Interactions between *Lactococcus ssp.* and Brewer’s Yeast Metabolism During the Fermentation and Storage of a Kefir-like Product: Growth Dynamic and Lactose Metabolism

Adriana PAUCEAN*, Carmen SOCACIU, Mirela JIMBOREAN, Elena MUDURA, Simona MAN

Dept. of Food Science and Technology, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine, 3-5 Mănăștur str., 400372 Cluj-Napoca, Romania,

*apaucean@yahoo.com

Abstract. Our objective was to study the growth dynamic and the lactose metabolism in a mixed culture, obtained by fermentation of two lactic acid bacteria (*Lactococcus lactis, Leuconostoc mesenteroides*) and spent brewer’s yeast in order to make use of the bioactive potential of brewer’s yeast in a kefir-type product, free of *Lactobacillus ssp.* and free of typical kefir yeasts. The fermentation was realized initially in a biofermentor than at larger scale (4l) in an upgraded installation. *Lactococcus* and *Saccharomyces* growth was synergistic and dominated the final composition of the product. We noticed a decrease of lactose content in the kefir, more intense than in control. The rate of lactose decrease during storage at low temperatures, was significantly more intense (P<0.05) than in fermentation conditions. We can assume that, the decrease of lactose content during storage was stimulated by the presence of brewer’s yeast in this kefir-like product. The decrease of L(+) lactic acid, especially during storage, was more intense than lactose, due to *Saccharomyces* metabolism, converting lactic acid to pyruvate, consumed by aerobic respiration and ethanol synthesis.

Keywords: *Lactococcus*, spent brewer’s yeast, metabolism, fermentation.

INTRODUCTION

Milk fermentation is an old process with a high metabolic complexity due to the interactions (antagonistic and/or synergistic) between microorganisms (bacteria or yeasts) difficult to manipulate in uncontrolled operation. Nowadays the use of selected starter cultures guarantee repeatable processes due the ability to grow rapidly in milk by high rate of lactose fermentation, accumulation of lactic acid with/without alcohol due to synergistic or antagonistic interrelations of microorganisms (Delaglio *et al.*, 1992). Each product type is obtained with specific microorganisms which interact by complex metabolic pathways involving the competition for the substrate and the elaboration of specific final products, including flavours, in spite of similar manufacturing technologies. The microorganisms in the starter culture determine the type of fermentation and the resulting products have individual characteristics, which derive from the metabolic activities of the starter bacteria (Tzanetaki & Tzanetaki 1999).

Fermented milk, depending on the proportion of bacteria strains and addition of yeasts can have a variety of sensorial properties and probiotic properties, dependent on the substrate bioconversions, mainly the competition for lactose and its metabolites. The most popular dairy products are produced by LAB (lactic acid bacteria) which are typical lactose fermenting microorganisms. By combining a fast- fermenting lactose bacteria with slow - fermenting lactose bacteria (e.g. *Lactococcus, Leuconostoc*) and non- lactose fermenting
yeasts, (*Debaryomyces, Saccharomyces*), we can obtain fermented milks with specific taste and flavour.

Kefir originates since centuries from the Caucasus Mountains where goat or cow’s milk was inoculated with „kefir grains” and fermented for days in goatskin leather bag. This dairy beverage has a creamy texture and refreshing sour flavor (Garrote et al., 2001). Kefir is a very popular dairy product in east Europe, traditionally made of cow milk with/without soy milk. Kefir contains several major strains of friendly bacteria not commonly found in yogurt, *Lactobacillus Caucasus*, *Leuconostoc*, *Acetobacter species*, and *Streptococcus* species. Lactococci and leuconostocs in kefir act synergistically in generating diacetyl from the milk citrate. It may also contain *Saccharomyces kefir* and *Torula kefir*, which dominate, control and eliminate destructive pathogenic yeasts in the body.

The mixed microflora in which yeasts and lactic acid bacteria coexist in a symbiotic association and are responsible for the acid-alcohol fermentation (Farnworth, 2005; Witthuhn et al., 2005a,b; Bolla et al., 2010). For the commercial production of large volumes of kefir, direct fermentation with kefir grains is impractical as this would demand a large quantity of grains (Gronnevik, H. et al., 2011). Kefir’s active yeast and bacteria provide more nutritive value than yogurt by helping digest easier the foods that you eat and by keeping the colon environment clean and healthy, preventing chronic fatigue and digestive disorders.

Nowadays, kefir is obtained by milk fermentation with starter cultures (prepared from kefir grains) which contain species of the genera *Lactobacillus, Leuconostoc, Lactococcus and Acetobacter*, a mix of lactose fermenting yeasts and non-lactose fermenting yeasts. According to Codex STAN 2423-2003 (Codex Alimentarius Commission, 2003), other microorganisms may be added to the specific starter cultures. In kefir, LAB are provided with essential amino acids and small peptides from yeasts metabolism (Roostita & Fleet, 1996; Paramithiotis et al., 2006; Gronnevik et al., 2011). The characteristic flavor of kefir products is a result of a complex interaction between the milk matrix and compounds formed during metabolic activity of the bacterial culture and the yeast culture. (Koroleva, 1988; Witthuhn et al., 2005a).

Brewer’s yeast (biomass) *Saccharomyces* is a microorganism with known biotechnological potential, used in food industry due to its functional properties and nutritional value (Steward, 1999; Ferreira et al., 2010). Many researches focused on kefir processing used *Saccharomyces* from kefir grains (Kwak et al., 1996; Tada et al., 2007; Golowczyc et al., 2009). Spent brewer’s yeast is obtained as a by-product from beer processing, a rich source of proteins and B-complex vitamins, nucleic acids, minerals and others biologically active compounds (chromium, cysteine, glycine, glutamic acid etc.). It is generally used as inexpensive animal feed after heat inactivation and conditioned as powders, flakes, tablets or in liquid form. These products can be mixed with milk or juices or used as a seasoning in soups or gravies (Ferreira et al., 2010).

The objective of the present work was to investigate the interactions of a lactose fermenting bacteria (*Lactococcus lactis*) with a non-lactose fermenting yeast (*Saccharomyces cerevisiae*) during the fermentation and storage of a “kefir-like” product. The growth dynamic and carbohydrate metabolism of *Lactococcus* vs Brewer’s yeast was evaluated by microscopy and biochemical parameters. By this study we intended to make use of the bioactive potential of brewer’s yeast in a dairy product free of typical kefir-yeast.
MATERIALS AND METHODS

Microorganisms used for fermentation
The microorganisms used to form the inoculum were represented by a bacterial starter culture and a yeast culture. We used a mesophilic bacterial culture FD-DVS CHN-22 (provided by Chr. Hansen) of Lactococcus lactis (ssp.cremoris, ssp. lactis and ssp lactis biovar diacetylactis) and Leuconostoc mesenteroides subsp. cremoris, (LC) containing $10^{10}$ colony forming units per mL (cfu/mL). This starter culture was freeze-dried powder and Direct Vat Set (DVS). The brewer’s yeast suspension (Saccharomyces cerevisiae) (BY) ($10^{10}$cfu/mL) was separated from the secondary fermentation of beer, provided by a local brewer, having a cellular viability of 96%. The cell density was determined by direct counting in the Thoma Chamber, while the viability of the brewer’s yeast was determined by differential coloration.

Preparation of the product
1. Short time fermentation under controlled parameters (biofermentor)
A Tryton- Pierre Guerin Technologies biofermentor, with a maximum capacity of 2L and a useful capacity of 1.5 L was used. The fermentation was supervised by a digital control unit (Tryton-sytem) with adjustment of the process parameters (temperature-time) in order to respect all the manufacturing stages of the dairy product. The volumetric ratio, expressed in mL, between 1.8% fat skimmed cow milk (M) and microorganisms was 2000:2:1 (M: LC: BY). This ratio was established after preliminary tests which aimed to find the best growth balance between LC and BY on selective media.

2. Preparation of the product in an upgraded installation
To obtain a larger quantity of the kefir-like product, in a second step we used an upgraded installation (4L capacity), at the Station (Pilot plant) of Dairy Products, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania. Pasteurized 1.8% fat skimmed, cow milk was cooled at 30°C and was inoculated with the same inoculum mixture, as presented before, the ratio between milk (M) and microorganisms (M: LC: BY) being 2000:2:1. After inoculation, the manufacturing operations included: incubation at 29-30°C for 12 hr, pre-cooling at 18-20°C for 1hr, cooling again at 4-6°C for 10 hr. The product was stored up to 21 days at 0-4°C. The control sample used an inoculum containing the commercial starter culture (LC) using a ratio of M: LC=4000:4, without brewer’s yeasts. Three replications of all batches and samples were performed.

Microbiological analysis
In order to monitor the density and viability of the lactic bacteria and brewer yeasts, a tryptone-water (Difco) mixture (1 g/L) was used to prepare the dilutions for the microbiological analysis. Lactococcus growth was counted in M17 agar (Biokar Diagnostics), a selective medium for lactococci (Terzaghi & Sandine, 1975), at pH 7.2 ± 0.2, after incubation under anaerobic conditions, 5% (v/v) CO$_2$, at 30 °C for 18–24 hr. Leuconostoc growth was counted in MSE agar (Biokar Diagnostics, Beauvais France), a selective medium for leuconostoc after incubation at 22°C for 4 days. Brewer’s Yeast, Saccharomyces, growth was followed at pH 5.5 ±0.3 in a selective medium WLN (Wallerstein Laboratoires Nutrient medium) after aerobic incubation at 25°C for 48hr. Three replications of all measurements were carried out using a Zeiss, Axio Observer microscope, examined at 40X magnification, for each sample.
Biochemical analysis and pH measurement

By UV spectrophotometry (Boehringer Mannheim R-Biopharm-Enzymatic BioAnalysis/Food Analysis) based on the measurement of the absorption ratios for NADH/NADPH at 340 nm, we determined the levels of lactose, glucose, D(-) and L(+) lactic acid and ethanol during fermentation. A Multi-Detection Microplate Reader SynergyTM-HT spectrophotometer manufactured by BioTek Instruments, Inc. USA was used. The data supervision, control and acquisition were performed through the Gen 5 software. The pH was measured using an electronic pH-meter (Hanna Instruments Inc.). At all sampling time, the measurements were made in triplicate.

Statistical analysis

In order to study significant differences between the different sampling points during the fermentation process and the shelf-life, a variance analysis (ANOVA, using the software Graph Pad Prism 5.00) was performed, with a confidence interval of 95% (P<0.05). The results were analyzed statistically through the two-way ANOVA- Bonferroni posttests.

RESULTS AND DISCUSSION

Growth dynamic

Figure 1 shows the growth dynamic of lactic bacteria and brewer’s yeasts by determination of their cellular density, during short-time fermentation (24h in biofermentor) and at upgraded scale, during fermentation and storage at 0-4°C (1-21 days) of kefir batches.

Fig. 1.A. The microbial density (log cfu/ml) of Lactococcus and Saccharomyces in their selective media (M17 and WLN, respectively) at different times of fermentation in biofermentor. All values represent the average of three batches. For details see Materials and methods.

In biofermentor (Fig.1.A), after 12 hours, in the selective M17 medium, the number of Lactococcus bacteria decreased from $10^{10}$ to $10^8$ cfu/ml and after 24 hours, to $10^6$ cfu/ml. Concomitantly, Saccharomyces cerevisiae yeasts count decreased to $10^7$ cfu/ml after 12 hours and $10^5$ cfu/ml after 24 hours.

In the upgraded installation (Fig.1B), the density of Lactococcus bacteria decreased to $10^6$ cfu/mL in 24 hrs and to a value of $10^5$ cfu/mL the first seven days of storage and kept constant up to 21 days, the expiry date. Comparatively, the density of brewer yeasts remained
$10^4$ cfu/mL for the first seven days and increased to $10^5$ cfu/mL up to day 14, then remained constant until the expiry date.

During storage, both *Lactococcus* and brewer yeast kept a constant density in the range $10^4 - 10^5$ cfu/mL.

![Figure 1.B. The microbial density (log cfu/ml) of *Lactococcus* and *Saccharomyces* in their selective media (M17 and WLN, respectively), at different times of fermentation and storage in the upgraded installation. After 24 hours the fermented milk was stored at 0-4°C (24-504 hrs). All values represent the average of three batches. For details see Materials and methods.](image1)

Fig. 2 shows, comparatively, the evolution of the *Lactococcus* bacteria cell density after fermentation with or without brewer’s yeasts, in the upgraded installation, followed by storage during the shelf-life (1 to 21 days at 0-4°C). Brewer yeast determined a slight decrease of density, but without significant differences (ns, p<0.05).

![Figure 2. Changes of *Lactococcus* cell density in the fermented milk containing brewer yeast vs control (without brewer yeast) during the storage period at 0-4°C (24-504 hrs). All values represent the average of three batches. For details see Materials and methods.](image2)

It was observed, according to Fig. 1 and 2 that the survival or growth of *Saccharomyces* yeast during fermentation and storage was not inhibited by the presence of lactobacilli. Their density dynamic was similar.
**Metabolic interactions**

To evaluate quantitatively their metabolic interactions we determined some biomarkers of fermentation, e.g. lactic acid, lactose, glucose and ethanol.

Table 1 includes the mean values of glucose, lactose, ethanol and lactic acid isomers (D and L) (g/100g) and their changes during short-time fermentation (24h, in biofermentor) and in the upgraded installation, for storage period at 0-4°C (1-21 days).

Changes of lactose, \( L(+) \) lactic acid and ethanol concentrations (g/100g) during short-time fermentation (24h, in the biofermentor) and in the upgraded installation, followed by a storage period at 0-4°C (1-21 days) of BY kefir samples vs control. Glucose concentrations (after 24 hrs) and D(−) lactic acid were non-detected.

<table>
<thead>
<tr>
<th>Fermentation and storage timing</th>
<th>( L(+) ) lactic acid</th>
<th>Lactose</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-time fermentation of BY samples in Biofermentor</td>
<td>n.a</td>
<td>4.72±0.38</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fresh Milk</td>
<td>0.318±0.04</td>
<td>4.36±0.22</td>
<td>0.005±0.002</td>
</tr>
<tr>
<td>12hrs</td>
<td>0.588±0.05</td>
<td>3.9±0.16</td>
<td>0.01±0.001</td>
</tr>
<tr>
<td>Samples containing BY manufactured in an upgraded installation during shelf-life</td>
<td>n.a</td>
<td>4.72±0.38</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fresh Milk</td>
<td>0.6±0.02</td>
<td>3.75±0.18</td>
<td>0.008±0.004</td>
</tr>
<tr>
<td>24 hrs</td>
<td>0.576±0.032</td>
<td>2.15±0.14</td>
<td>0.018±0.006</td>
</tr>
<tr>
<td>7 days</td>
<td>0.54±0.01</td>
<td>1.9±0.09</td>
<td>0.025±0.005</td>
</tr>
<tr>
<td>14 days</td>
<td>0.458±0.01</td>
<td>1.4±0.1</td>
<td>0.032±0.002</td>
</tr>
<tr>
<td>21 days</td>
<td>0.592±0.01</td>
<td>4.014±0.16</td>
<td>0.007±0.001</td>
</tr>
<tr>
<td>Control samples (without BY) fermented and stored in an upgraded installation</td>
<td>n.a</td>
<td>4.014±0.16</td>
<td>0.007±0.001</td>
</tr>
<tr>
<td>24 hrs</td>
<td>0.58±0.015</td>
<td>3.68±0.09</td>
<td>0.018±0.0015</td>
</tr>
<tr>
<td>7 days</td>
<td>0.572±0.02</td>
<td>3.20±0.1</td>
<td>0.028±0.006</td>
</tr>
<tr>
<td>14 days</td>
<td>0.535±0.012</td>
<td>2.91±0.158</td>
<td>0.029±0.003</td>
</tr>
</tbody>
</table>

The lactose content decreased significantly (p<0.05) during the first 24 h of fermentation and during the shelf-life. As seen in Table 1, lactose initially present in the raw milk was consumed during the fermentation process by the lactic bacteria as follows: after 24 hours of fermentation in the biofermenter, lactose concentration decrease 17.4% comparing to its initial concentration in milk, while in the upgraded installation, decreased 20.55% of the initial lactose amount after 24 hours and 70% after storage. In the same conditions, in the control (non-BY kefir), lactose decreased 8% and 18%, respectively.

We found, in the biofermentor, a maximum L (+) lactic acid concentration after 12 hours of fermentation, namely 0.60% which decreased to 0.458% during storage in the BY-containing kefir. During the first seven days of shelf life, the L (+) lactic acid content was constant, but decreased significantly from 14 to 21 days. In the control sample, although the L(+) lactic acid formed after 24 hours was close to the BY containing kefir (0.592%), the concentration during storage was almost constant (up to 0.535%). In conclusion, the decrease of lactic acid in BY-kefir during storage was 24% (vs 10% in control).

We noticed that Lactococcus and BY growth (densities of 10^4 to 10^5 CFU/ml) were synergistic and dominated the final composition of the product. Their density dynamic during fermentation and storage was similar to other data. For example, for yeasts, the fermented Milk Codex (Codex Alimentarius Commission, 2003) mentions 10^4 cfu/g as minimum yeast density.
density for kefir. Other authors reported values range from $10^3$ to $10^6$ (Duitschaever et al., 1988; Farnworth, 2005; Guzel-Seydim et al., 2005, Kuo & Lin, 1999, Simova et al., 2002). Meanwhile, the growth of *Leuconostoc* on selective medium was found to be significantly inferior, due to *Lactococcus* and BY domination in the fermented sample (data not shown).

The lactose content of the end product was lower than the typical values for kefir, comparing to some literature data (Irigoyen et al., 2005; Garcia Fontan et al., 2006). Finally the decrease of lactose concentration from 4.72% to 1.4% in BY-containing samples, was more intense than in control fermented sample (without brewer yeast), where lactose reached finally mean values of 2.91%. The rate of lactose decrease during storage period at lower temperatures was significantly more intense (p<0.05) than in fermentation conditions.

The change of lactose content is related to low, non-detectable glucose concentrations in BY containing kefir. Glucose, formed by β-galactosidase hydrolysis of lactose (due to lactic bacteria) was immediately metabolized by the other microbial species of the mix, and mostly by the brewer yeasts. Such low glucose concentrations were reported by other authors when *Lactococcus lactis* and *Leuconostoc mesenteroides* was fermenting milk (Beshkova et al., 2003).

We can assume that, the decrease of lactose content during the product’s shelf life and the total consumption of glucose was stimulated by the presence of BY in this kefir-like fermented milk. Similar values were displayed by the fermentation process in the biofermentor and in the upgraded installation.

Regarding lactic acid production, only L(+) lactic acid was detected, but not D(-) lactic acid, which is synthesized specifically by *Leuconostoc mesenteroides*. The absence of the D(-) lactic acid is related to the low concentration of *Leuconostoc* after fermentation, comparatively to *Lactococcus*, as mentioned also by other authors (Baron et al., 2000; Garcia Fontan et al., 2006).

Comparing with the decrease of lactose, we can assume that lactic acid decreased more intense than lactose, due mainly to BY metabolism, converting lactic acid to pyruvate which is metabolized by aerobic respiration and ethanol synthesis. The lactic acid decrease was negatively correlated with the pH: after 24 hrs of fermentation, the pH value of 4.31 was correlated with the highest lactic acid formation, then, during storage, the pH increased, while the lactic acid content decrease. This variation is likely to be also caused by lactic acid consumption by BY, as noticed by other authors (Viljoen & Heard, 1999).

The ethanol content is low after 24 hrs fermentation (0.01%) but increase significantly (4 times; P<0.05) during storage, having similar concentrations for control and BY-kefir, and falls within the normal values for kefir products. These values are higher than those reported by Beshkova et al. (2003) for *Lactococcus lactis C1*, single-strain culture, but close to the values reported by Garcia Fontan et al. (2006) for kefir manufactured using commercial starter culture. Considering that no differences were noticed for BY-kefir and control, we assume that for ethanol synthesis, microorganisms from lactic starter culture were the main contributors and not BY.

The sensorial analysis of the new BY-kefir, free of *Lactobacillus ssp.*, containing *Lactococcus ssp.* and *Leuconostoc* showed its good tasty quality (Paucean, unpublished data) and promote its functionality as probiotic. The combination of two lactose fermenting bacteria and a non-fermenting lactose yeast resulted in a kefir-type fermented milk with special taste and aroma. Also, the low lactose, low lactic acidity and pH, combined with a pleasant aroma are convenient for consumers, considering as well the good balance of yeasts and lactic bacteria with probiotic potential against pathogenic microbiota.
Further studies will be focused on the analysis of aroma volatile compounds released during cow milk fermentation by the synergic metabolic activity of these microorganisms.

CONCLUSION

The growth dynamic and carbohydrate metabolism of Lactococcus vs Brewer’s yeast (Saccharomyces) was evaluated by microscopy and biochemical parameters. To evaluate quantitatively their metabolic interactions we determined some biomarkers of fermentation, e.g. lactic acid (L, D), lactose, glucose and ethanol. Lactococcus and Saccharomyces growth were synergistic. The survival of Saccharomyces yeast during fermentation and storage was not inhibited by the presence of lactic bacteria. The rate of lactose consumption was high during the fermentation and increased significantly during the storage period at refrigerated conditions. Glucose concentrations were not detected. Only L(+) lactic acid was detected. A marked decrease of L(+) lactic acid during storage period was founded due to brewer’s yeast metabolism. The ethanol synthesis during fermentation and storage was low, mainly due to lactic starter.

ACKNOWLEDGMENT

The authors thank The University of Agricultural Sciences and Veterinary medicine Cluj-Napoca for the financial support of this study and the company Chr. Hansen for providing the microorganisms used in experiments.

REFERENCES


