



## Comparative study of the biochemical changes and volatile compound formations during the production of novel whey-based kefir beverages and traditional milk kefir

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### ABSTRACT

Cheese whey (CW) and deproteinised cheese whey (DCW) were investigated for their suitability as novel substrates for the production of kefir-like beverages. Lactose consumption, ethanol production, as well as organic acids and volatile compounds formation, were determined during CW and DCW fermentation by kefir grains and compared with values obtained during the production of traditional milk kefir. The results showed that kefir grains were able to utilise lactose from CW and DCW and produce similar amounts of ethanol (7.8–8.3 g/l), lactic acid (5.0 g/l) and acetic acid (0.7 g/l) to those obtained during milk fermentation. In addition, the concentration of higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, 2-methyl-1-propanol, and 1-propanol), ester (ethyl acetate) and aldehyde (acetaldehyde) in cheese whey-based kefir and milk kefir beverages were also produced in similar amounts. Cheese whey and deproteinised cheese whey may therefore serve as substrates for the production of kefir-like beverages similar to milk kefir.

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### 1. Introduction

In the past few years there has been an increased interest in the production of fermented dairy beverages containing probiotics due to several health claims that have been associated with their consumption (Özer & Kirmaci, 2010). Probiotics are usually defined as live microorganisms that, when ingested in adequate amounts, confer a health benefit on the host (Vasiljevic & Shah, 2008). Many of these microorganisms have been identified as lactic acid-producing bacteria and are usually consumed in the forms of fermented milks, yogurt or kefir (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000; Zajek & Gorek, 2010).

Kefir is a refreshing, naturally carbonated fermented dairy beverage with a slightly acidic taste, yeasty flavour and creamy consistency (Powell, Witthuhn, Todorov, & Dicks, 2007). The traditional production of kefir is initiated by the addition of small (0.3–3.5 cm in diameter), irregularly shaped, yellow–white kefir grains to fresh milk (Garrote, Abraham, & De Antoni, 1997; Güzel-Seydim, Seydim, Greene, & Bodine, 2000). Kefir grains are mostly composed by proteins and polysaccharides and enclose a

complex microflora. Lactic acid bacteria (LAB) and yeasts exist in a complex symbiotic relationship and are responsible for alcoholic and lactic acid fermentation, respectively. Since kefir grains are able to metabolize lactose, they can be used to ferment cheese whey, a lactose-rich waste of negligible cost (Papapostolou, Bosnea, Koutinas, & Kanellaki, 2008).

Cheese whey, the yellow–green liquid remaining after the precipitation and removal of milk casein during cheese making, has been considered as one of the major problems in the dairy industry. It represents an important environmental pollution, exhibiting a biochemical oxygen demand (BOD) equal to the maximum allowable limits of 50,000 mg/l and chemical oxygen demand (COD) equal to the maximum allowable limits of 80,000 mg/l (Siso, 1996). Furthermore, deproteinised cheese whey or whey permeate, the liquid fraction obtained through the ultrafiltration or diafiltration of raw cheese whey, account for more than 70% of total whey solids and is mostly responsible for the whey polluting load. This liquid therefore generates disposal problems, in terms of volumes produced and polluting load, almost equal to the disposal of raw whey (Guimarães, Teixeira, & Domingues, 2010).

In recent years, considerable efforts have been undertaken to find new ways of using cheese whey and reduce environmental pollution. The lactose content of cheese whey and the presence

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of other essential nutrients for microbial growth make this dairy by-product a potential feedstock for the production of valuable compounds through fermentation processes (Panesar, Kennedy, Gandhi, & Bunko, 2007). Besides bio-ethanol fermentation by *Kluyveromyces marxianus* (Sansonet, Curcio, Calabrò, & Iorio, 2009; Zafar & Owais, 2006), *Candida pseudotropicalis* (Ghaly & El-Taweel, 1995) and genetically modified *Saccharomyces cerevisiae* yeasts (Domingues, Guimarães, & Oliveira, 2010; Domingues, Lima, & Teixeira, 2001; Guimarães, François, Parrou, Teixeira, & Domingues, 2008), the production of alcoholic beverages, including distilled beverages (Dragone, Mussatto, Oliveira, & Teixeira, 2009) and kefir-like whey beverages (Paraskevopoulou et al., 2003), has also been considered as an interesting alternative for cheese whey valorisation.

Recently, we characterized the microbiota of kefir grains and beverages obtained from milk and raw/deproteinised cheese whey using microscopy and molecular techniques (Magalhães, de M Pereira, Dias, & Schwan, 2010). However, scientific information on chemical changes occurring during cheese whey (mainly deproteinised cheese whey) fermentation by kefir grains is still scarce. Therefore, the objective of this work was, for the first time, to evaluate the biochemical changes, organic acids production and volatile compounds formation during deproteinised cheese whey (DCW) fermentation by kefir grains, and compare their performance with that obtained during the production of raw cheese whey (CW) kefir beverage and traditional milk kefir.

## 2. Materials and methods

### 2.1. Kefir grains and inoculum preparation

Kefir grains isolated from Brazilian milk kefir beverages were used in the experiments. The inoculum was prepared by cultivating kefir grains in pasteurized whole milk, renewed daily, for a duration of 7 days. After this time, the grains were washed with sterile distilled water and subsequently, the grains (12.5 g) were inoculated in the different fermentation media.

### 2.2. Media and fermentation conditions

Pasteurized whole cow's milk, as well as CW powder solution and DCW powder solution, were used as fermentation media for the production of traditional milk kefir and whey-based kefir beverages, respectively. CW powder solution was prepared by dissolving cheese whey powder (Lactogal, Porto/Portugal) in sterile distilled water to the same lactose concentration as in whole milk (46 g/l). DCW powder solution was obtained by autoclaving the CW powder solution at 115 °C for 10 min, followed by aseptic centrifugation (2220g for 20 min) to remove proteins.

Kefir grains were cultivated under static conditions in 1-l Erlenmeyer flasks, containing 250 ml of medium at 25 °C for 48 h. The fermentation runs were assessed through periodic sampling in order to determine lactose consumption, ethanol and organic acids production, as well as the formation of volatile compounds.

### 2.3. Protein determination

The protein content of the different samples was assessed, at both the beginning and at the end of the fermentation process, using the nitrogen content, based on the Kjeldahl method (AOAC, 1995). The protein content was calculated by multiplying the total nitrogen by 6.38. All protein contents were expressed as g/l.

### 2.4. HPLC analysis

Lactose and ethanol were quantified by high performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with a refractive index (RI) detector (Jasco 830-RI). Lactic acid and acetic acid were also quantified by high-performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with UV-Vis detector (Jasco 870-UV-visible) and a Chrompack column (300 × 6.5 mm) at 60 °C, using 5 mM sulfuric acid as the eluent, at a flow rate of 0.5 ml/min and a sample volume of 20 µl.

### 2.5. GC/FID analysis

Higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, 2-methyl-1-propanol, and 1-propanol), ester (ethyl acetate) and aldehyde (acetaldehyde) in milk kefir and whey-based kefir beverages were determined by extraction with dichloromethane, and subsequent analysis of the extracts by gas chromatography using a Chrompack CP-9000 gas chromatograph equipped with a Split/Splitless injector and a flame ionization detector. A capillary column (50 m × 0.25 mm i.d., 0.2 µm film thickness; Chrompack), coated with CP-Wax 57 CB was used. The temperature of the injector and detector was set to 250 °C. The oven temperature was held at 50 °C for 5 min, then programmed to run from 50 °C to 220 °C at 3 °C/min, before being held at 220 °C for 10 min. Helium was used as the carrier gas at 125 kPa, with a split vent of 15 ml/min. Injections of 1 µl were made in the splitless mode (vent time, 15 s); 4-nonanol (internal standard) was added to the sample to give a final concentration of 122.05 mg/l. The volatile compounds were identified by comparing retention indices with those of standard compounds. Quantification of volatile compounds was performed with the Varian Star Chromatography Workstation software (Version 6.41) and expressed as 4-nonanol equivalents, after determining the detector response factor for each compound.

### 2.6. Statistical analysis

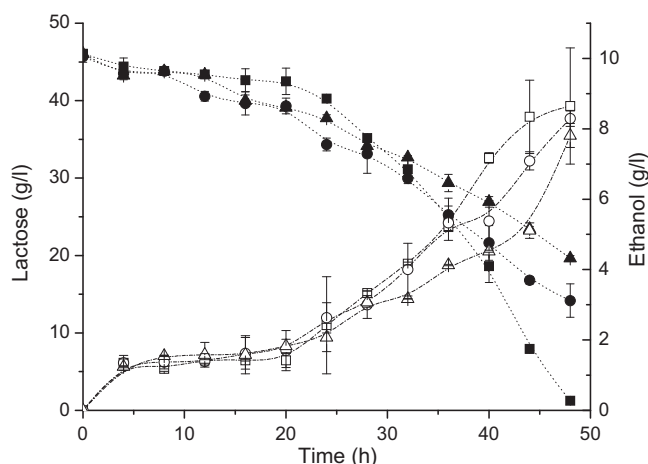
Each fermentation was carried out in duplicate and mean values are reported. The Tukey's test using Statgraphics Plus for Windows 4.1 software (Statistical Graphics Corp., 1999) was performed to evaluate statistical significance of differences between the beverages and to compare the means among the samples.

## 3. Results and discussion

### 3.1. Fermentation performance of kefir grains cultivated in milk, CW and DCW

Fig. 1 shows the time evolution of lactose and ethanol during the fermentation of milk, CW and DCW by kefir grains. It can be observed that most of the lactose present in milk was metabolized within 48 h, resulting in the formation of 8.65 g/l (1.1%) ethanol. Similar results were reported earlier by Papapostolou et al. (2008) during lactose fermentation at 30 °C by thermally dried kefir cells using a conventional drying method at 38 °C. On the other hand, the use of CW and DCW as substrates for the production of a whey-based beverage resulted in lower lactose consumption than that observed during milk fermentation.

The higher lactose utilisation during milk fermentation by kefir grains could probably be due to the characteristics of milk that, being richer in nutrients (primarily proteins) than CW and DCW (Table 1), allowed an improved growth for microorganisms. Table 1 shows that, despite the higher lactose consumption during milk fermentation, there was no statistically significant difference

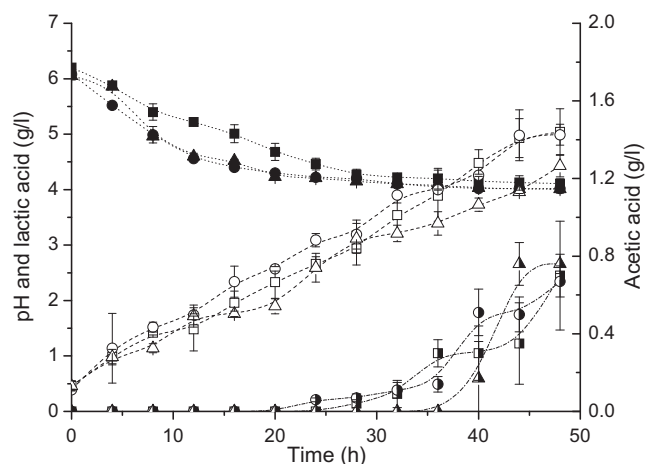


**Fig. 1.** Lactose consumption (closed symbols) and ethanol production (open symbols) during kefir grain cultivation at 25 °C, using milk (square), cheese whey (circle) and deproteinised cheese whey (triangle) as substrates. Bars represent the standard deviation.

( $p < 0.05$ ) among the final ethanol concentrations in the three beverages. A higher lactose utilisation for cell growth could explain the lower ethanol yield obtained at the end of milk fermentation by kefir grains.

The final ethanol concentrations ( $8.7 \pm 1.6$  g/l,  $8.3 \pm 0.2$  g/l and  $7.8 \pm 0.3$  g/l for milk kefir, CW-based kefir and DCW-based kefir, respectively) were within the range of ethanol contents, 0.5% v/v (3.9 g/l) to 2.4% (18.9 g/l), reported previously by Papapostolou et al. (2008) for the production of kefir using lactose and raw cheese whey as substrates. Although yeasts such as *Kluyveromyces* sp. are primarily responsible for the conversion of lactose to ethanol during kefir fermentation, some heterofermentative bacteria (e.g. *Lactobacillus kefir*) are also capable of producing ethanol (Güzel-Seydim et al., 2000). The presence of *K. marxianus* and *Lactobacillus kefiranofaciens* in grains and kefir beverages (milk, CW and DCW) were recently identified by our group using culture-independent methods (PCR–DGGE) (Magalhães et al., 2010).

The mean changes in pH values during cultivation of kefir grains in the three different substrates are depicted in Fig. 2. A sharp decrease in the pH was observed during the first 28 h, from an initial value of about 6.1 to 4.3 at 28 h, for all the substrates. Afterwards, the pH decreased slightly, reaching a final value of nearly 4.0. After 48 h of incubation, pH values of the fermented milk kefir and whey-based beverages were not significantly different ( $p < 0.05$ ). These pH values were similar to those previously reported for milk kefir (García Fontán, Martínez, Franco, & Carballo, 2006). Athanasiadis, Paraskevopoulou, Blekas, and Kiosseoglou (2004), suggested an optimal pH of 4.1 for a novel beverage obtained from cheese whey fermentation by kefir granules. According to these authors the flavour of the fermented product was improved at a final pH value of 4.1, due to the higher profile of volatile by-products than for other final pH values.



**Fig. 2.** Time evolution of pH (closed symbols), lactic acid (open symbols) concentration and acetic acid (half-closed symbols) concentration during milk (square), cheese whey (circle) and deproteinised cheese whey (triangle) fermentation by kefir grains. Bars represent the standard deviation.

Production of lactic acid has been linked with lactic acid bacteria metabolism and is of great importance due to its inhibitory effect on both spoilage and pathogenic microorganisms in kefir milk (Magalhães et al., 2010). As expected, while the pH decreased, the lactic acid concentration increased progressively during milk, CW and DCW fermentations, from a mean value of 0.5 g/l at 0 h to 5.0 g/l at 48 h. This agrees with the finding of Güzel-Seydim et al. (2000) that kefir has a lower lactic acid content than yogurt (8.8–14.6 g/l) probably due to the preferential use of the heterofermentative pathway, rather than the homofermentative pathway, with a resultant production of  $\text{CO}_2$ .

The mean concentration of acetic acid was practically zero during the first 24 h of milk, CW and DCW fermentation (Fig. 2), before it increased slightly during the period from 24 to 48 h, reaching a final concentration of 0.7 g/l; this value is similar to those observed by other authors (Rea et al., 1996) during skim milk fermentation by different Irish kefir grains. The presence of acetic acid in the fermented beverages could be attributed to heterofermentative lactic acid and acetic acid cultures present in kefir grains microflora (Magalhães et al., 2010).

### 3.2. Volatile by-products identified by GC-FID

Volatile compounds are important contributors to the flavours of beverages, as they determine different desirable sensory characteristics (Arrizon, Calderón, & Sandoval, 2006). Previous studies have shown that the formation of volatile higher alcohols and esters during kefir fermentation is influenced by the composition of the medium (Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001). In our study, a total of seven flavour-active compounds, including five higher alcohols, one ester and one aldehyde, were identified by gas chromatography coupled with flame ionization

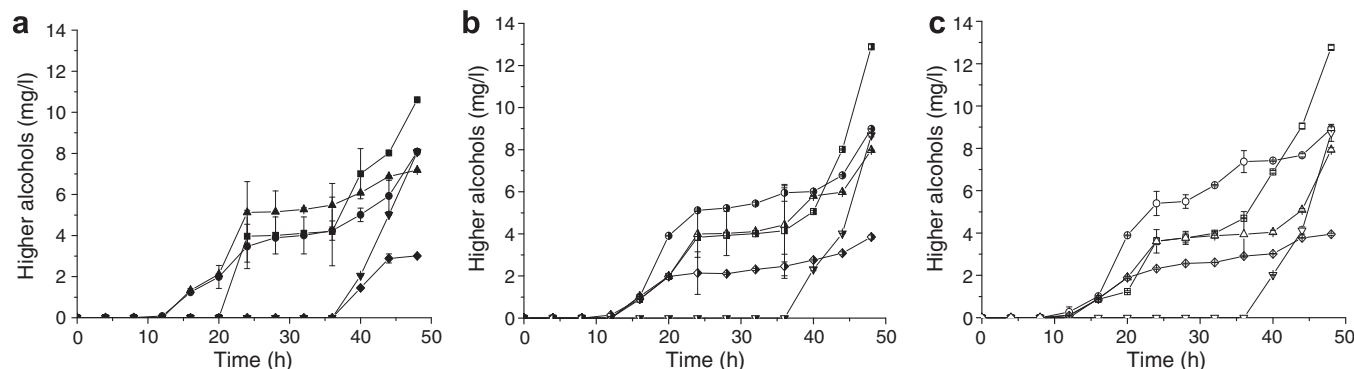
**Table 1**

Lactose consumption, ethanol production, ethanol yield factor ( $Y_{P/S}$ )<sup>a</sup>, protein utilisation and increment of kefir grains weight after 48 h of kefir grains cultivation in different fermentation media.

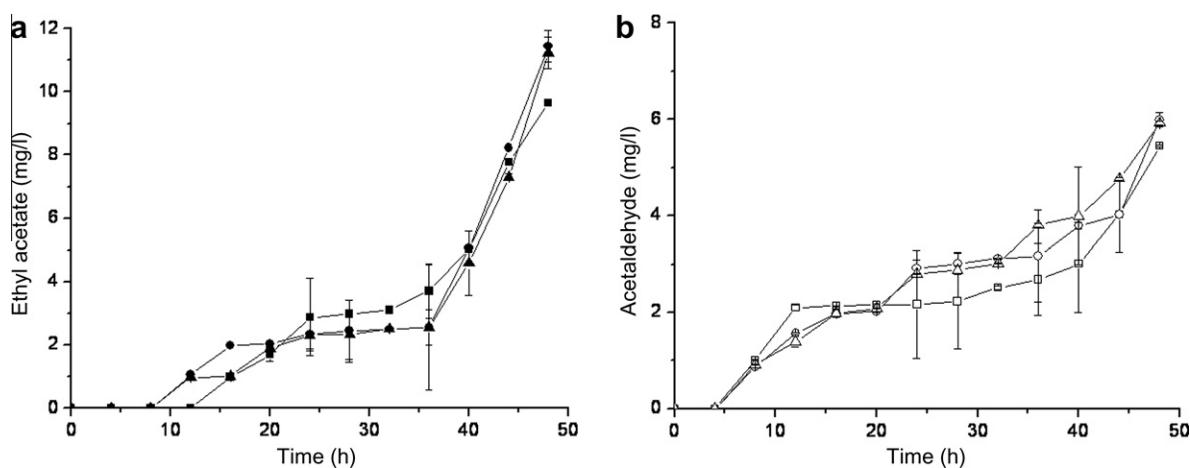
Media	Lactose consumption (g/l)	Ethanol production (g/l)	$Y_{P/S}$	Initial protein conc. (g/l)	Final protein conc. (g/l)	Initial kefir grains weight (g)	Final kefir grains weight (g)
Milk	44.8a	8.7a	0.19	34.7a	0	12.5	15.0a
Cheese whey	31.5b	8.3a	0.26	19.1b	0	12.5	14.1b
Deproteinised cheese whey	26.4c	7.8a	0.30	0	0	12.5	14.2b

Means within the same column with different letters are statistically different at 95% confidence level.

<sup>a</sup>  $Y_{P/S}$  was defined as the ratio between the ethanol concentration (g/l) and lactose consumed (g/l).



**Fig. 3.** Formation of higher alcohols – 2-methyl-1-butanol (square), 3-methyl-1-butanol (circle), 1-hexanol (down-triangle), 2-methyl-1-propanol (up-triangle), and 1-propanol (lozenge) – during kefir grains cultivation, using: (a) milk, (b) cheese whey and (c) deproteinised cheese whey as substrates. Bars represent the standard deviation.



**Fig. 4.** (a) Production of ethyl acetate (closed symbols) and (b) acetaldehyde (open symbols) during milk (square), cheese whey (circle) and deproteinised cheese whey (triangle) fermentation by kefir grains. Bars represent the standard deviation.

detection (GC-FID), and analysed during 48 h of kefir grain cultivation in different media (milk, CW and DCW).

The evolution of each group of volatile compounds during the production of milk kefir and whey-based kefir beverages are illustrated in Figs. 3 and 4.

The higher alcohols identified during milk, CW and DCW fermentations were 2-methyl-1-butanol (active amyl alcohol), 3-methyl-1-butanol (isoamyl alcohol), 1-hexanol (hexyl alcohol), 2-methyl-1-propanol (isobutyl alcohol), and 1-propanol (propyl alcohol) (Fig. 3a–c). The levels of these alcohols increased from the beginning until the end of the fermentation period, for the three different substrates.

The volatile higher alcohol identified, 2-methyl-1-butanol, attained the highest concentration at the end of CW and DCW fermentations (12.8–12.9 mg/l) and milk fermentation (10.6 mg/l). This volatile compound is produced during the catabolism of the branched chain amino acid (BCAA) isoleucine, or is synthesized *de novo* during the biosynthesis of the BCAA (Schoondermark-Stolk et al., 2006). Therefore, the higher concentration of 2-methyl-1-butanol in the whey-based beverages could be related with the higher isoleucine content in CW (0.31–0.69 mg/100 g powder; (Mavropoulou & Kosikowski, 1973) in comparison with that found in milk ( $0.14 \pm 0.08$  mg/100 g milk; (Albert, Mándoki, Csapó-Kiss, & Csapó, 2009). To our knowledge, no previous scientific results are available concerning the presence of 2-methyl-1-butanol in kefir beverages obtained from deproteinised cheese whey ( $0.12 \pm 0.01$  mg/100 g).

Despite the different evolution patterns observed for 1-hexanol and 3-methyl-1-butanol (Fig. 3), both higher alcohols achieved similar concentrations (nearly 9 mg/l) at the end of fermentation, for the different substrates. These alcohols have a positive influence on the aroma of the fermented beverage when they occur in concentrations up to 20 mg/l. On the contrary, increased concentration of these alcohols, having a volatile description of “coconut-like”, “harsh” and “pungent”, can contribute negatively to the product aroma (Dragone et al., 2009; Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007).

Within the group of higher alcohols, 1-propanol, associated with ripe fruit and alcohol aromas, showed the lowest concentration in the different fermented beverages. The final content of this compound in milk kefir (3.0 mg/l) was lower than those found in whey-based kefir beverages (3.9 mg/l). However, these values were well below the odour threshold of 306 mg/l (Peinado, Mauricio, & Moreno, 2006). Similar levels of 1-propanol were also reported in the continuous fermentation of raw cheese whey, using delignified cellulosic-supported kefir yeast at 27 °C (Kourkoutas et al., 2002).

Only one ester, characterized by fruity attributes, namely ethyl acetate, was detected during milk, CW and DCW fermentations by kefir grains. The concentration of this volatile compound increased slowly for the first 36 h, and then increased markedly until the end of fermentation (Fig. 4a). No statistically significant differences ( $p < 0.05$ ) were found in the final concentrations of ethyl acetate (9.7–11.5 mg/l) for the different fermented beverages, using milk,



CW and DCW as substrates. Kourkoutas et al. (2002), showed that kefir yeasts, immobilized on delignified cellulosic material, were capable of producing ethyl acetate from raw cheese whey in a wide range of concentrations (from traces to 95 mg/l). According to these authors, such concentrations are typical of fermented beverages.

Acetaldehyde, which imparts nutty and pungent aromas, was found in milk kefir and whey-based kefir beverages at low concentrations (6.0 mg/l) after 48 h of fermentation (Fig. 4b). These results were consistent with those reported by Ertekin and Güzel-Seydim (2010) for whole and non-fat milk kefir fermented at 25 °C during 18 ± 2 days and stored at 4 °C for 1 day. According to these authors, acetaldehyde is considered the major yogurt-like flavour in fermented milks. Acetaldehyde can be formed by group N streptococci. These microorganisms degrade lactose to galactose and glucose. According to Geroyiannaki et al. (2007) the glucose can be metabolized by the homofermentative Embden–Meyerhof–Parnas pathway to pyruvate, where 2 mol of lactate is formed per glucose molecule. Residual pyruvate, catalyzed by an  $\alpha$ -carboxylase, is then converted to diacetyl and acetaldehyde. An aldehyde dehydrogenase may also generate acetaldehyde from acetyl-CoA which is formed from pyruvate by the action of a pyruvate dehydrogenase. Nitrogen metabolism can also result in acetaldehyde formation. Threonine aldolase catalyzes the cleavage of the amino acid threonine to acetaldehyde and glycine (Zourari, Accolas & Desmazeaud, 1992).

#### 4. Conclusion

Although a lower lactose utilisation was observed during the production of the cheese-whey based beverages, in comparison with that obtained during the traditional cultivation of kefir grains in milk, no significant differences were found among samples at the end of the fermentations when considering final ethanol content, pH, lactic acid and acetic acid concentrations, as well as major volatile formation. Therefore, the results of the present study provided evidence indicating that cheese whey and deproteinised cheese whey may serve as substrates for the production of kefir-like beverages similar to milk kefir. The use of deproteinised cheese whey as a substrate in kefir fermentation processes can be considered as a new whey valorisation strategy.

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