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Sugary Kefir: Microbial Identification and Biotechnological Properties

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Received: 31 July 2019; Accepted: 8 October 2019; Published: 15 October 2019

Abstract: *Background:* The aim of the present study was to assess the microecosystem composition of three different fruit kefir grains used for the fermentation of apple juice (NAJ), cherry juice (SCN), and a solution of sugary water, enriched with plums (BSS). *Methods:* Yeast and bacterial populations were enumerated using classical microbiological techniques, clustered by RAPD-PCR genotyping, and identified by sequencing of the D1/D2 region of 26S-rRNA gene and the V1-V3 region of 16S-rRNA gene, respectively. The caseinolytic/lipolytic activities and the production of antimicrobial compounds were assessed by well diffusion assays. The proteolytic and lipolytic capacity were further assessed by SDS-PAGE and titrimetric assay, respectively. *Results:* Yeast and bacterial populations were enumerated at 6.28, 6.58, and 6.41 log CFU/g and at 4.32, 4.85, and 4.34 log CFU/g, regarding BSS, NAJ, and SCN, respectively. *Saccharomyces cerevisiae* dominated all three sugary kefir grains; *Kluyveromyces marxianus* formed a secondary microbiota in BSS and NAJ. *Bacillus amyloliquefaciens* dominated NAJ and SCN; *Lactobacillus rhamnosus* dominated BSS. Four bacteria and nine yeast isolates exhibited proteolytic activity. Forty bacteria and 45 yeast isolates possessed lipolytic activity. No antibacterial activity was detected. *Conclusions:* Prevalence of yeast over bacterial populations was evident in all samples assessed. Several bacterial and yeast strains exhibited proteolytic and lipolytic activities, making them suitable candidates for inclusion in starter cultures for milk and sugary kefir fermentation.

Keywords: sugary kefir; apple; cherry; brown sugar; *Lactobacillus rhamnosus*; *Bacillus amyloliquefaciens*; *Saccharomyces cerevisiae*; *Kluyveromyces marxianus*

1. Introduction

Kefir is a self-carbonated, slightly acidic and alcoholic fermented beverage, with yeasty flavor, originating from Caucasian and para-Caucasian regions. It is typically manufactured by transferring kefir grains into a milk-based substrate. Kefir grains are yellowish-white, gelatinous, irregularly formed, variable in size and morphologically resembling small cauliflowers. These grains include a consortium of lactic acid bacteria (LAB) (10^8 CFU/g), yeasts (10^6–10^7 CFU/g), and occasionally acetic acid bacteria (10^5 CFU/g), attached to a polysaccharide matrix, known as kefiran [1].

In addition to using milk, an alternative substrate for kefir production has been the brown sugar solution. Sugary kefir beverage is based on sucrose solution with the addition of kefir grains, dried fruit and optionally some slices of lemon [2]. Although an association of sugary kefir grains with the

ginger beer plants that were transferred from the Crimean War in 1855 has been documented, the roots of sugary kefir remain reasonably unclear [3]. Pidoux [4] used the term "sugary kefir grains" to distinguish them from the typical kefir grains.

Sugary kefir grains share plenty similarities with milk kefir grains in relation to their morphological characteristics and microcommunity composition. Many scientific reports have documented the presence of *Lactobacillus casei*, *Lb. hordei*, *Lb. kefir*, *Lb. nagelii*, *Lb. parabuchneri*, *Lb. paracasei*, *Lb. rhamnosus*, *Leuconostoc citreum*, and *Ln. mesenteroides* in sugary kefir grains [2,5,6]. As far as yeasts were concerned, the microcommunity includes *Zygotorulaspora florentina*, *Kluyveromyces lactis*, and *Saccharomyces cerevisiae* as the most prevalent species, being accompanied by *Hanseniaspora valbyensis*, *Kazachstania aerobia*, *Lachancea fermentati*, and *L. meyersii* [2,5].

The aim of this study was to identify the microorganisms present in three different samples of sugary kefir grains, produced by water enriched with sugar and plums, apple juice, and cherry juice. Furthermore, the technological properties associated with its production were studied, such as proteolytic and lipolytic capability, acidification potential, and antimicrobial activity against foodborne pathogens.

2. Materials and Methods

2.1. Origin and Types of Sugar Kefir Grains

The sugary kefir grains that were used in the present study were obtained via the conversion of classical milk kefir grains following gradual acclimatization to different substrates. The initial acclimation process from milk to sucrose fermentation lasted 5 days, during which lactose (whole fat milk) was progressively replaced by sucrose (brown sugar solution, BSS). For the preparation of the BSS, 46.5 g of commercial brown sugar (97% purity) was dissolved in 1 L of tap water and was supplemented with 10 g of dry prunes, with net sugar content 38%, resulting thus to a solution of 50.3 ± 0.2 g/L total soluble sugars. The final fermentation test that was used for the isolation of microorganisms (sucrose derived) was conducted with 100% BSS as fermentation media. Subsequently, the sugar kefir grains were used for the fermentation of two different types of commercial juices i.e., sour cherry nectar (SCN) and natural apple juice (NAJ), after gradual acclimation to each new substrate according to the protocol described above.

2.2. Fermentation Tests

All fermentation tests were conducted at static batch conditions, at 25.0 ± 0.2 °C, without initial pH adjustment. Kefir grains, priory acclimated to each substrate for 5 days, were introduced to fresh medium at a ratio of 5%, (w/v, wet basis). After 24 h of fermentation, kefir grains were drained and their weight increase was estimated. The fermentation broth was analyzed in terms of final pH and concentration of fermentation products.

2.3. Yeasts and Lactic Acid Bacteria Enumeration and Isolation

Kefir grains (10 g) were homogenized with 90 mL sterile Ringer solution; serial decimal dilutions were prepared in the same diluent. Enumeration of lactic acid bacteria (LAB) and yeasts was carried out in De Mann, Rogosa, Sharpe agar (MRS) (LABM, Lancashire, UK) and in Rose-Bengal Chloramphenicol agar (RBC) (LABM) after pouring and spreading of serial dilutions and anaerobic (GasPak, BBL, Cockeysville, MD, USA) incubation at 30 °C for 48 h and aerobic incubation at 25 °C for 5 d, respectively. From each sample, all colonies present in the final dilution were selected and purified by successive subculturing. LAB and yeast isolates were stored at −20 °C in Nutrient broth (LABM), supplemented with 50% glycerol.

2.4. Clustering and Identification of the Isolates

Clustering of both yeast and LAB isolates was performed by PCR-RAPD using UBC155 as primer. More accurately, DNA was extracted according to Doulgeraki et al. [7]; each PCR mix consisted of 0.2 mM dNTPs (Peqlab, Erlangen, Germany), 2.5 mM $MgCl_2$, 1.5 μM primer UBC155 (5′-CTG GCG GCT G-3′) and 2 U *Taq* polymerase (Kapa Biosystems, MA, USA). Thermocycling conditions included initial denaturation step at 95 °C for 2 min, 10 cycles of (95 °C for 1 min, 45 °C for 1 min decreasing by 1 °C at each cycle to 36 °C, 72 °C for 2 min), then 30 cycles of (95 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min), and a final extension step at 72 °C for 10 min [8]. PCR products were separated by electrophoresis in 1.5% agarose gel in 1.0 × TAE at 100 V for 1.5 h, visualized by ethidium bromide staining and pictured using a GelDoc system (BioRad, Hercules, CA, USA). Bionumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium) was used for conversion, normalization, and further analysis of the genotypic profiles obtained; clustering was obtained using the Dice coefficient and UPGMA analysis. In agreement with Doulgeraki et al. [7], one to three representative bacteria strains from each cluster were subjected to sequencing of the V1-V3 region of 16S-rRNA gene and the D1/D2 region of 26S-rRNA gene, for LAB and yeast isolates, respectively, for species identification.

2.5. Assessment of Technological Properties

2.5.1. Proteolytic Activity

Proteolytic activity of LAB and yeasts isolates was initially screened by an agar well diffusion assay and, in the case of positive result, further analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

In the first case, LAB and yeast isolates were grown in MRS broth and BHI broth (LAB M) at 30 and 25 °C, respectively, for 24 h. An amount (50 μL) of the cell-free supernatant obtained by centrifugation (12,500 rpm; 15 min; 4 °C) was placed into the wells that were punched into a solidified medium consisting of casein 1.0%, tryptone 0.5%, yeast extract 0.25%, glucose 0.1%, and agar 1.5% (pH 6.9). Incubation took place at 25 and 30 °C for 48 h, for yeasts and LAB, respectively. After incubation, plates were stained with Coomassie Brilliant Blue R (Sigma, Chemical Co., St. Louis, MO, USA); proteolytic activity was indicated by the presence of a clear zone surrounding the inoculated wells. Test was performed in triplicate.

The strains that exhibited proteolytic activity were further tested by SDS-PAGE. Actively growing LAB and yeast cells were inoculated into a broth medium consisted of tryptone 0.5%, yeast extract 0.25%, glucose 0.1%, casein 1% (pH 6.9), and incubated at 30 and 25 °C, respectively, for 48 h. Uninoculated broth served as a blank. Then, the cell-free supernatants were analyzed by SDS-PAGE according to Drosinos et al. [9]. Tests were performed in triplicate.

2.5.2. Lipolytic Activity

Lipolytic activity of LAB and yeasts isolates was initially screened by an agar well diffusion assay and, in the case of positive result, further analyzed by a titrimetric assay. In the first case, LAB and yeast isolates were grown in MRS broth and BHI broth (LAB M) at 30 and 25 °C, respectively, for 24 h. An amount (50 μL) of the cell-free supernatant obtained by centrifugation (12,500 rpm; 15 min; 4 °C) was placed into the wells that were punched into a solidified medium consisting of nutrient agar, tributyrine (1%) and DMSO (0.25%). Incubation took place at 30 and 25 °C for bacterial and yeast strains, respectively, for 10 days. Presence of a transparent zone around the wells was an indicator of lipolysis. Test was performed in triplicate.

The strains that exhibited lipolytic activity were further analyzed by the titrimetric method proposed by Kamzolova et al. [10] with slight modifications. More accurately, actively growing cells of LAB and yeast strains were inoculated into a broth medium consisted of lecithin 0.5%, tributyrine 1%, meat extract 3 g/L and peptone 5 g/L. Incubation took place at 30 and 25 °C for bacterial and yeast strains, respectively, for 7 days. Uninoculated broth served at blank. After incubation, 10 mL of the

cell-free supernatant obtained by centrifugation (12,500 rpm; 15 min; 4 °C) was titrated with 50 mM NaOH (to final pH value of 9.0). One unit (U) of lipase activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of fatty acids per mL. The test was performed in triplicate.

2.5.3. Acidification Capacity

LAB acidification capacity was assessed in sterile reconstituted skim milk. More accurately, 10 mL of sterile skim milk was inoculated with overnight bacterial cultures and incubated at 30 °C for 24 h. The pH changes were assessed with a pH meter (WTW, Weilheim, Germany). The acidification capacity was calculated as $\Delta pH = pH_{24h} - pH_{initial}$. The test was performed in triplicate.

2.5.4. Antimicrobial Activity

The antimicrobial activity of the lactic acid bacteria isolates was carried out with the well diffusion assay according to Drosinos et al. [9]. In brief, cell-free supernatant was obtained from overnight bacterial cultures, neutralized, treated with catalase, and added to the wells that were punched in freshly prepared lawns containing a mixture of 5 strains of *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* sp. [11,12]. Growth inhibition exceeding 5 mm around the wells was used as an indicator of antimicrobial activity of the supernatant applied. The test was performed in triplicate.

2.6. Analytical Methods

Sugars were quantified according to DuBois et al. [13]. The pH was measured by a Metrohm 744 pH meter. Ethanol and acetic acid were quantified via HPLC-RI (Shodex) with an Aminex HPX–87H column (Biorad) at 60 °C and a cation H micro-guard cartridge (Biorad), with H_2SO_4 0.006 N as mobile phase at a flow rate of 0.7 mL/min. Lactic acid was quantified spectrophotometrically at 340 nm using a D-/L-Lactic Acid Assay Kit (Megazyme). The test was performed in triplicate.

3. Results

3.1. Fermentation Tests

Table 1 summarizes the results of fermentation of the three sugary substrates that were tested in this study. The main difference among substrates is the initial pH of the cultures, which was neutral for BSS and quite acidic for SCN and NAJ and the initial concentration of sugar, which was much higher for the juices. As shown, in the case of BSS, 80% of the sugar content was fermented whereas in the cases of SCN and NAJ the uptake of sugars was less than 50%. The distribution of the fermentative products were similar with ethanol being the prominent final product, reaching maximum concentration of 2.14 ± 0.12% (v/v) for SCN. Lactate was also detected in all three beverages in similar concentrations, whereas the presence of acetic acid was not detected.

Table 1. Change of the pH value, carbohydrate consumption and metabolite production after 24 h fermentation at 25 °C of the three sugary substrates employed in this study.

Substrate	pH_0	pH_f	S_0 Sugars (%, w/v)	S_f Sugars (%, w/v)	Ethanol (%, v/v)	Lactic Acid (%, w/v)	Acetic Acid (mg/L)
BSS	6.9 ± 0.01	4.1 ± 0.01	5.03 ± 0.2	0.93 ±0.11	1.96 ± 0.11	162.12 ± 12.09	nd
SCN	3.9 ± 0.01	3.5 ± 0.02	13.2 ± 0.2	8.92 ± 0.1	2.14 ± 0.12	148.35 ± 9.46	nd
NAJ	4 ± 0.01	3.7 ± 0.01	9.2 ± 0.1	6.96 ± 0.1	0.85 ± 0.04	134.13 ± 8.78	nd

nd: not detected.

3.2. Yeast and Bacterial Enumeration in Kefir Grains

Bacterial and yeast populations were enumerated in BSS, NAJ and SCN kefir grains at 4.32, 4.85 and 4.34 log CFU/g, and at 6.28, 6.58, and 6.41 log CFU/g, respectively.

A total of 50 bacterial and 83 yeast isolates were obtained, subjected to RAPD-PCR analysis and effectively separated into several clusters (Figures 1 and 2). More accurately, bacteria were separated into five clusters, designated I to V (Figure 1). The majority of the clusters contained isolates from the same kefir sample with the exception of clusters II and III, which consisted of isolates from apple (NAJ) and cherry (SCN) kefir samples. One to three representative isolates from each cluster were subjected to sequencing of the V1-V3 region of the 16S rRNA gene and the resulting phylogenetic affiliation is exhibited in Table 2. Similarly, yeasts were separated into seven clusters, defined I to VII (Figure 2). The clusters involved yeast isolates from more than one sugary kefir grains, apart from cluster IV, which comprised of isolates from apple (NAJ) kefir sample.

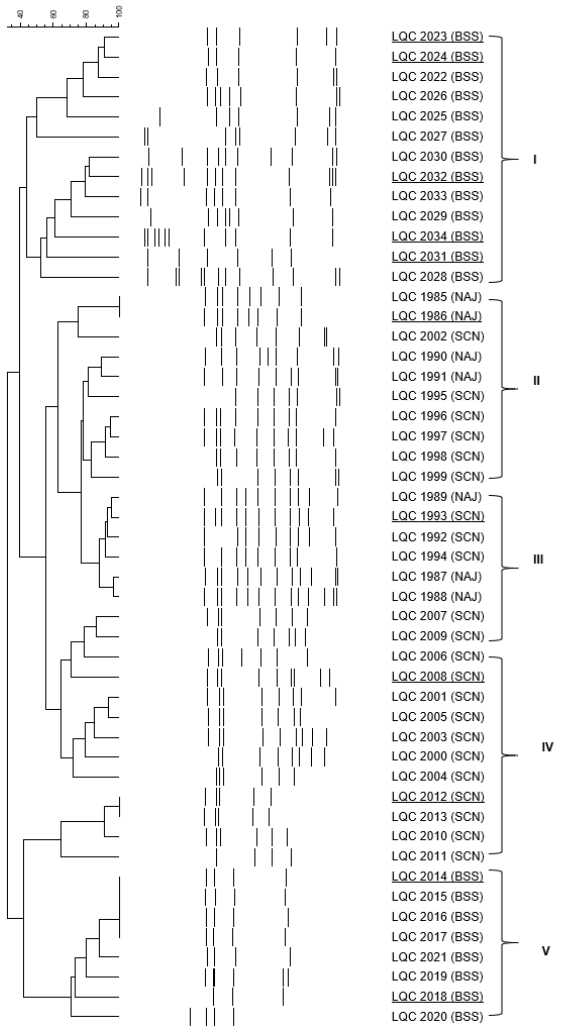

Figure 1. Cluster analysis of RAPD-PCR patterns of bacterial isolates, obtained from apple (NAJ), cherry (SCN) and sugary water (BSS) kefir grains. Distance is indicated by the mean correlation coefficient [r (%)] and clustering was performed by UPGMA analysis. The representative strains selected for 16S rRNA gene sequencing are underlined. Latin numerals designate bacterial species (I, and V: *Lb. rhamnosus*, II, III and IV: *B. amyloliquefaciens*).

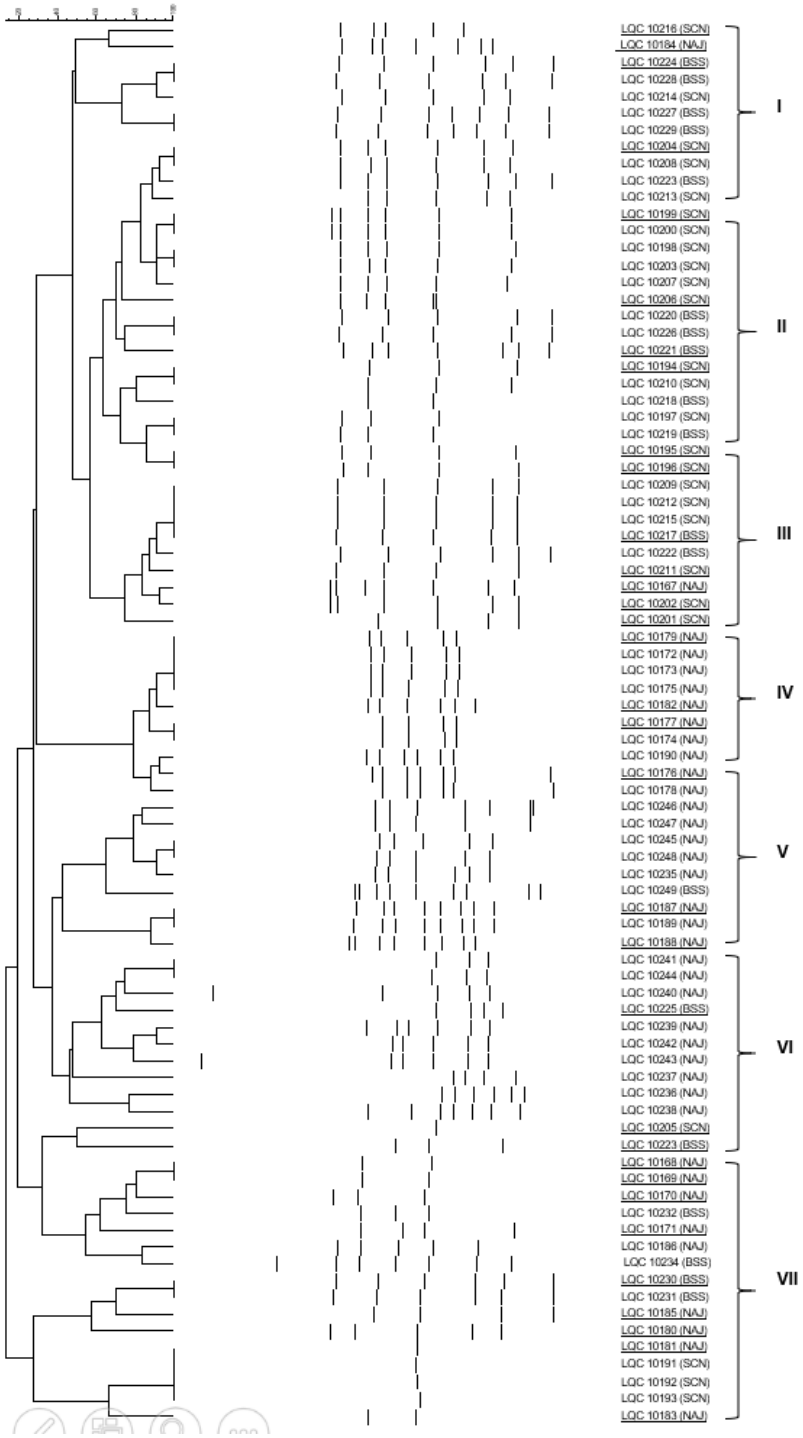

Figure 2. Cluster analysis of RAPD-PCR patterns of yeast isolates, obtained from apple (NJA), cherry (SCN) and sugary water (BSS) kefir grains. Distance is indicated by the mean correlation coefficient [r (%)] and clustering was performed by UPGMA analysis. The representative strains selected for 26S rRNA gene sequencing are underlined. Latin numerals designate yeast species (I, II, III, VI and VII: *S. cerevisiae*, IV and V: *K. marxianus*).

Table 2. Phylogenetic position of yeast and bacterial strains based on sequencing of the D1/D2 region of 26S-and V1-V3 region of 16S rRNA gene, respectively.

	Strain Number	Closest Relative	Identity (%)	Accession Number
Yeasts	LQC 10194	*S. cerevisiae*	99	MG017543.1
	LQC 10195	*S. cerevisiae*	99	MG017587.1
	LQC 10196	*S. cerevisiae*	99	MG017572.1
	LQC 10199	*S. cerevisiae*	99	MG017572.1
	LQC 10201	*S. cerevisiae*	99	MG017587.1
	LQC 10202	*S. cerevisiae*	99	MG017588.1
	LQC 10204	*S. cerevisiae*	99	MG017572.1
	LQC 10205	*S. cerevisiae*	100	JQ964228.1
	LQC 10206	*S. cerevisiae*	100	MG017572.1
	LQC 10211	*S. cerevisiae*	100	MF406146.1
	LQC 10216	*S. cerevisiae*	100	KR069090.1
	LQC 10167	*S. cerevisiae*	100	MG017572.1
	LQC 10168	*S. cerevisiae*	99	JQ964228.1
	LQC 10170	*S. cerevisiae*	100	MF406146.1
	LQC 10171	*S. cerevisiae*	100	MF406146.1
	LQC 10176	*S. cerevisiae*	99	KJ660850.1
	LQC 10177	*K. marxianus*	99	KJ491106.1
	LQC 10179	*K. marxianus*	99	KJ491106.1
	LQC 10180	*K. marxianus*	100	KJ491106.1
	LQC 10181	*S. cerevisiae*	100	MF406146.1
	LQC 10182	*K. marxianus*	100	MH244202.1
	LQC 10183	*S. cerevisiae*	100	KJ660848.1
	LQC 10184	*S. cerevisiae*	100	MG017572.1
	LQC 10185	*S. cerevisiae*	99	MF406146.1
	LQC 10187	*K. marxianus*	99	KJ491106.1
	LQC 10188	*K. marxianus*	100	FJ896141.1
	LQC 10217	*S. cerevisiae*	100	MG017572.1
	LQC 10221	*S. cerevisiae*	99	MG017572.1
	LQC 10224	*S. cerevisiae*	100	MG017587.1
	LQC 10225	*S. cerevisiae*	99	MG017585.1
	LQC 10230	*S. cerevisiae*	100	MF406146.1
	LQC 10233	*S. cerevisiae*	100	MF406146.1
Bacteria	LQC 1986	*B. amyloliquefaciens*	99	KY392912.1
	LQC 1993	*B. amyloliquefaciens*	98	MH045777.1
	LQC 2012	*B. amyloliquefaciens*	99	KY072769.1
	LQC 2014	*Lb. rhamnosus*	99	CP020464.1
	LQC 2018	*Lb. rhamnosus*	100	HQ774719.1
	LQC 2023	*Lb. rhamnosus*	99	CP020464.1
	LQC 2024	*Lb. rhamnosus*	99	CP020464.1
	LQC 2031	*Lb. rhamnosus*	99	LC333198.1
	LQC 2032	*Lb. rhamnosus*	99	LC333198.1
	LQC 2034	*Lb. rhamnosus*	99	LC333198.1
	LQC 2008	*B. amyloliquefaciens*	99	MF953984.1

The majority of bacterial isolates (29 of 50) were identified as *Bacillus amyloliquefaciens*, while the rest 21 were classified as *Lactobacillus rhamnosus*. Regarding yeasts, the majority of the isolates (64 of 83) were identified as *Saccharomyces cerevisiae* while the remaining 19 were classified as *Kluyveromyces marxianus*.

Regarding the bacterial and yeast microecosystem composition of the BSS, NAJ and SCN kefir samples, a total of 50 bacterial isolates were obtained, 7 of which were retrieved from NAJ, 22 from SCN, and 21 from BSS kefir grains. *B. amyloliquefaciens* was the only species isolated from NAJ and SCN kefir grains; on the contrary, *Lb. rhamnosus* was the only species isolated from BSS kefir grains. As far as yeasts were concerned, a total of 83 yeast isolates were obtained; 38 from NAJ, 26 from SCN, and 19

from BSS kefir grains. *S. cerevisiae* was the only species isolated from SCN kefir grains. A consortium consisting of *S. cerevisiae* and *K. marxianus* was evident in BSS and NAJ kefir grains; in the first case 94.7% of the isolates were assigned to *S. cerevisiae* and the remaining 5.3% to *K. marxianus*. Regarding the yeast microbiota of NAJ kefir grains, it consisted of *S. cerevisiae* (55.3%) and *K. marxianus* (44.7%).

3.3. Assessment of Technological Properties

3.3.1. Proteolytic Activity

The agar well diffusion assay was performed for 50 bacterial and 83 yeasts isolates and those that provided with positive results were further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In the first case, a clear zone of casein hydrolysis, around the wells, indicated the presence of proteolytic activity. The agar plate assay revealed that 4 bacterial and 9 yeast isolates were proteolytic. Regarding the bacterial isolates, three were identified as *Lb. rhamnosus* (LQC 2014, 2015, 2030) and one as *B. amyloliquefaciens* (LQC 1990) and originated from BSS and NAJ kefir grains, respectively. As far as yeasts were concerned, three *S. cerevisiae* (LQC 10184, 10235, 10237) and six *K. marxianus* isolates (LQC 10179, 10182, 10245, 10246, 10247, 10248) were found proteolytic, all originating from NAJ kefir grains. Then, SDS-PAGE electrophoresis was performed on the positively indicated proteolytic isolates. The protein patterns of the uninoculated broth serving as a blank solution and the ones inoculated with microbial isolates were identical. More accurately, the protein bands corresponding to caseins as1, as2, β, and κ, with molecular weights 23, 25, 24, and 19 kDa, respectively, were clearly visible in all samples, including the blank, indicating absence or partial hydrolysis.

3.3.2. Lipolytic Activity

A total of 50 bacterial and 83 yeast isolates were tested for lipase secretion, by employing a screening technique on agar plates and a subsequent titrimetric analysis, for those assessed as lipolytic. According to the agar plate assay 40 bacterial isolates appeared to possess lipolytic activity, 29 of which were identified as *B. amyloliquefaciens* and the rest 11 as *Lb. rhamnosus*. As far as yeasts were concerned, 45 isolates were revealed to possess lipolytic capacity, 29 were assigned to *S. cerevisiae,* and 16 to *K. marxianus.* Those having exhibited lipolytic activity were tested for lipase production in liquid medium containing tributyrine and lecithin as emulsifier. The results in AU/mL are presented in Figures 3 and 4 for yeasts and bacteria, respectively. Bacterial isolates showing the highest lipase activity were *B. amyloliquefaciens* LQC 1990 and *Lb. rhamnosus* LQC 2023 (10.50 and 10.05 AU/mL, respectively), originating from NAJ and BSS kefir grains, respectively. Bacterial isolates from SCN kefir grains displayed the lowest lipase activity, with the values ranging from 1.05 to 5 AU/mL. Yeast strains retrieved from NAJ and SCN kefir grains presented both, the highest and lowest lipase capabilities. In other words, yeast isolates *S. cerevisiae* LQC 10237 and LQC 10186, originating from NAJ kefir grains, exhibited lipase activities of 20.85 and 1.05 AU/mL, respectively. Similarly, yeast isolates *S. cerevisiae* LQC 10199 and LQC 10198, which were obtained from SCN kefir grains, showed lipase activities of 16.05 and 1.45 AU/mL, respectively.

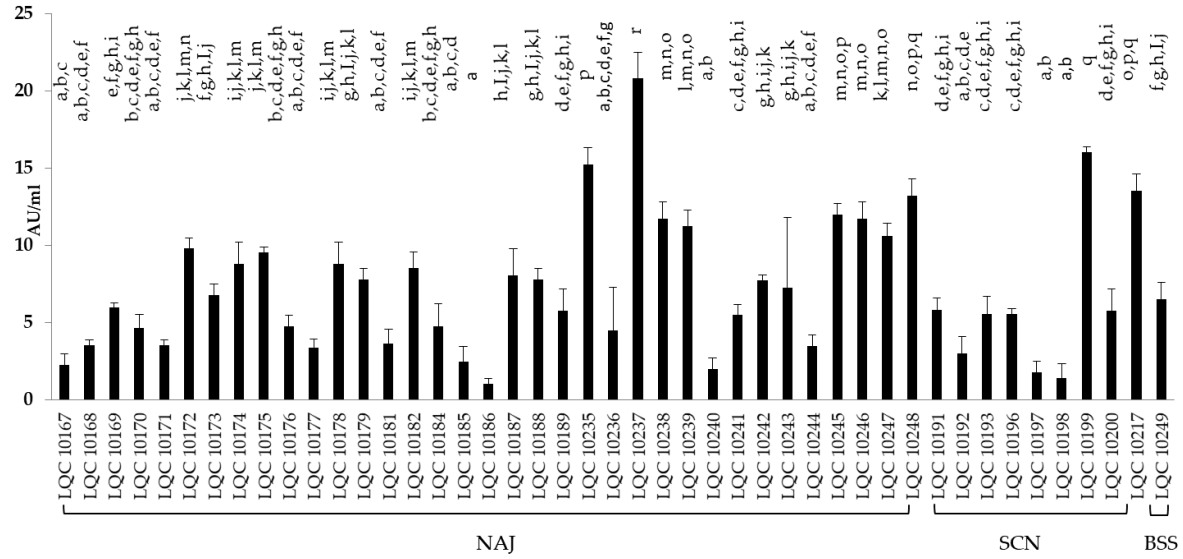

Figure 3. Lipase activity in AU/mL of the yeast isolates. Different letters (a–r) indicate statistically significant differences ($p < 0.05$).

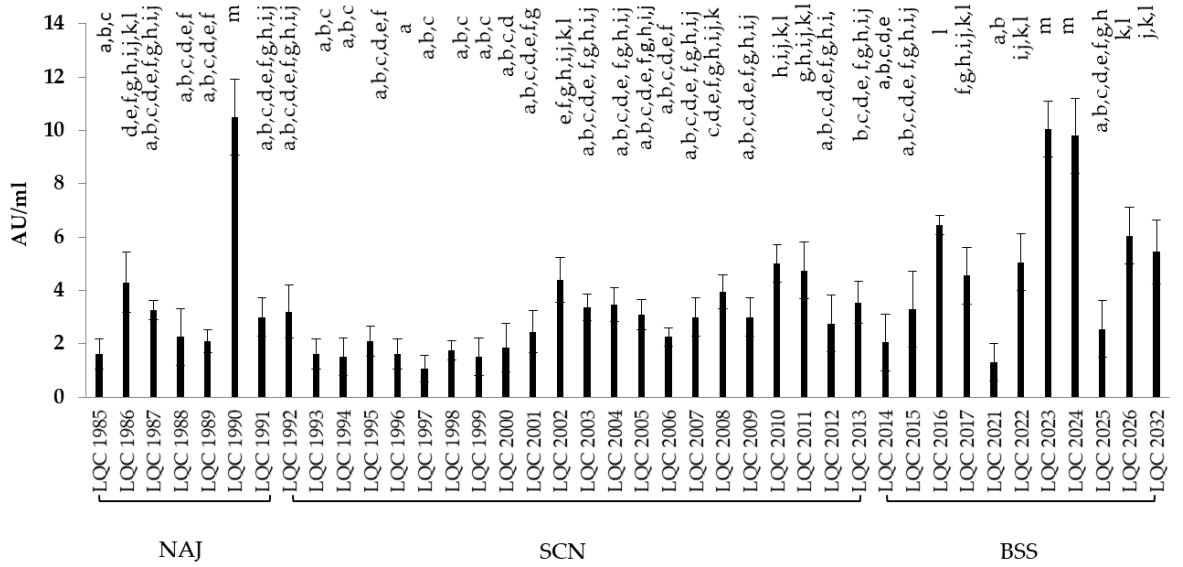

Figure 4. Lipase activity in AU/mL of the bacterial isolates. Different letters (a–m) indicate statistically significant differences ($p < 0.05$).

3.3.3. Acidification Capacity

The acidification capacity of the bacterial isolates after 24 h of incubation in reconstituted skim milk, is exhibited in Figure 5. The most prominent acidification, with ΔpH ranging between 0.78 and 1.07 pH units, was displayed by the 21 isolates identified as *Lb. rhamnosus*. In particular, two *Lb. rhamnosus* isolates, namely LQC 2018 and LQC 2022, achieved the highest acidifying capacity, with a ΔpH of 1.06 and 1.07, respectively. In contrast, the 29 *B. amyloliquefaciens* isolates, showed significantly ($p < 0.05$) lower acidifying activity, with ΔpH values, ranging between 0.12–0.28 pH units.

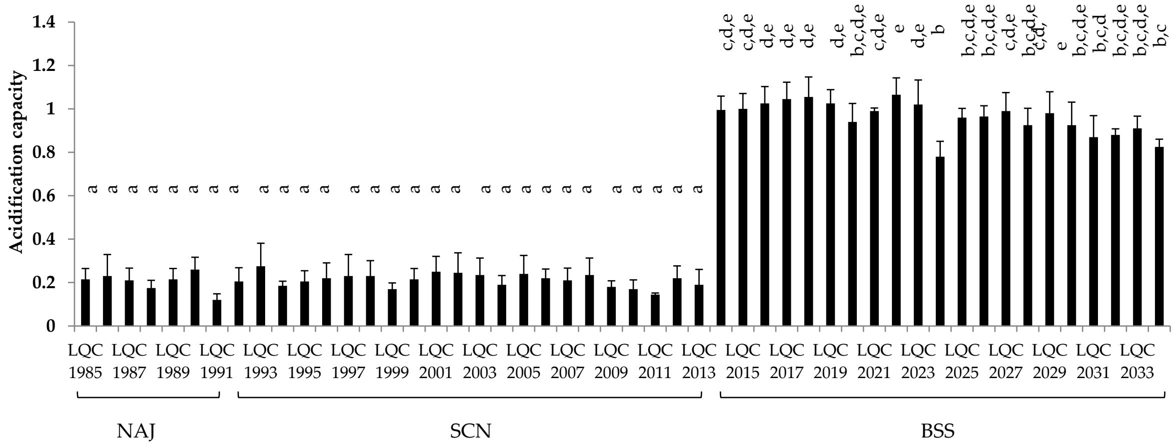

Figure 5. Acidification capacity of the bacterial isolates after 24 h of incubation in reconstituted skim milk. Different letters (a–e) indicate statistically significant differences ($p < 0.05$).

3.3.4. Antimicrobial Activity

A total of 50 bacterial isolates were screened for the production of antimicrobial compounds against a mixture of 5 strains of *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* sp. No such activity was detected.

4. Discussion

In general, the results obtained in the present study regarding the carbohydrate consumption and the metabolite production of BSS, SCN and NAJ are in good agreement with previous studies on sugary kefir beverages, according to which ethanol is the main metabolic product followed by lactate and acetate and in some cases citrate [3]. In the present study citric acid was not quantified but according to the mass balances of sugars to metabolites conversion the presence of other metabolites should indeed be expected. Differences in the final concentrations could be attributed to differences in the microbial consortia but also to the fermentation conditions. For instance, in the study of Randazzo et al. [14], in which the production of kefir beverages from different fresh fruit juices was investigated, higher ethanol concentrations (up to 4.96%, v/v) were reported and also acetate detection was noted in similar concentrations with lactate. It should be noted that Randazzo et al. [14] performed backslopping inoculation instead of direct fermentation via water kefir grains whereas fermentation lasted 48 h and as such sugar consumption was much higher. In the present study fermentations were ceased after 24 h, and consequently lower consumption of carbohydrates and lower concentration of fermentation products were indeed expected.

Previous studies have demonstrated the microbial complexity of sugary kefir grains, mainly composed of LAB, namely *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp. and *Streptococcus* spp., yeasts, including *Saccharomyces* spp., *Kluyveromyces* spp. and *Candida* spp. and occasionally acetic acid bacteria. The diverse microbial profile of sugary kefir grains has been attributed to the various geographical regions of the grain preparation and the fermentation conditions [2,3,5,14].

In general, LAB population has been reported to exceed that of yeasts in both milk and sugary kefir grains [1,2,5]. However, there are studies that have either reported similar populations or exhibited the exact opposite, i.e., dominance of yeast population over LAB [15]. The latter was also the case in the present study; yeasts population exceeded the one of LAB by 2 log CFU/g. This can be attributed to the increased sugar concentration due to the gradual replacement of milk with brown sugar solution enriched with plums (BSS) and then apple (NAJ) and cherry juice (SCN).

In the present study, *S. cerevisiae* was present in all three sugary kefir grains as the predominant yeast, while *K. marxianus* formed a secondary microbiota in two out of three sugary kefir grains, namely BSS and NAJ kefir grains. Regarding *S. cerevisiae*, these findings are in agreement with previous

studies [3,5,6,16], which confirmed the high prevalence of this yeast species in sugary kefir grains. On the other hand, although *K. marxianus* presence in sugary kefir grains has been previously reported [16], this yeast is isolated from milk kefir grains more frequently as part of the secondary microbiota [17]. Compared to *S. cerevisiae*, which cannot ferment lactose, *K. marxianus*, a lactose-fermenting yeast, may possess a comparative advantage in milk-based substrates. However, in sugary solutions, *S. cerevisiae* converts sucrose into glucose and fructose, and in milk substrates presence of other microbial species that convert lactose into glucose and galactose may favor its dominance [3]. The presence of both *S. cerevisiae* and *K. marxianus* in Tibetan kefir grains was previously demonstrated by Lu et al. [17], who reported *S. cerevisiae* predominance over time, due to its adaptation to low pH, ability to survive with or without oxygen and its alcohol tolerance. As for *K. marxianus*, the necessity for oxygen uptake and the antagonistic relationship with *S. cerevisiae* contributed to its decrease over time. Despite the predominant character of *S. cerevisiae* in various sugary kefir grains, Gulitz et al. [2] after employing PCR-RAPD analysis and 16S rRNA gene sequencing, reported *S. cerevisiae* presence as secondary yeast biota in water kefir grains, originating from different regions, while the majority of yeast isolates were identified as *Zygotorulaspora florentina*. Thus, the geographical origin of the kefir grains seems to determine the microbial profile revealed.

As for bacterial isolates, those originating from NAJ and SCN kefir grains were identified as *B. amyloliquefaciens*, while the ones from BSS kefir grains were classified as *Lb. rhamnosus*. *B. amyloliquefaciens* presence in any type of sugary or milk kefir grains has not been reported so far. On the other hand, presence of *Lb. rhamnosus* in Thai sugary kefir grains and beverage has been reported by Sarikkha et al. [6]. However, *Lb. rhamnosus* was identified with less than 97% similarity, compared to those of *B. cereus* and *Gluconobacter japonicus*, ranging from 99% to 100%, respectively.

The presence of *Lactobacillus* spp., *Lactococcus* spp. and *Leuconostoc* spp. in sugary kefir grains has been previously reported. Dominance of *Lb. hordei* and *Lb. nagelii* and a secondary microbiota consisting of *Ln. mesenteroides* and *Ln. citreum* in sugary kefir grains, was reported by Gulitz et al. [2]. Hsieh et al. [18] used a combined approach of culture-dependent and -independent methods and reported the dominance of *Ln. mesenteroides* in kefir grains fermented with brown sugar, followed by *Lb. hordei* and *Lb. mali* at lower levels. Isolation of *Lb. kefiri*, *Lb. kefiranofaciens* and *Ln. mesenteroides* from milk kefir grains, as well as *Lb. perolens*, *Lb. parafarraginis*, *Lb. diolivorans*, and *Oenococcus oeni* from sugary kefir grains was reported by Zanirati et al. [19]. The microbial complexity of sugary kefir grains was also demonstrated by Magalhães et al. [5], reporting *Lb. paracasei* as the predominant species, accompanied by a secondary microbiota consisting of *Acetobacter lovaniensis*, *Lb. parabuchneri*, *Lb. kefiri,* and *Lactococcus lactis*.

B. amyloliquefaciens is a phylogenetically close relative of *B. subtilis*, whose presence has been verified via phenotypic and genotypic approaches from Tibetan kefir grains [20]. Both *B. subtilis* and *B. amyloliquefaciens* species, are often isolated from fermented soy products [21], dairy products [22] and soil as well [23]. Their antibiotic and antimicrobial activity as well as probiotic potential have also been studied [24]. Interestingly, Kort et al. [25] reported that the microbial consortium of *S. thermophilus* C106 and *Lb. rhamnosus* yoba 2012 was based on the proteolytic character of the former and its capacity to digest lactose and casein, thus aiding the latter to grow in milk substrates. *B. amyloliquefaciens* has also been reported as one of the most potent producer of extracellular proteases [21], whose interaction with *Lb. rhamnosus* could probably overcome the limitations highlighted by Kort et al. [25] and Parker et al. [26].

Acidifying capacity constitutes a determining test for the selection of bacterial strains as starter cultures in dairy fermentations. In our study *Lb. rhamnosus* isolates exhibited higher capacity than *B. amyloliquefaciens* strains. Despite the fact that *Lb. rhamnosus* isolates are not preferred as primary starter cultures in the dairy industry, this species involves some extremely interesting strains, such as *Lb. rhamnosus* GG, whose probiotic potential has been extensively studied [27]. In a study by Ayad et al. [28] *Lb. rhamnosus* acidifying activity was characterized as slow, since it is grouped with facultative heterofermentative lactobacilli. In agreement with these results, Sodini et al. [29] confirmed

its poor acidification activity, by reporting that more than 20 h were needed for *Lb. rhamnosus* to reach pH 5.0. In contrast Marroki et al. [30] reported two fast acidifying *Lb. rhamnosus* strains, which showed values of ΔpH 0.63 and 0.69 pH units after 6 h, as well as 1.87 and 1.96 pH units after 24 h. As for *B. amyloliquefaciens*, although its probiotic potential has been investigated [21], its acidifying capacity has not been thoroughly assessed.

Proteolysis is considered as one of the most important technological properties, actively participating in texture and flavor development of fermented products. Proteolytic activity of LAB has been studied for a variety of dry fermented meat products during the ripening process [31], dairy products [32] and sourdoughs [33]. In the present study, the initial screening with agar well diffusion assay revealed four proteolytic bacterial isolates, three of which were identified as *Lb. rhamnosus* and one as *B. amyloliquefaciens*, as well as nine proteolytic yeast isolates, three of which were identified as *S. cerevisiae* and six as *K. marxianus*. However, further analysis by SDS-PAGE revealed no difference in the protein profiles between the uninoculated samples, serving as control, and the ones inoculated with the microbial isolates. Similar results were reported by Kirilov et al. [34] and Dallas et al. [35]. In both studies, the inability of SDS-PAGE to properly depict caseinolytic activity was highlighted and longer incubation times were suggested. On the other hand, SDS-PAGE, applied on LAB isolated from dairy products, after the initial proteolytic screening by an agar well diffusion method, revealed the presence of 3 highly proteolytic isolates identified as *Enterococcus faecalis* (strains FT132 & FT522) and *Lb. paracasei* FT700 [36]. SDS-PAGE analysis confirmed their proteolytic capacity, showing the degradation of a-and β-caseins, as well as β-lactoglobulin and α-lactalbumin. In agreement with these findings, El-Ghaish et al. [37] using an agar well diffusion assay, as a screening proteolytic method on lactococci isolated from Egyptian dairy products, followed by further analysis by SDS-PAGE, reported six highly proteolytic isolates, four identified as *E. faecalis* and two as *E. faecium*. The proteolytic capacity of *Lb. rhamnosus* [38,39] as well as *B. amyloliquefaciens* [40–42] have been studied to some extent. In the first case, Moslehishad et al. [38] demonstrated the high protease capacity of *Lb. rhamnosus* PTCC 1637 in fermented cow and camel milk. In addition, the presence of probiotic cultures *Lb. rhamnosus* HN001 and *Lb. acidophilus* NCFM enhanced proteolysis in Edam cheese [39].

Bacillus species produce a wide range of extracellular and intracellular proteases, with subtilisins, a family of alkaline serine endoproteases, being the most extensively studied ones. The extracellular protease capacity is mainly attributed to an alkaline serine protease (subtilisin) and a neutral metalloprotease, while less than 1% to an esterase. As for intracellular proteases, at least two homologous genes have been reported in the genome of *B. subtilis* coding for serine proteases, intracellular serine protease and secretory subtilisin [43]. Samad et al. [42] reported the protease production by *B. amyloliquefaciens* B7, originating from fermented fish sauce and its purification with the use of an aqueous two-phase system (ATPS). Wang et al. [40] reported the cloning of a neutral protease encoding gene, *Banpr*, from *B. amyloliquefaciens* strain K11. Finally, high protease activity of *B. amyloliquefaciens* UEF01 endophytic to carnivorous plant *Utricularia exoleta* R. Br. was also demonstrated by Chaudhuri et al. [41], with the enzyme sharing similarities with alkaline proteases, previously isolated from *B. amyloliquefaciens*.

As far as yeasts were concerned, they constitute an extremely diverse group, with significant contribution to the formation of aroma and flavor precursors, during fermentation and maturation processes. Their ability to degrade residual lactose, lactate utilization, symbiosis with LAB, and their protein hydrolytic capacity necessitate their use as adjunct microbiota in the production of fermented dairy products. The proteolytic intracellular system of *S. cerevisiae* includes three categories of proteases, cytosolic proteasome, vacuolar proteases, and finally proteases within the secretory pathway [44]. Several studies have reported the poor proteolytic capacity of *S. cerevisiae* strains [45]. On the other hand, Klein et al. [46] reported that some yeast isolates, including *S. cerevisiae*, showed greater peptidase capacity compared to most bacterial species and were more capable of hydrolyzing β-casein phosphorylated peptides. The degradation of as1 and β-casein components of the *S. cerevisiae* FB7, isolated from blue veined cheeses, has also been stated [47]. Finally, *Seredyński* et al. [48] *reported*

that S. cerevisiae strains secreted diverse proteases, depending on the availability and quality of the protein substrate.

K. marxianus, an interesting yeast in biotechnology, has been regularly isolated from dairy products due to its ability to assimilate a wide range of sugars, such as lactose. Beniwal et al. [49] reported *K. marxianus* 6C17 ability to hydrolyze galactose into ethanol, thus verifying its variability in sugar fermentation and thereby its alignment with Crabtree-positive yeasts. However, little is known about its proteolytic capacity. Ramírez-Zavala et al. [50] demonstrated the presence of a *K. marxianus*-derived serine-carboxypeptidase, an exopeptidase, hydrolyzing C-terminal amino acids from peptides and proteins and proposed its usage as substitute for animal-originating enzymes in food industry. They also reported the isolation of a lysine aminopeptidase, originating from *K. marxianus*, which catalyzed the removal of *n*-terminal alkaline residues, mainly lysine. Its strong suppression by bestatin, o-phenanthroline and EDTA, has led to its characterization as a metalloprotease.

Lipolysis, the hydrolysis of triacylglycerols to free fatty acids (FFA) mono- and diglycerides, has been attributed to the presence of lipases and esterases, with the former hydrolyzing esters of longer chain fatty acids, FA, and the latter preferentially acting on esters of shorter chain FFA [51]. Further FFA catabolism leads to the production of impact-aromatic compounds, namely esters, methylketones, lactones and secondary alcohols. Lipolysis in fermented meat products, such as dry-fermented sausages, has been partly attributed to members of *Staphylococcus* genus and partly to muscle and fat tissue enzymes and is essential for the development of dry-cured sausage flavor [52]. Lipolysis during cheese ripening has also been studied by many authors [53]. However, milk fat lipolysis does not happen in a great range of circumstances, which is the reason why many authors have focused on studying the proteolysis and FFA catabolism, as determining factors for the formation of aroma compounds. Lipolysis has been investigated in fermented products, such as wine [54] and fermented olives [55]. In our study, the lipolytic activity was initially assessed by a screening agar well diffusion assay and further by quantitative evaluation of the lipase activity, a procedure that has been also applied previously [10]. Seven *B. amyloliquefaciens* and 33 *Lb. rhamnosus* isolates as well as 29 *S. cerevisiae* and 16 *K. marxianus* isolates showed halos around the well and were further analyzed with titrimetric assay. The bacterial strains with the highest lipase activity were LQC 1990 (10.50 AU/mL) and 2023 (10.05 AU/mL), identified as *B. amyloliquefaciens* and *Lb. rhamnosus*, respectively. The best lipase producing yeast isolates were LQC 10237 (20.85 AU/mL), 10199 (16.05 AU/mL), 10235 (15.25 AU/mL) and 10248 (13.25 AU/mL), the former two identified as *S. cerevisiae* and the latter as *K. marxianus*. At yeast level, *S. cerevisiae* and *K. marxianus* strains have been characterized as poor lipase producers [54,55]. Hansen & Jakobsen [47] demonstrated that *S. cerevisiae* strains isolated from blue veined cheeses could ferment tributyrine and low chain FFA, but not C:14 FFA, thus indicating no lipase activity.

Few studies have elucidated the lipolytic system of *K. marxianus*. *S. cerevisiae* and *K. marxianus* lipolytic activity were investigated by Hernández et al. [56]. In that study the majority of the yeast strains were able to hydrolyze tributyrine, thus exhibiting esterase capability. However, less strains exhibited lipase activity when olive oil was used as substrate. In contrast, Cardoso et al. [57], studying the proteolytic and lipolytic profile of yeasts isolated from traditional Brazilian cheese, revealed that *K. marxianus* 83F achieved the highest lipase capacity (0.06 ± 0.04 U/mL), while *K. marxianus* 60P showed β-galactosidase activity.

As far as bacterial isolates were concerned, several authors have reported moderate to none lipolytic activity of *Lb. rhamnosus* strains [58]. However extended ripening period, contributes to the hydrolysis of considerable amounts of FFA [59]. Weak lipolytic capacity constitutes a beneficial attribute for fermented food products, such as cheese, by inhibiting the development of bitter and rancid aroma-compounds. Falcinelli et al. [60] demonstrated that *Lb. rhamnosus* administration caused transcriptional modifications in TGs biosynthesis associated genes, namely *agpat4*, *dgat2,* and *fit2* and probably induced lipolysis. An inhibition of *agpat4* and *dgat2* transcription, and thus a decline in triglyceride capacity and a reduction of LD aggregation, stimulated by lower levels of *fit2* gene, showed the triggering of lipolysis by *Lb. rhamnosus*.

Bacillus spp. is a well-known producer of valuable extracellular enzymes, such as lipases. In most cases, lipases are induced from lipidic carbon substrates, namely oils, fatty acids and glycerol. From an industrial point of view, lipase production is gaining much attention due to its use as cheap and versatile catalysts in biodiesel processing. Due to the rigid regulation of lipase synthesis, the cloning of lipase genes is often preferred. The first documentation of lipase gene from *B. amyloliquefaciens* cloning and application in biodiesel production was reported by Cai et al. [61]. *B. amyloliquefaciens* Nsic-8 lipase gene, *lipBA*, was isolated from a stinky tofu brine and successfully cloned in *E. coli*. Saengsanga et al. [62] also reported the cloning of another *lip* gene from *B. amyloliquefaciens* E1PA in *E. coli* and showed the affinity of the inducible alkaline E1PA lipase with wider range of substrates.

Bacteria may produce a wide variety of antimicrobial compounds, such as organic acids, ethanol, hydrogen peroxide and bacteriocins. The latter have been in the epicenter of intensive study over the last decades; knowledge on the factors that affect their production, their biochemical properties, as well as their applicability within the context of the hurdle theory, is constantly expanding [63]. The production of bacteriocins from *Lb. rhamnosus* and *B. amyloliquefaciens* strains has been reported [64]. Bacteriocin production and sensitivity are strain-dependent properties [65]; the latter may partially explain their in situ limited use. In order to address this limitation, a mixture of strains is increasingly used in screening studies so that detection of antimicrobial compounds of broader range is facilitated. In the present study, no such compounds were detected.

In conclusion, according to the results obtained in the present study, dominance of yeast over bacterial population was evident in all sugary kefir samples assessed and attributed to the elevated carbohydrate concentration, compared to milk kefir. Regarding the technological properties of the isolates, *Lb. rhamnosus* strains LQC 2014 and 2015 that exhibited potent acidification capacity as well as proteolytic and lipolytic activities along with *S. cerevisiae* strains LQC 10184, 10235, and 10237 and *K. marxianus* strains LQC 10179, 10182, 10245, 10246, 10247, and 10248 that exhibited proteolytic and lipolytic activities are suitable candidates for the formation of the microbial consortium that is necessary to drive milk and sugary kefir fermentations.

Author Contributions: Conceptualization, M.P., I.N., S.P., E.H.D.; apply of analytical methods, M.K.S., I.N., S.P.; Writing–Original Draft Preparation, M.K.S., M.P., I.N., S.P., Review & Editing M.K.S., M.P., I.N., S.P., E.H.D.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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