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Interaction between *Galactomyces geotrichum* KL20B, *Lactobacillus plantarum* LAT3 and *Enterococcus faecalis* KE06 during Milk Fermentation

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Abstract: Microbial interactions are fundamental during milk fermentation, determining the product final characteristics. *Galactomyces geotrichum*, *Lactobacillus plantarum* and *Enterococcus faecalis* are among the most common microorganisms in the Colombian Kumis. The aim of the research was to evaluate the yeast–bacteria interactions in milk fermentation at 28 °C. UHT (Ultra-High Temperature) milk was inoculated with single- or multiple-strains associations and analysed periodically to determine the microbial counts, organic acids and total free amino acids (FAA). The results evidenced different growth performance of the strains in single or co-culture, with a positive effect of *G. geotrichum* KL20B on the lactic acid bacteria (LAB) growth performance. All the strains consumed citric acid after 6 h of incubation with *E. faecalis* KE06 as the major consumer; however, all the co-cultures showed an early metabolism of citrate but with a low intake rate. In addition, the interaction between *G. geotrichum* KL20B and *E. faecalis* KE06 led to a low accumulation of acetic acid. Formic acid fluctuated during fermentation. The strains interaction also led to an increase in ethanol content and a lower accumulation of FAA. In conclusion, the three strains co-culture enhances the LAB viability, with high production of lactic acid and ethanol, as a consequence of adaptation to the environment and substrate exploitation. To our knowledge, this is the first time in which it is showed that *G. geotrichum* KL20B could be used to compensate for the slow acid-producing ability of *Lb. plantarum* and *E. faecalis* in milk, underlining that this consortium applies some mechanisms to regulate the growth and milk composition in acids and ethanol content.

Keywords: *Galactomyces geotrichum*; *Lactobacillus plantarum*; Interaction lactic acid bacteria (LAB) and yeast; organic acids; milk fermentation

1. Introduction

During milk fermentation, the composition and frequency of species within the microbiota, as well as the amount of final bio-products are influenced by the environmental conditions encountered by microbial cells, the available energy sources and also the growth-stimulating or -inhibiting factors. Thus, during milk fermentation, microbial interactions play an important modulatory role in this particular ecosystem. On the other hand, the impact of the process variables and their changes during continuous propagation enables the microbial association to endure even for years [1].

In fermented milks such as Kefir, Koumis, Kumis, Viili, Longfil, Laban, Amasi and Kurut, the co-occurrence of yeasts and lactic acid bacteria confers particular treats to the products. Yeasts are believed to be essential in the production of these fermented milks, since their presence is usually in a high number. In Kumis, a traditional low alcoholic fermented cow milk produced in rural and urban

areas of southwest Colombia, yeast counts reach values from 6.2 ± 0.17 to 8.6 ± 0.21 log CFU/mL [2]. This population is composed by strains belonging to 14 species shared among 11 genera, indicating that the selective pressures of tropical environments may favor yeasts biodiversity [3]. In particular, *Galactomyces geotrichum* was the most frequent and most abundant isolated species, representing 22.58% of the total yeasts [1]. Although in lower percentages, *G. geotrichum* has been isolated also from other products obtained by spontaneous fermentation of milk, as the African Nunu, realized with cow milk and the Kazakhstani Shubat, made of camel milk [4,5]. In previous studies [2–6], many lactic acid bacteria and enterococci have been detected in traditional Kumis, by culture-dependent method. In particular, *Lactobacillus plantarum* and *Enterococcus faecalis* were the most frequent species found among the samples collected from different geographic regions of Colombia.

In a previous study, we explored the effect of several strains isolated from Colombian Kumis inoculated alone and in co-culture of three different strains, to produce milk with high content of ACE inhibitory (ACEI) peptides and free from bitter taste [7]. The complex nature of these interactions has been emphasized by the fact that the co-cultures of *G. geotrichum* KL20B, *E. faecalis* KE06 and *Lb. plantarum* LAT3 together, showed high production of peptides with high ACEI activity.

Considering that acid production is an important phenomenon associated with fermented milks manufacture, in this work we evaluated the effect of the interaction of the same strains on the production of organic acids, ethanol and amino acids during the fermentation of milk at 28 °C for 48 h, in order to better understand the real contribution of each strain on growth dynamics and metabolites production. In fact, as the interaction between yeasts and bacteria involves mechanisms of stimulation or inhibition and the specific mode of interaction is dependent on both microorganisms [8,9], the purpose of this work was to obtain more information concerning yeast–bacteria interactions in milk.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

In this study, microorganisms previously isolated from Colombian Kumis were used [7]. They were formerly selected for their capability to produce Kumis with high ACE inhibitory activity and with a pleasant taste. In detail, the strains were: *Lactobacillus plantarum* LAT3, *Enterococcus faecalis* KE06 and *Galactomyces geotrichum* KL20B. *Enterococcus faecalis* KE06 has been selected as free from virulence factors [2].

The strains belonged to the Faculty of Bioscience and Technology for Food, Agriculture and Environment of the University of Teramo (Italy) and were stored at −80 °C in specific culture media added with 25% of glycerol (Sigma-Aldrich, Milan, Italy). The strains were activated by cultivating the yeast on YPD (1% yeast extract, 2% peptone, 2% dextrose) added with chloramphenicol (0.1 g/L) for 48 h at 30 °C, the lactic acid bacterium on de Man Rogosa Sharp agar (MRS, Oxoid Thermofisher, Milan, Italy) for 48 h at 30 °C, and the enterococcus on M17 (Oxoid Thermofisher, Milan, Italy) and Kanamycin Aesculin Azide agar (KAA, Oxoid Thermofisher, Milan, Italy) for 48 h at 37 °C. After a first step in liquid media, two more transfers were performed on plates of the same culture media and in the same conditions.

2.2. Milk Preparation and Fermentation Conditions

Whole UHT (Ultra-High Temperature) milk was used. Microbial inocula were prepared from isolated colonies grown overnight on the appropriate culture media, as reported in Section 2.1. A single colony was transferred in 1 mL of appropriate broth and incubated at 28 °C for 24 (bacteria) or 48 h (yeasts). Afterwards, the inocula were aseptically centrifuged at 5000 rpm for 10 min in a refrigerated centrifuge, then the pellet was suspended in 1 mL of sterile saline and washed three times, then suspended again in sterile saline, and finally the inocula were standardised at O.D.600 values of 0.900. Hence, 100 µL of these suspensions were inoculated in 100 mL of UHT milk and incubated at 28 °C for 24 h. The milk was fermented at 28 °C for 18 h for the bacteria and 48 h for the yeast. Each culture

sample was used as pre-inoculum (1% *v*/*v*) for 400 mL of milk. Un-inoculated milk was used as control. Samples were incubated at 28 °C for 48 h.

Different single and co-cultures were inoculated, with the aim of verifying the impact on the fermented milk characteristics. In detail: (a) *E. faecalis* KE06; (b) *Lb. plantarum* LAT3; (c) *G. geotrichum* KL20B; (d) *Lb. plantarum* LAT3 + *E. faecalis* KE06; (e) *E. faecalis* KE06 + *G. geotrichum* KL20B; (f) *Lb. plantarum* LAT3 + *G. geotrichum* KL20B; (g) *Lb. plantarum* LAT3 + *E. faecalis* KE06 + *G. geotrichum* KL20B.

2.3. Microorganisms Enumeration

Before fermentation (time 0) and after 6, 24 and 48 h of fermentation, the number of microorganisms in milk was determined by means of viable counts on the suitable culture media and incubation conditions reported in Section 2.1. In detail, aliquots of 10 mL of each fermented milk were serially diluted with sterile saline solution (0.85% NaCl) and plates of the specific media were inoculated with 100 µL of the opportune dilutions. The plates were incubated at 28 °C respectively for 24 h and 48 h for the bacteria and the yeast.

2.4. pH Measurement

The pH of all the fermented milk samples was measured by means of Crison-micro pH 2001-Instruments Lab Control pH-meter (Hach Lange Spain, S.L.U., L'Hospitalet de Llobregat, Barcelona, Spain) after 0, 6, 24 and 48 h of incubation. The measurement was performed on 5 mL of milk at each sampling time. Also, the pH of un-inoculated milk in the same incubation conditions was registered at each sampling time.

2.5. Determination of Organic Acids Content

At each sampling time (0, 6, 24, and 48 h), fermented milk samples were centrifuged at 10,000 rpm for 10 min at 4 °C, afterwards the supernatant was diluted 1:10 with 0.08 M H_2SO_4 (Sigma-Aldrich) and filtered with HPLC filters. Aliquots of 20 µL were injected directly in HPLC (Perkin-Elmer, Milan, Italy), using an Aminex HP87 H Ion Exclusion, ionic exchange column (Bio-Rad, Hercules, CA, USA), 300 nm × 7.8 mm. The column was warmed at 35 °C, then the following working conditions were applied: flux 0.4 mL/min, mobile phase H_2SO_4 0.08 M, run length 40 min. Acid concentration was determined by means of calibration curves performed with standards of citric, lactic, formic and acetic acids.

2.6. Determination of Ethanol Content

Ethanol content was determined using the kit EnzyPlus® Ethanol (BioControl Systems Inc., Bellewue, WA, USA) according to the manufacturer's instruction. The analyses were performed at each sampling time (0, 6, 24 and 48 h).

2.7. Determination of Amino Acids Content

Method of Cd-Ninhydrin, proposed by Folkertsma and Fox [10] was applied to determine amino acids content. Ninhydrin (0.8 g) was dissolved in 80 mL of absolute ethanol, then 10 mL of glacial acetic acid and 1 g of $CdCl_2$ in 1 mL of distilled water were added. The reactive was stored at 4 °C in the dark. A calibration curve was prepared with Leucine 0.4 mM. All reagents were from Sigma-Aldrich. Fermented milk samples were centrifuged at 13,000 rpm, then the volume was adjusted to 500 µL with distilled water, and 1 mL of ninhydrin reactive was added. The sample was then incubated at 84 °C for 5 min, immediately cooled in ice and absorbance at 507 nm was determined. The determination was performed at each sampling time (0, 6, 24 and 48 h).

2.8. Statistical Analysis

All the experiments were carried out in triplicate, and the analyses in duplicate. Data from the growth dynamics, production of organic acids, ethanol and amino acids content during time were analyzed by Analysis of Variance (ANOVA), and the Tukey post hoc test at $p < 0.05$ was used for means comparison. For data processing, the Statistics software for Windows version 5.1. (STATISTICA, 1998, Statsoft, Tulsa, OK, USA) was used.

3. Results

3.1. Growth Performance of the Co-Cultures

It is well known that the outcome of fermented milks depends not only on the growth of microbiota but also on its metabolic activity. Thus, the interactions among lactic acid bacteria and yeast during milk fermentation have a direct effect on the quality of the product [7,11–14].

The fermentation dynamics of different milk samples were studied in this work and are reported in Figure 1.

The behavior of *Lb. plantarum* LAT3 and *E. faecalis* KE06 in single-strain cultures was different, with *Lb. plantarum* LAT3 showing a ΔCFU/mL at 48 h of 2.09 ± 0.17, while the value for *E. faecalis* KE06 was 1.02 ± 0.14 CFU/mL. The scarce growth rate observed for *E. faecalis* KE06 might be due to a lower lactose consumption during the exponential growth phase compared to that of *Lb. plantarum* LAT3. As regards *G. geotrichum* KL20B, it showed an average growth of ΔCFU/mL 0.32 ± 0.14; this very poor performance could depend on the fact that it is a not-fermenting lactose species. Very few are the lactose fermenting yeasts, with *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Torula kefir* as the most important species involved in milk fermentation.

Microbial interactions in mixed cultures occur via multiple mechanisms and the effects of such interactions on the fitness of the strains involved may either be positive, neutral or negative. When bacteria were grown in co-culture with *G. geotrichum* KL20B, significant differences ($p < 0.05$) in cell counts were observed among the single-strain and co-culture assays. In fact, an increase ($p < 0.05$) of the bacterial cell counts was detected already after 12 h (Figure 1a,b). As evidenced, the mean of cells increase of the co-cultures with respect to the single culture was 1.34 ± 0.21 log CFU/mL for *E. faecalis* KE06 and 0.90 ± 0.15 log CFU/mL for *Lb. plantarum* LAT3, at the end of the fermentation process. It is generally believed that the yeasts excrete nutrients of which the LAB benefit, such as pyruvate, amino acids and vitamins [13–15]. For example, the trophic interaction between *Lactobacillus hordei* and yeasts such as *Saccharomyces cerevisiae* and *Zygotorulaspora florentina*, is constituted by the release of amino acids and vitamin B6 from the yeasts, whereas *Lactobacillus nagelii* growth is supported by their production of amino acids, in particular arginine, which is essential for *Lb. nagelii* [16]. These results could in part explain our data. However, the reduction of yeast counts in presence of *Lb. plantarum* (0.75 ± 0.10 log CFU/mL) and of *E. faecalis* KE06 (0.24 ± 0.15 log CFU/mL), with respect to the single-strain culture, suggested that other mechanisms, such as autolysis, could be probably involved during the first 24 h (Figure 1c). A previous study on mixed cultures of kefir microorganisms reported that the production of CO_2, pyruvate, propionate, acetate and succinate by *Saccharomyces florentinus* supported the survival and lactic acid production of *Lactobacillus hilgardii*, but also in this case the growth of *Saccharomyces florentinus* was drastically reduced [17]. Also in grape must, the simultaneous presence of lactic acid bacteria and yeasts led to an accelerated yeasts lysis promoted by bacterial enzymes, with the consequent release of vitamins, amino acids and peptides, that exerted a growth stimulation of the lactic acid bacteria [18].

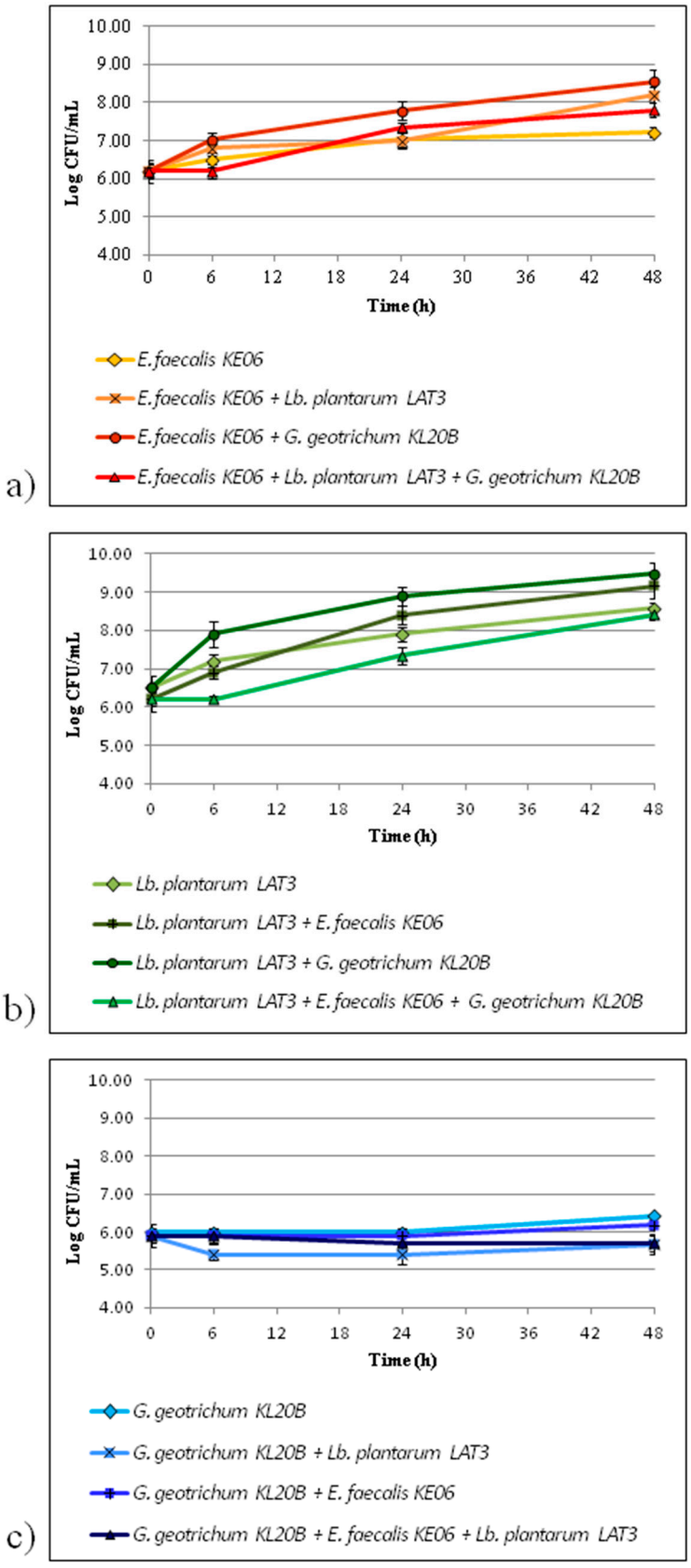

Figure 1. Growth dynamics of (**a**) *E. faecalis* KE06; (**b**) *Lb. plantarum* LAT3; (**c**) *G. geotrichum* KL20B during milk fermentation at 28 °C for 48 h.

A question can be raised regarding the viability reduction of *G. geotrichum* KL20B during milk fermentations. In fact, its decrease in all the co-cultures could be probably due to the fact that its exposure to the acids could have caused a significant loss of cell viability and vitality. *Lb. plantarum* produces antimicrobial compounds including organic acids such as phenyllactic acid that exerts inhibitory activity against contaminating yeasts [19]. Also Durlu-Özkaya et al. [20] showed that *Lb. casei*, to some extent, was effective in the delay of *Rhodotorula mucilaginosa* growth. Álvarez-Martín et al. [12] reported strong inhibition of *Candida famata* 1AD5, in presence of *Lactococcus lactis*, *Leuconostoc citreum* and *Lb. paracasei*. In this context, Stadie et al. [16] suggested that the co-culture of LAB and yeast partially affects autolysis in yeasts or triggers other mechanisms of (selective) nutrient release. In fact, the induction of autolysis of yeast cells plays an important role on the one hand for nutrient exchange, while on the other hand for species regulation in the consortium, preventing organisms overgrow. In addition, although the antifungal activity of LAB is based on several mechanisms such as synergistic activity of organic acids and pH or pH-dependent antifungal proteins [21], it is likely that during the acid-adaptation phase, *G. geotrichum* begins to grow again asynchronously, as a consequence of the normal heterogeneity within the high initial cell density culture [22]. Further analyses on the level of the acid stressed *G. geotrichum* cells are needed to confirm this hypothesis. In any case, it is important to underline that changes in the co-culture metabolism could have determined the success of the yeast survival in milk, which is the result of the energy-efficient growth and of the aptitude to react to changing environment.

In the co-culture containing *Lb. plantarum* LAT3 + *E. faecalis* KE06, the interaction was beneficial for *Lb. plantarum* which increase was of about 1 log with respect to the single-strain culture. In this interaction, the growth of *E. faecalis* KE06 was characterized by two exponential phases, from 0 to 6 and from 24 to 48 h. This diauxic growth probably could be due to a catabolic repression of citrate metabolism. In this regard, Rea and Cogan [23] observed that citrate metabolism occurred for *E. faecalis* E-239, only after the total consumption of glucose, while glucose concentrations higher than 25 mM inhibited citrate metabolism for several hours after glucose had been consumed.

In the co-culture with three microorganisms, a significant reduction of *Lb. plantarum* LAT3 and *E. faecalis* KE06 was observed after 6 h of incubation with respect to the single-strain growth, while binary co-cultures suggested an evident stimulation of the metabolism of lactose, with lactic acid production and consequent pH reduction.

3.2. Organic Acid Accumulation

It is well known that the organic acid profile of the fermented milks is the result of both production and consumption activities, and this balance contributes to give a particular sensorial profile to the product. In general, during fermentation of the different cultures here studied, the concentration of some organic acids such as lactic and acetic was subjected to an increase, while the concentration of citric acids decreased (Figure 2).

All the strains showed the capability to reduce the milk pH at 28 °C after 48 h from the inoculum. In particular, *Lb. plantarum* LAT3 reduced the pH values to 5.5 ± 0.09, while in the case of *E. faecalis* KE06 culture, the pH value was 4.4 ± 0.12. Surprisingly the *G. geotrichum* strain used in this study reduced the values to 4.90 ± 0.20. In line with our results, Álvarez-Martín et al. [12] reported similar values of pH for *Geotrichum candidum*, anamorph form of the old complex *Galactomyces geotrichum*/*Geotrichum candidum* [24]. In co-cultures, the final pH ranged between 4.3 and 4.7; evidently the interactions compensated the slow acid-producing ability of *Lb. plantarum* LAT3, especially in the early exponential growth phase.

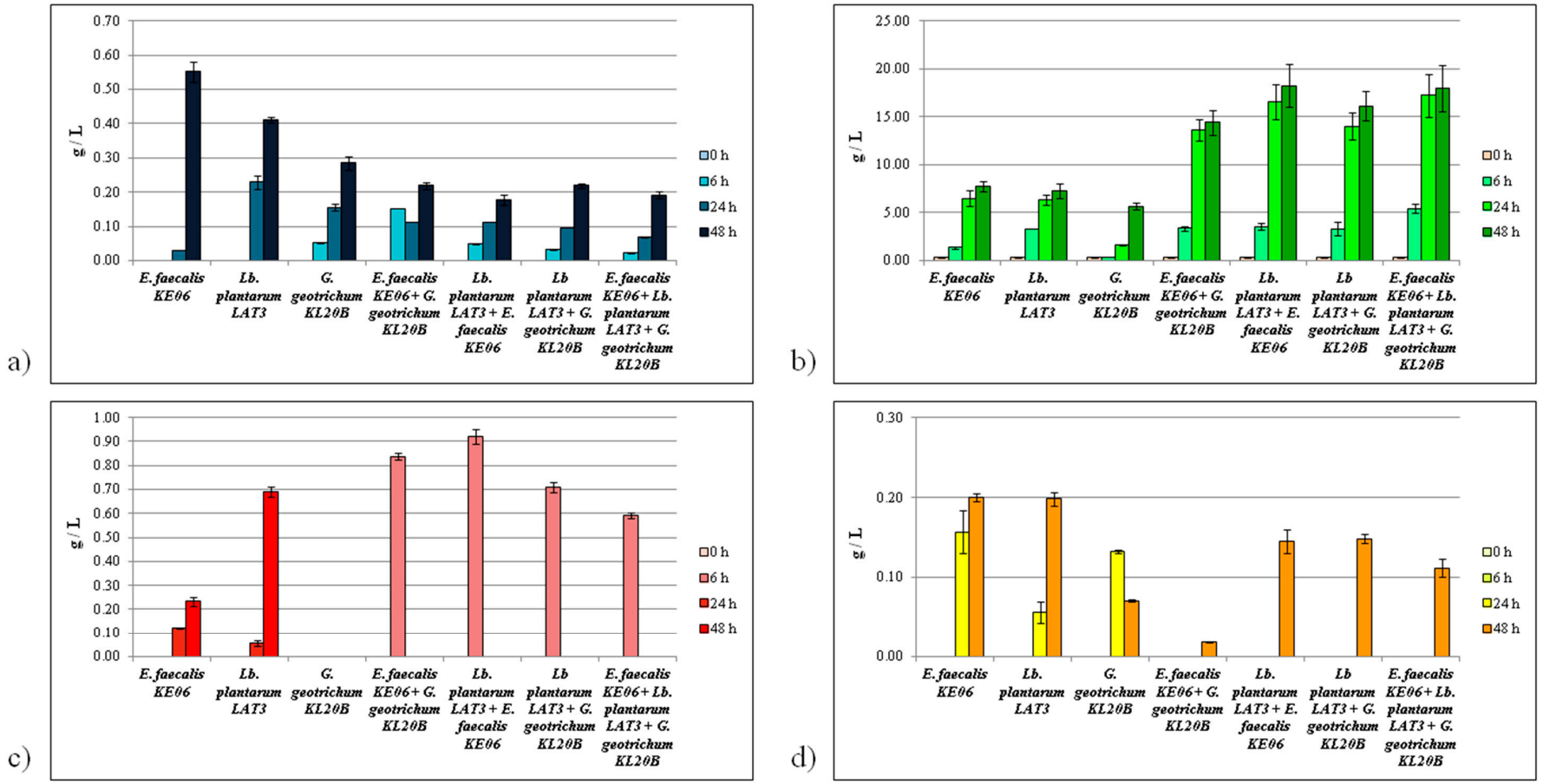

Figure 2. Dynamics of organic acids accumulation during milk fermentation at 28 °C for 48 h: (**a**) Citric acid consumption; (**b**) Lactic acid production; (**c**) Formic acid production; (**d**) Acetic acid production.

3.2.1. Citric Acid Consumption

It is well known that bacterial metabolism of milk citrate contributes to the sensory properties of fermented dairy products. The values presented in Figure 2a were calculated subtracting the amounts of citric acids present in un-inoculated milk samples. In general, in all the single-strain culture and co-cultures samples, together with lactic acid production, a reduction in citric acid content was observed.

In our work, statistically significant differences were revealed between single-strain cultures and co-cultures, either in citric acid consumption and in the microbial growth phase in which it was consumed (Figure 2a). In particular, while in milk fermented with *G. geotrichum* KL20B, in which citrate metabolism was evident during early exponential phase (during the first 6 h of fermentation), for the samples inoculated with bacteria strains, the consumption was clear after this period. Few lactic acid bacterial species, such as *Lactococcus lactis* subsp. *lactis* biovar diacetylactis, *Leuconostoc mesenteroides* subsp. *cremoris,* and *Lactobacillus plantarum*, have the capacity to use citrate as co-substrate [25]. As evidenced in Figure 2a, the single-strain cultures of *Lb. plantarum* LAT3 and *E. faecalis* KE06 apparently co-metabolized lactose and citrate around 24 h of incubation. The major consumption was revealed for *E. faecalis* KE06 culture, in which citrate was metabolized with the production of acetate. The co-metabolism of lactose and citrate has been previously reported for *E. faecalis* FAIR-E-229 [26], *E. faecium* ET C9 and *E. durans* Ov 421 [27].

In contrast, all the co-cultures showed an early metabolism of citrate, indicating a possible co-metabolism with lactose or galactose, but with a low intake rate. On the other hand, the citric acid consumption observed for bacterial co-cultures suggests that the contribute of *G. geotrichum* to the citrate consumption in three strains co-culture was reduced. This could be due to the reduction of yeast cell count. Moreover, most yeasts rely on the growth of LAB to support their growth, based on the breakdown of lactose [28] and on the capability to metabolize lactic acid [29,30]. However, as evidenced by the single-strain culture, the survival of *G. geotrichum* KL20B in co-culture could be in part due to the consumption of citric acid. In an interesting work, Álvarez-Martín et al. [12], observed that citric acid utilization was partially inhibited in the co-cultures of *Lb. paracasei* with *G. candidum* and *Pichia fermentans*.

3.2.2. Lactic Acid Production

The kinetics of lactic acid production is shown in Figure 2b, where the relationship between the lactic acid mass accumulation and fermentation time are represented. In single-strain cultures, the production of lactic acid is clear after 6 h of incubation with *Lb. plantarum* LAT3 as the major producer strain. At the same time, *E. faecalis* KE06 produced this acid at slower rates than *Lb. plantarum* LAT3. However, after six hours of fermentation, *E. faecalis* showed a major lactic acid rate production. As regards *G. geotrichum* KL20B, a not-lactose fermenting species, the analysis revealed a production of lactic acid of 5.68 $\pm$ 0.38 g/L at 48 h of incubation. In line to our results, Álvarez-Martín et al. [12] reported in *Geotrichum candidum* high quantities of lactic acid.

An increase in lactic acid production was observed in all the binary co-cultures and in the co-culture with three strains. The positive interaction between *E. faecalis* KE06 and *Lb. plantarum* LAT3 that led to an increase in the cell counts (Figure 1a,b) was also evidenced in the lactic acid production, particularly after 24 h of incubation. Also, Starke et al. [31] previously reported a positive interaction between *E. faecalis* and *Lactobacillus* species, suggesting that bacterial cross-feeding mechanisms may explain this response. On the other hand, the presence of *G. geotrichum* KL20B led to an increase in lactic acid that resulted to be from 2-fold more than that revealed in the single-strain cultures. This value could be justified by the increase of cell counts, approximately of 0.6 and 1.3 log CFU/mL for *Lb. plantarum* LAT3 and *E. faecalis* KE06, respectively. It is well known that the efficiency of lactic acid production will be affected in some extent by the enzymatic properties of the different lactate dehydrogenase (LDH) enzymes of the bacteria. Nevertheless, in the co-culture *G. geotrichum* + *E. faecalis* could have probably occurred: (i) glucose catabolism at

higher rate, producing higher lactic acid concentrations than in single-strain culture; or (ii) additional lactic acid production from the pyruvate formed by the pathway of citrate, via citrate lyase and oxaloacetate decarboxylase enzymes. This latter hypothesis could be true also for the co-cultures in which *G. geotrichum* + *E. faecalis* + *Lb. plantarum* were present; in fact, as evidenced above, in this co-culture, bacterial counts were reduced of about 1 log with respect to the binary co-cultures. Further analyses are necessary to better explain this reduction.

3.2.3. Formic Acid Production

As evidenced in Figure 2c, the content of formic acid fluctuated during fermentation either in single and in co-culture, suggesting its production and consumption by the bacterial and yeast metabolism. Several species of mesophilic lactobacilli metabolise citrate with concomitant production of diacetyl and formic acid; in our case, *Lb. plantarum* LAT3 and *E. faecalis* KE06 displayed the production of formic acid in single-strain cultures after 24 h of fermentation. During fermentations, the relations that occur among the consortium members are at the base of the performances of the single microorganisms [32]. Thus, since the positive effect of formic acid on the growth of some LAB is related to the biosynthesis of purines, the marked consumption observed after 24 h of fermentation could be related to the increase of *Lb. plantarum* LAT3 and *E. faecalis* KE06 counts.

3.2.4. Acetate Production

Many LAB species are able to metabolise the citrate present in milk into flavor compounds such as acetate, diacetyl, acetaldehyde and acetoin [33] that mainly increase during the stationary growth phase. In addition, some *Lactobacillus* strains produce acetic acid from the degradation of lactic acid [34] or from heterofermentative pathways [35]. Among the different batch cultures, the main differences in acetate accumulation corresponded to the first 24 h of fermentation: in fact, in the single-strain cultures, acetate production was clear after 24 h of incubation, while the accumulation in co-cultures was appreciable after 48 h of fermentation, although in different proportions depending on the strain. In this case, *E. faecalis* KE06 was the major producer in the first 24 h (Figure 2d). It is well known that a desirable sensory characteristic in a fermented milk is the production of small amounts of acetic acid, in comparison to lactic acid [36]. The lactic acid/acetic acid ratio obtained in the fermented milk produced by these co-cultures was 18:0.1 for the co-culture in which the three microorganisms were present, thus indicating the potential of this mix of microbial co-culture. The major concentration of acetic acid production suggested that the pathway pyruvate formate-lyase (PFL) and the system pyruvate-dehydrogenase and acetate-kinase were the enzymatic pathways expressed during the fermentation of milk in which *E. faecalis* KE06 and *Lb. plantarum* LAT3 were inoculated. In contrast with our results, Álvarez-Martín et al. [12], observed a strong reduction of acetic acid production when *Lactobacillus citreum* was in co-culture with *Candida famata* and *Yarrowia lypolytica*.

3.3. Ethanol Accumulation

Ethanol is the major volatile compound produced by yeasts and it is very important for determining the properties of fermented milk [11]. In this work, all the strains produced small quantities of ethanol in single-strain culture and in co-culture.

The kinetics of ethanol production during fermentation is shown in Figure 3.

As evidenced, in single-strain cultures, the ethanol concentration increased slightly over approximately the early exponential phase, and then it showed a sharp increase, reaching a final mean value of ethanol of from 1.29 mg/L in *G. geotrichum* KL20B to 4.30 mg/L for *E. faecalis* KE06. Ethanol is a common fermentation product for many LAB and is produced by the reduction of acetaldehyde [37] or by the reduction of acetate to ethanol. Acetate kinase is the enzyme involved in these steps, which had been previously revealed in cell-free extracts of *Lb. plantarum* [38]. During fermentation in co-culture, the quantities of ethanol were remarkably increased; probably the not-lactose-fermenting *G. geotrichum* could have utilized the galactose produced by LAB lactase, enhancing the ethanol

production. However, also in this case, the LAB contribute in ethanol formation could have accounted for the ethanol accumulation, reaching values of approximately 11 mg/L in the co-cultures with the three strains. In line with our results, the study of Sudun et al. [11] showed that in the mixed co-culture of 4C with *Leuconostoc mesenteroides* subsp. *dextranicum* 6B2081, or *Lb. reuteri* 940B3, or *Lb. helveticus* 130B4, ethanol production was enhanced if compared to the single culture.

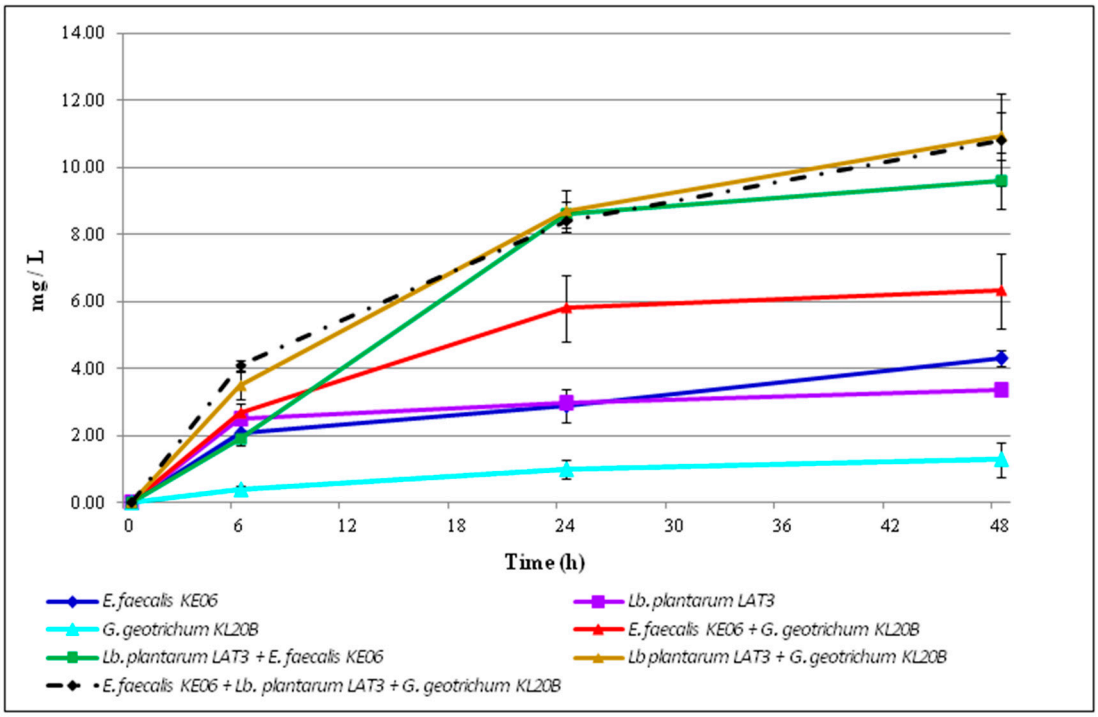

Figure 3. Dynamics of ethanol production during 48 h of fermentation.

The ethanol concentration during the co-cultures fermentation here studied is lower than the range of values (1.0–2.0%) reported by Chaves-López et al. [39]; in that case, besides not-fermenting yeast species like *Saccharomyces cerevisiae*, that had been reported to produce moderate amounts of ethanol in co-cultures with *Lactobacillus* strains in fermented milk [11], also lactose-fermenting yeast species such as *Kl. marxianus* were observed [1].

3.4. Total Free Amino Acids Content (FAA)

During milk fermentation, the proteolytic system of lactic acid bacteria and yeasts degrades casein into peptides and free amino acids. These compounds are essential for the growth of the strains. Most of the volatile flavor components derive from the metabolism of the FAAs released during proteolysis, and the products derived from the amino acid catabolism give a great contribute to flavor [40].

Table 1 shows the influence of the strains on the accumulation of amino acids in fermented milk, comparing their concentration in single culture with the co-cultures, during the incubation time. *E. faecalis* KE06 alone was the best FAA producer, with values of 0.119 ± 0.031 mM Leucine after 24 h of incubation. The species *E. faecalis* has been previously demonstrated to exert proteolytic and aminopeptidase activity in milk, contributing to the technological and sensory properties of fermented milk and cheeses [41]. However, this species has the paradoxical position of being useful in dairy fermentations, and being considered as a potential human pathogen, usually harbouring virulence genes and being involved in nosocomial infections, thus its intentional use in foods should be associated with preceding safety evaluations [42].

Table 1. Dynamics of total free amino acids production during milk fermentation.

Total Free Amino Acids (mM Leucine)	0 h	6 h	24 h	48 h
E. faecalis KE06	0.047 ± 0.003 a	0.074 ± 0.012 b	0.119 ± 0.031 c	0.202 ± 0.042 d
Lb. plantarum LAT3	0.047 ± 0.003 a	0.022 ± 0.010 b	0.029 ± 0.007 b	0.060 ± 0.010 c
G. geotrichum KL20B	0.047 ± 0.003 a	0.058 ± 0.004 b	0.063 ± 0.016 b	0.196 ± 0.022 c
E. faecalis KE06 + *G. geotrichum* KL20B	0.047 ± 0.003 a	0.111 ± 0.033 b	0.068 ± 0.003 c	0.061 ± 0.008 c
Lb. plantarum LAT3 + *E. faecalis* KE06	0.047 ± 0.003 a	0.053 ± 0.005 ab	0.057 ± 0.006 b	0.057 ± 0.003 b
Lb plantarum LAT3 + *G. geotrichum* KL20B	0.047 ± 0.003 a	0.037 ± 0.004 b	0.048 ± 0.004 a	0.059 ± 0.002 b
E. faecalis KE06 + *Lb. plantarum* LAT3 + *G. geotrichum* KL20B	0.047 ± 0.003 a	0.037 ± 0.002 b	0.064 ± 0.003 c	0.053 ± 0.002 d

Mean and standard deviation of three replicates. Different letters among the rows indicate significant differences between the fermentation time.

The analysis of the cultures in which *Lb. plantarum* LAT3 was present shows a statistically significant reduction of free amino acids (FAA) content in the milk after inoculation, indicating that the cells start growing exponentially using the amino acids that are freely available. Subsequently, in single-strain culture and in binary co-culture with *G. geotrichum* KL20B, in which a slowdown of the growth rate is clear (Figure 1), the FAA content remained almost constant, suggesting that the synthesis of extracellular proteases probably began during this period. After 24 h, the proteolytic system of *Lb. plantarum* determined an increase of FAA content in milk, to supply sufficient peptides for exponential growth.

Although the co-cultures with *E. faecalis* KE06 and *G. geotrichum* KL20B showed a marked production of FAA during the first 6 h of incubation, after that a sharp consumption was revealed, hence explaining why *G. geotrichum* KL20B did not decrease its cell counts in this co-culture (Figure 1c). The LAB proteolytic system is a very complex system in which the proteinases associated to the cell wall are able to disassemble casein into oligopeptides able to enter into the cytoplasm, where they are further dismantled into small peptides and amino acids [43]. The presence of extracellular and intracellular proteases has also been reported in yeast species. Previously, we observed the ability of *G. geotrichum* KL20B proteases to produce peptides in milk [1]; in this work, the activity of the amino peptidases was able to produce about of 0.196 mM of Leucine in single-strain culture and in co-cultures, probably providing FAA for *Lb. plantarum* LAT3 growth.

In a recent review, Grygier et al. [44] underlined the importance of *G. geotrichum* in the production of fermented foods as it makes possible to obtain a product with peculiar characteristic aroma and taste. In this study, we underline the contribution of this not-fermenting lactose yeast on the lactic acid bacteria growth, as well as on the increase of some metabolic compounds content.

In conclusion, during this study we explained the metabolic interactions among LAB and yeast in fermented milk and we observed that the use of co-cultures involving *E. faecalis* KE06, *Lb. plantarum* LAT3 and *G. geotrichum* KL20B provides a mean to enhance the viability of lactic acid bacteria and the production of lactic acid, ethanol and amino acids, as an adaptive response to the environment. To our knowledge, this is the first time in which it is showed that *G. geotrichum* KL20B could be used to compensate for the slow acid-producing ability of *Lb. plantarum* and *E. faecalis* in milk, underlining that this consortium applies some mechanisms to regulate the growth and milk composition in acids and ethanol content.

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