previous authors, 6–6.5 log cfu mL^{-1} at the end of storage (Grønnnevik 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^{-1} at 24 h of fermentation that remained constant after 28 days of storage.

The counts of *L. mesenteroides* ssp. *cremoris* had initial values of 4.5 log cfu mL^{-1} (Fig. 2c), then significantly increased ($P < 0.05$) during the whole fermentation process, mainly between 6 and 24 h of fermentation, with final values of 6.3 log cfu mL^{-1} at the end of fermentation. These data were in line with those reported in traditional kefirs, with an increase during the first hour of fermentation, reaching final values of 7.5 log cfu mL^{-1} (Fontán, Martínez, Franco, & Carballo, 2006). During the entire refrigerated storage period, levels of this microorganism remained unaltered in all prepared kefirs.

Finally, the initial yeast content in the studied kefir samples was 2.0 log cfu mL^{-1} (Fig. 2d) and remained constant during the first 6 h of fermentation and then increased up to values of 2.3 log cfu mL^{-1} at 12 h 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) that reports a minimum of 4 log cfu mL^{-1} in kefir. Nevertheless, other studies on kefir production also reported low values (3.3 log cfu mL^{-1} and lower than 3 log cfu mL^{-1}) at the end of the fermentation period (Fontán et al., 2006; Grønnevik et al., 2011).

Overall, time is an important variable to consider in the viability of lactic acid bacteria (*Lactococcus* ssp., *S. thermophilus*, *Lactobacillus* ssp. and *L. mesenteroides* ssp. *cremoris*) and fermenting yeasts (*K. marxianus* and *S. 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 metabolised by the Embden-Meyerhoff-Parnas pathway (EMP), promoting the growth of all lactic acid bacteria. This consumption seems to take place mainly between 6

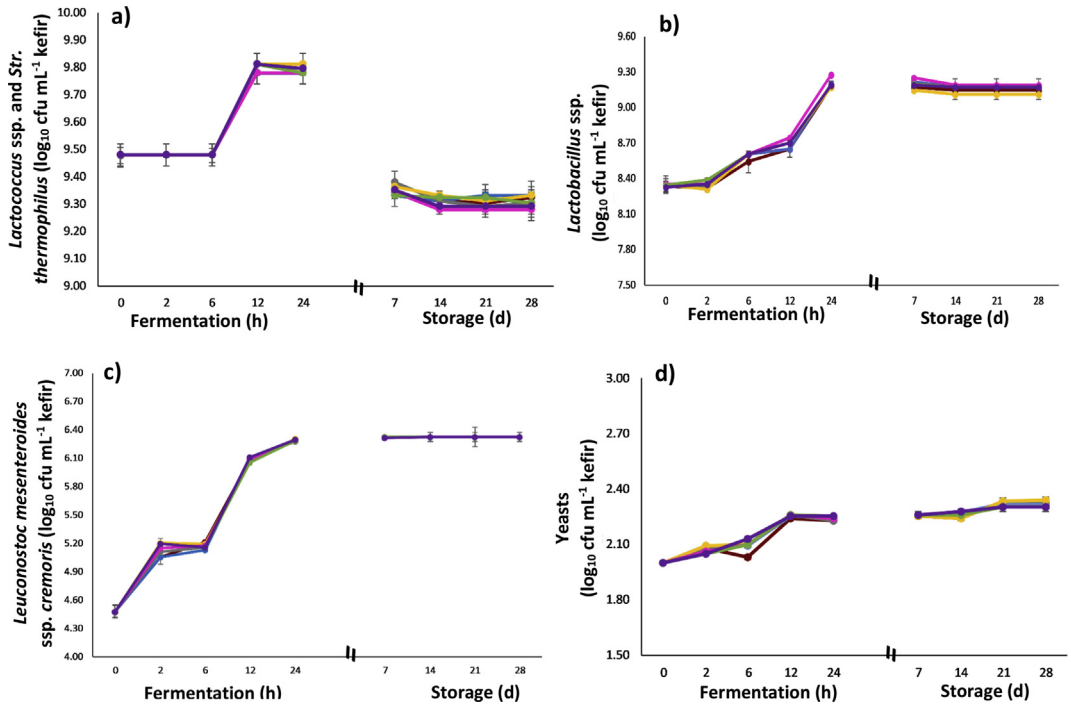

Fig. 2. Viability of (a) *Lactococcus* ssp. and *Streptococcus thermophilus*, (b) *Lactobacillus* ssp., (c) *Leuconostoc mesenteroides* ssp. *cremoris* and (d) yeasts in control (●) and prebiotic supplemented kefirs (●, 2% OsLu; ●, 4% OsLu; ●, 2% lactulose; ●, 4% lactulose; ●, 2% GOS; ●, 4% GOS) during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

and 24 h of fermentation, according to the significant increases observed in *Lactococcus* ssp., *S. thermophilus, Lactobacillus* ssp. and *L. mesenteroides* ssp. *cremoris* (Fig. 2a–c), which is, in turn, concomitant with the sharp pH decrease previously described (Fig. 1).

3.3. Formation of organic acids 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 (Fig. 3). As could be expected considering lactose consumption by kefir microbiota, lactic acid was the major organic acid detected in all kefirs (Fig. 3a). The initial content at the beginning of fermentation was approximately 0.1 g 100 g^{-1} kefir. During the fermentation process, the level of this acid increased significantly ($P < 0.05$) from the sixth hour, reaching levels of 0.6–0.64 g 100 g^{-1} kefir at 24 h of fermentation. These data are in accordance with the observed decreases in pH and viable cell counts of microorganisms. During cold storage, the content of lactic acid significantly increased during the first week of storage and then remained constant, reaching final values of 0.68 g 100 g^{-1} kefir at the end of the period studied. Previous studies have reported similar lactic acid content after 21 days of cold storage (0.77 g 100 g^{-1} kefir) (Güzel-Seydim et al., 2000).

The content of citric acid, originally present in milk, at the beginning of fermentation was approximately 0.14 g 100 g^{-1} kefir and decreased significantly after 12 h of fermentation, reaching values of approximately 0.09 g 100 g^{-1} kefir at the end of the fermentation process in all kefirs (Fig. 3b). Then, citric acid also decreased significantly ($P < 0.05$) after 7 days of cold storage (approximately 0.04 g 100 g^{-1} kefir) and remained constant until the end of storage with final values of approximately 0.03 g 100 g^{-1} kefir in all samples (a total decrease of approximately 76%). The decrease in citrate content can be attributed to the metabolism of lactic acid bacteria that converts citric acid to pyruvate and acetic acid; pyruvate can then be transformed into lactic acid and other volatile compounds, such as acetoin and diacetyl (Verhue & Tjan, 1991). Grønnevik et al. (2011) also reported a decrease in citric acid in kefir, reaching final values of approximately 0.010 g 100 g^{-1} kefir after 28 days of storage.

SCFA (acetic, formic, propionic and pyruvic acids) formation was also studied during elaboration of control and prebiotic supplemented kefirs. With respect to acetic acid (Fig. 3c), the initial levels were rather moderate in all cases approximately 0.01 g 100 g^{-1} kefir. During fermentation process, the content of acetic acid began to increase significantly ($P < 0.05$), mostly after 12 h. Nevertheless, during cold storage, acetic acid remained constant, with values about approximately 0.03 g 100 g^{-1} 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 et al. (2011) also observed an increase in acetic acid from 12 to 24 h of fermentation to remain, then, constant until 28 days of storage, reaching final values of 0.08 g 100 g^{-1} kefir. These results were in agreement with those found by Gul et al. (2015) in kefir stored for 21 days.

Formic acid was detected in all kefirs, with values of approximately 0.02 g 100 g^{-1} kefir at the beginning of fermentation process remaining the same throughout the fermentation and storage periods, indicating that it is not metabolised 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 *Lactobacillus lactis, Lb. acidophilus, S. thermophilus* and *K. marxianus* can transform pyruvate directly to lactic acid by the EMP pathway. The presence of propionic, pyruvic (Güzel-Seydim et al., 2000; Puerari et al., 2012) and formic acids (Leite et al., 2013; Oh et al., 2013) is not always described in kefirs, 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

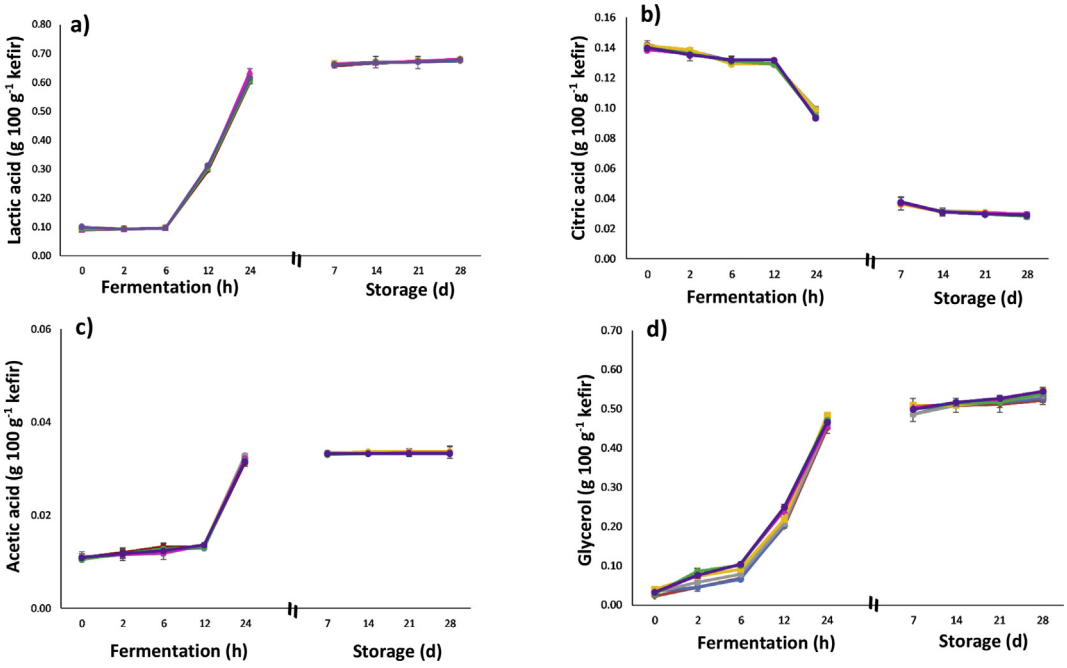

Fig. 3. Evolution of (a) lactic acid, (b) citric acid, (c) acetic acid and (d) glycerol in control (●) and prebiotic supplemented kefirs (●, 2% OsLu; ●, 4% OsLu; ●, 2% lactulose; ●, 4% lactulose; ●, 2% GOS; ●, 4% GOS) during fermentation and cold storage. Error bars represent standard deviation ($n = 6$).

low (less than 0.10 g 100 g^{-1} kefir) (Fig. 3d). 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, with levels of 0.54 g 100 g^{-1} kefir at the end of studied period. These low levels of glycerol in control and prebiotic supplemented kefirs were consistent with those detected by Puerari et al. (2012) at 72 h of fermentation. Glycerol is the main secondary product in alcoholic fermentations by *Saccharomyces* ssp. (mainly *Saccharomyces cerevisiae*). In kefirs here elaborated, *S. unisporus* could metabolise glucose derived from the hydrolysis of lactose and produce glycerol, ethanol and esters as final products (Fiorda et al., 2017). This process can take place mainly during fermentation, when the hydrolysis of lactose and the consumption of glucose mostly occurs.

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 either the production of organic acids (lactic and citric acids and SCFAs) or the glycerol during the fermentation and storage processes (Fig. 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 h (Fig. 4a). This fact could be explained by the hydrolysis of lactose carried out by lactic acid bacteria and *K. marxianus*, releasing galactose and glucose. Galactose is subsequently metabolised by the Leloir pathway or by tagatose pathway (PKP) to produce lactic acid, among other products (Srinivas, Mital, & Garg, 1990). During storage, the concentration of galactose remained stable in all prebiotic supplemented kefirs. Grønnevik et al. (2011) also reported low values of galactose in traditional kefirs during 28 days of storage.

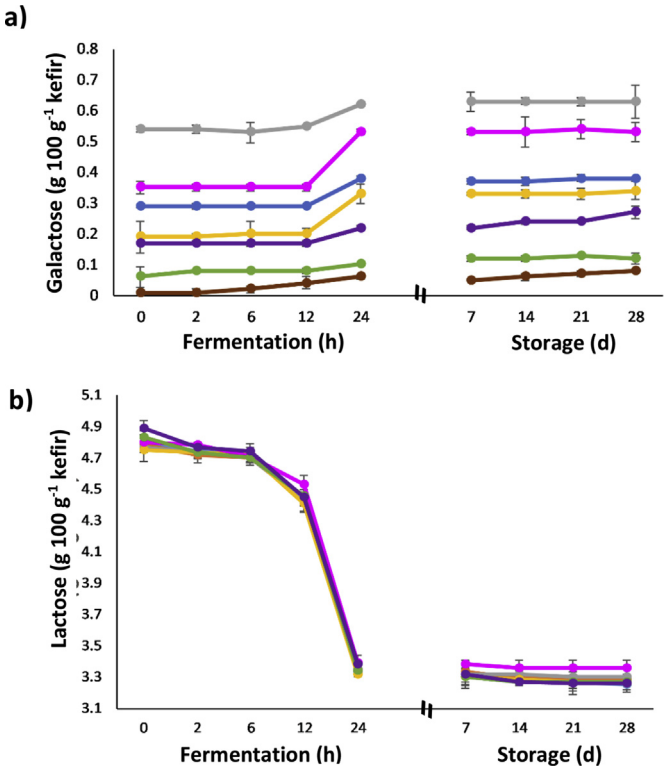

Fig. 4. Changes in the content of (a) galactose and (b) lactose in control (●) and prebiotic supplemented kefirs (●, 2% OsLu; ●, 4% OsLu; ●, 2% lactulose; ●, 4% lactulose; ●, 2% GOS; ●, 4% GOS) during fermentation and cold storage. Error bars represent standard deviation ($n = 6$).

Glucose was also found in small quantities (<0.1 g 100 g^{-1} kefir) 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 g 100 g^{-1} kefir, respectively, were found and these values significantly decreased until 0.17 and 0.38 g 100 g^{-1} kefir, respectively. This different behaviour could be due to the higher initial levels of glucose in kefirs with added GOS, as compared with the rest of the kefirs assayed. The glucose released from lactose hydrolysis could be metabolised by EMP pathway to produce lactic acid or via hexose monophosphate pathway (HMP) to produce lactic acid, glycerol, acetic acid, ethanol and CO_2 (Puerari et al., 2012).

Finally, fructose was only found in kefir with OsLu, at levels of 0.44 and 0.79 g 100 g^{-1} kefir at the beginning of fermentation process. During fermentation and cold storage, fructose was slightly degraded by kefir microflora being the final content 0.16 g 100 g^{-1} kefir and 0.55 g 100 g^{-1} kefir in kefirs with 2 and 4% of OsLu, respectively.

3.4.2. Lactose

The major carbohydrate in the kefir matrix is lactose, which is hydrolysed into two monosaccharides (galactose and glucose) by lactic acid bacteria and *K. marxianus* during fermentation and storage of kefirs. The initial concentration of lactose in all samples was 4.8 g 100 g^{-1} kefir (Fig. 4b); there was a significant decrease ($P < 0.05$) during fermentation, mainly between 12 and 24 h, achieving a final lactose concentration of 3.3–3.4 g 100 g^{-1} 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^{-1} kefir at the end of the fermentation process (Fontán et al., 2006; Grønnnnevik et al., 2011).

During cold storage, the concentration of lactose in the kefirs studied did not change significantly after 28 days of storage, this fact agreed with the stability of the pH during storage (Fig. 1). Grønnevik et al. (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 *K. marxianus*, as compared with the control kefir.

3.4.3. Prebiotic oligosaccharides

The evolution of prebiotic carbohydrate content in kefirs during fermentation and storage is shown in Figs. 5 and 6. Both GOS (Fig. 5a and b) and OsLu (Fig. 5c and d) remained unaltered ($P > 0.05$) during fermentation and storage in all kefir samples regardless of the degree of polymerisation and 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 show the 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 of their inability to hydrolyse these complex carbohydrate structures in the presence of lactose and monosaccharides. A similar behaviour has been described following the addition of GOS in yoghurts (Delgado-Fernandez, Corzo, Olano, Hernández-Hernández, & Moreno, 2019; Vénica, Wolf, Bergamini, & Perotti, 2016).

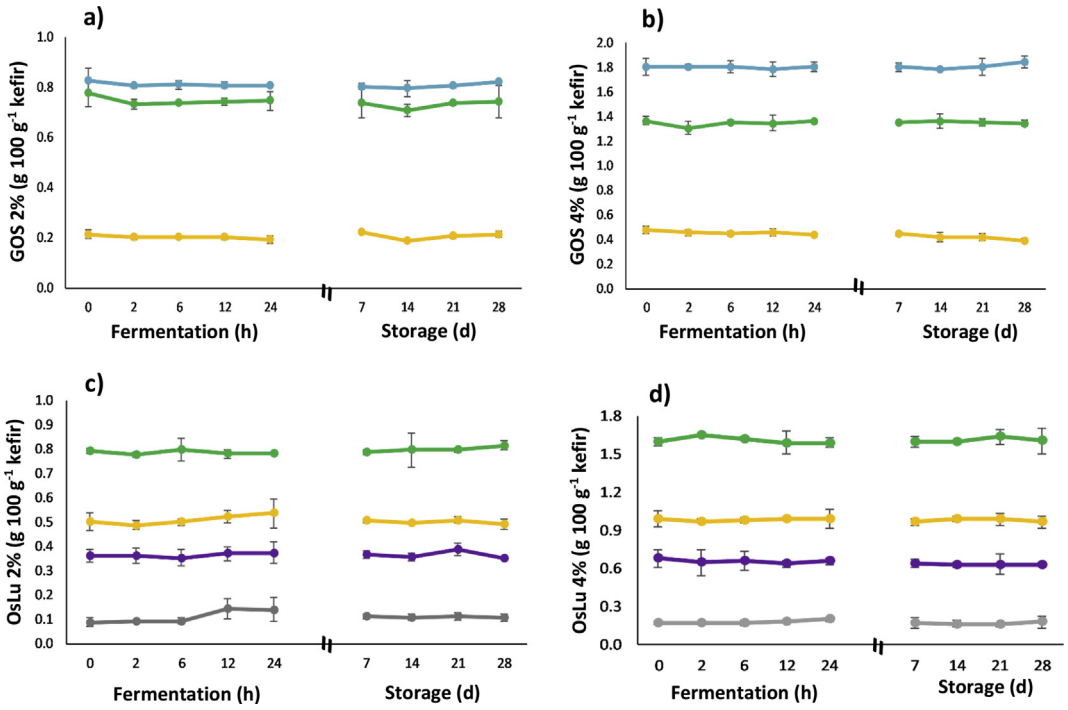

Fig. 5. Changes in the content of GOS (a,b: ●, GOS-disaccharides; ●, GOS-trisaccharides; ●, GOS-tetrasaccharides) and of lactulose-derived oligosaccharides (c,d: ●, lactulose; ●, OsLu-disaccharides; ●, OsLu-trisaccharides; ●, OsLu-tetrasaccharides) following their addition at 2% (a,c) and 4% (b,d) during fermentation and cold storage of kefirs. Error bars represent standard deviation ($n = 6$).

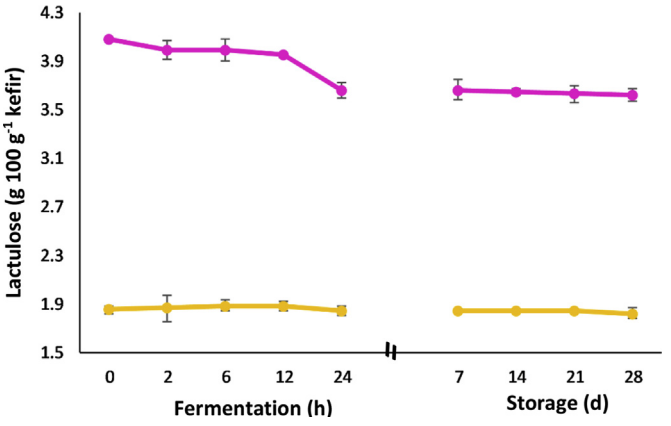

Fig. 6. Changes in the content of lactulose after its addition at (●) 2% and (●) 4%, during fermentation and cold storage of kefirs. Error bars represent standard deviation ($n = 6$).

The evolution of lactulose content in kefir samples was dependent on its concentration (Fig. 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 hydrolysed by certain species of *Lactobacillus*, such as *L. acidophilus* (Oliveira, Florence, Perego, De Oliveira, & Converti, 2011).

4. Conclusions

Findings described in this work point out the feasibility to elaborate kefirs using either the emerging prebiotic, OsLu, as well-recognised prebiotics GOS and lactulose, added at two concentrations (2 or 4%). Despite the complex composition of microbial species of the kefir starter culture, lactose assimilation prevailed over the prebiotic consumption during the fermentation period and subsequent cold storage; 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 or modify the production of glycerol, lactic acid, pH and SCFAs, while the stability of prebiotics was not jeopardised.

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 a variety of prebiotics, including those from of novel syntheses such as OsLu, widening their potential food applications.

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