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Production and Biochemestry-Molecular Analysis of Microbial Community Fermenting Whey as a Potential Probiotic for Use Animals

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ABSTRACT

The aims of this work were: To achieve a simple and low cost propagation of potential probiotic agents using plain whey as a culture medium, study the diversity of the members of the bacterial community (MC) in plain whey and to evaluate the probiotic capacity of this MC. After a systematic selection of agents according to their growing capacity in whey, the constituted MC was considered as a unit. Biochemical characterization of the lactic acid bacteria were performed using the API system. Molecular characterization of the lactic acid bacteria was realized using AFLP™ DNA-fingerprinting, partial 16S rDNA sequence analysis and PCR-denaturing gradient gel electrophoresis (PCR-DGGE). The physiological characterization of yeast was determined with the automated microplate method Allev/Biolomics and using yeast characterization system based on standard taxonomic criteria. The identification molecular was realized by PCR-fingerprinting. The resistance of MC to pH and bile salts were evaluated. The MC was composed of agents from different separated Dominium like Bacteria (*Lactobacillum*) and Eukaria (*yeast*). They are multispecies and also multistrain assuring high biodiversity. The MC grew at low pH and different concentrations bile salts.

Keywords: Probiotic; Multispecies; Multistrain; Plain Whey

1. Introduction

Whey is a cheese industry effluent and a powerful environmental contaminant, in most cheese making regions in Argentina. In recent years, the recovery and marketing of whey components like proteins, minerals and lactose has diminished the quantities of this effluent discarded to the environment [1]. The fermentation of whey by different procedures as an alternative to produce single cell protein, beverages, ethanol and methane has been communicated by various authors [2-4]. In our case the production of a microbial community fermenting whey could be utilized as potential probiotic in animals. A probiotic is a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" [5]. Thus, using a probiotic means an intervention on the intestinal ecosystem. Probiotic bacteria would have antagonistic impact against intestinal infectious bacteria just by competitive exclusion [6], modulating the intestinal medium, favoring growth of friendly bacteria or producing natural antibiotics or bacteriocines [7].

The aim of this work was: To conform a microbial community of simple and low cost propagation with potential probiotic agents using plain whey as a culture medium, to analyze the species that constitute the microbial community (MC) by means isolating and identifying strains through the application biochemistry and molecular techniques and to evaluate the probiotic capacity of this MC.

2. Materials and methods

Development of the microbial community: Fresh pooled whey from cheese producing companies located in Tandil, Argentina, was collected and transported to destination in food grade stainless steel tanks. Average

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protein concentration was 3.5 ± 0.5 mg/ml and pH 5.7 ± 0.3. Immediately after arriving, whey was centrifuged up to 2800 xg keeping a continuous flow of 4.167 l/min, at room temperature. Fat free whey was sterilized by micro filtering successively through 1 µ, 0.5 µ and 0.1 µ pore size Koch membranes and was used for MC development.

We examined whey fermentation capacity of some Caucasian Kefir agents and then added local lactic acid bacteria obtained from pooled whey in local cheese producing plants. Criteria for the addition of new local strains were as follows: The final pH had to be lower and the biomass (g/l) had to be equal or larger than obtained by Kefir agents. It was considered a satisfactory community fermenting whey which consistently drops pH down to 3.6 ± 0.1 and biomass increases to 6 ± 0.5 g/l after 24 h culture. Cultures were incubated 24 h in stationary conditions at 37˚C. Biomass was harvested by centrifugation at 10˚C and 2800 xg keeping a continuous flow of 4.167 l/min.

Freeze-drying: Compact biomass obtained by MC centrifugation was suspended (1:2) in 10% (w/v) reconstituted skim milk and 5% (w/v) sodium glutamate (pH was adjusted to 5 with 1M NaOH). Bacterial suspension was freeze-drier. After the freeze-drying cycle has been complete, dried MC was stored at 4˚C under vacuum.

Isolation and biochemical characterization of the lactic acid bacteria were carried out by the Centro de referencia de Lactobacillus, Tucumám, Argentina (CERELA).

Lactic acid bacteria isolation: A twenty grams sample of the compact biomass obtained by MC centrifugation were added to 180 ml of saline-2 peptone water (2% of NaCl, 0.1% of bacteriological peptone and 1% of Tween 80) and mixed for 3 min into a sterile stomacher bag (Stomacher Lab-Blender 400, A. J. Seward Lab., London, England)

Serial dilutions were made in 1‰ peptone water and plated on MRS [8] agar, MRS pH 5.4, LAPTg agar [9], KF agar and ST medium, all supplemented with cycloheximide (25 µg/ml) to suppress yeast growth. Plates were incubated at 30˚C and 37˚C anaerobically for 6 days. Isolated colonies that differed microscopically were randomly selected. The isolates were propagated in MRS broth and purified. Gram positive rods and negative to catalase reaction were selected for further studies as presumptive *Lactobacillus* species.

Biochemical characterization: Bacteria were first clustered on the basis of cellular morphology, growth at 15˚C, 30˚C, 37˚C and 45˚C, 0.1% and 0.3% methylene blue, nitrate reduction, indol production, ammonia production from arginina, esculin hydrolysis, CO_2 production from glucose and gluconate, ability to form diacetyl from citrate. Carbohydrate fermentation tests of selected lactobacilli were performed using the API 50CH system (bioMerieux, Marcyl' Etoile France) according to the manufacturer instructions. Tests preparations were incubated at 37˚C and final readings were made after 48 h. Fermentation profiles were analyzed by APILAB Plus version 4.0 program (bioMerieux, Marcyl' Etoile France).

Molecular characterization of the lactic acid bacteria: AFLP™ DNA-fingerprinting, partial 16S rDNA sequence analysis and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) were carried out by the BCCM/LMG Bacteria Collection (Ghent University. Belgium). AFLP™ (The AFLP™ technology is subject to patents and patent applications and AFLP is a registered trademark, all owned by Keygene N. V., the Netherlands) is a PCR based technique for whole genome DNA fingerprinting via the selective amplification of restriction fragments [10].

Five lyophilized individual specie biochemically characterized and 2 lyophilized samples of the whole MC, obtained 4 months apart, were sent to the BCCM/LMG. The twelve lyophilized cultures were recovered and checked for purity on MRS (Oxoid CM361) after anaerobic incubation at 37˚C for 72 h. One well isolated colony was picked for further cultivation and subsequent analyses. Elementary bacteriological tests (cell morphology, gram stain, oxidase and catalase reactions) were performed for purity check.

DNA was prepared using the method of Gevers *et al*. 2001 [11], slightly modified. Purified total DNA was digested by two restriction enzymes (4 and 6 base cutter). In this way, only a limited number of fragments with two different ends and of suitable size for efficient PCR were obtained. Small ds DNA molecules (15 - 20 bp) containing one compatible end were ligature to the appropriate "sticky end" of the restriction fragments. Both adaptors are restriction half site-specific and have different sequences. These adaptors serve as binding sites for PCR primers. In the current analyses, the following restriction enzymes and adaptors were used:

Restriction enzyme: EcoR I [hexacutter]
Adaptor: 5'-CTCGTAGACTGCGTACC-3'
3'-CTGA CGCATGGTTAA-5'
Restriction enzyme: Taq I [tetracutter]
Adaptor: 5'-GACGATGAGTCCTGAC-3'
3'-TACT CAGGACTGGC-5'

Selective amplification of some of the restriction fragments: PCR primers were specifically hybridized with the adaptor ends of the restriction fragments. Since the primers contain at their 3'-end one or more so-called "selective bases" that extend beyond the restriction site into the fragment, only those restriction fragments that have the appropriate complementary sequence adjacent to the restriction site will be amplified. Following primer combination was used:

E01:5'-GACTGCGTACCAATTCA-3', T11:5'-GTTT

CTTATGAGTCCTGACCGAA-3'. PCR products were separated according to their length using an ABI Prism® 3130XL Genetic Analyzer. Fragments that contain an adaptor specific for the restriction half site created by the 6-bp cutter are visualized due to the 5'-end labeling of the corresponding primer with the fluorescent dye FAM.

The resulting electrophoreses patterns were normalized and subjected to a band pattern recognition procedure using the GeneMapper 4.0 software (Applera, USA). A normalized table of peaks, containing fragments of 20 to 600 base pairs, was transferred into the BioNumerics™ 4.61 software. For numerical analysis, a data interval was delineated between the 40 and 580 bp bands of the internal size standard. The profiles were compared with the reference profiles of the lactic acid bacteria (including bifidobacteria) as currently available in BCCM database. Clustering of the patterns was done using the Dice coefficient and the Upgma algorithm.

Partial 16S rDNA sequence analysis and phylogenetic study: DNA prepared for the PCR-DGGE analyses was used. 16S rRNA genes were amplified by PCR using the forward primer 16F27 (pA) 5'AGA GTT TGA TCC TGG CTC AG 3' and 6R1522 (pH) 5'AAG GAG GTG ATC CAG CCG CA 3' (hybridizing position referring to E. coli 16S rRNA gene sequence numbering 8-27 and 1541-1522 respectively). PCR amplified 16S rDNAs were purified using the NucleoFast® 96 PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit and purified using the BigDye® XTerminatorT Purification Kit Sequencing was performed using an ABI Prism® 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The following forward and reverse primers were used to get a partial overlap of sequences, ensuring highly reliable assembled data: 16F358 (Gamma) 5'CTC CTA CGG GAG GCA GCA GT 3', 16R339 **(**Gamma) 5'ACT GCT GCC TCC CGT AGG AG 3', 16R519 (BKL1) 5' GTA TTA CCG CGG CTG CTG GCA 3' (hybridizing position referring to E. coli 16S rRNA gene sequence numbering 339-358, 358-339 and 536-516). Sequence assembly was performed by using the program Autoassembler™ (Applied Biosystems, Foster City, CA, USA).

A similarity matrix was created using the software package Bio Numerics (Applied Maths, Belgium) by homology calculation with a gap penalty of 0% and after discarding unknown bases and is based on pairwise alignment using an open gap penalty of 100% and a unit gap penalty of 0%.

Phylogenetic analysis was performed using the software package Bio Numerics after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. A resulting tree was constructed using the neighbor-joining method.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE): PCR-DGGE analysis was carried out as described in Van Hoorde *et al*., 2008 [12].

Physiological and molecular characterization of yeast.

Each sample of freezer-died power of MC after addition of an equal amount of peptone water were cultured on dextrose yeast extract peptone agar (DYPA) with 0.02% of chloramphenicol. The physiological and morphological profiles of the yeast isolates were determined with the automated microplate method Allev/Biolomics (BioAware SA, Hannut, Belgium) [13], and using yeast characterization system based on standard taxonomic criteria [14,15]. The identification molecular was realized according to Kutzman and Robnett 1998 [16] and Barnett *et al*., 2000 [17].

For DNA sequence analyses, the primer pair LR0R-LR6 was used to amplify the D1/D2 region of the large subunit (LSU) of the ribosomal gene complex [18]. Successful PCR amplifications resulted in a single band observed on a 0.8% (w/v) agarose gel, corresponding to approximately 600 bp. PCR products were cleaned using the QIAquick PCR purification kit, following the manufacturer's protocol. Sequencing reactions were performed using a CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, California, USA), according to the manufacturer's recommendations, with the primers LR0R and LR3. Nucleotide sequences were determined using a CEQ 2000 XL capillary automated sequencer. The sequences were assembled and edited in Sequencher 4.8 (Gene Codes Corp., Ann Arbor, Michigan, USA). After initial BLAST searches for the most similar sequences, alignments were performed to compare type strain sequences with the query sequences. The number of substitutions and potential insertions or deletions (indels) was determined from these pairwise comparisons using BioEdit 7.0.5.3 [19]. The sequences were deposited in the EMBL databank (http://www.ebi.ac.uk/EMBL; Hinxton, UK) with accession numbers DIV/08 182 A, 182 B, 182 C, 182 D.

High-molecular-mass DNA for PCR-fingerprint analyses was extracted using the Invisorb® Spin Plant Mini Kit (Invitek GmbH, Berlin, Germany) as described by Groenewald *et al*., 2008 [20]. PCR-based fingerprinting with the single primer M13, a ubiquitous microsatellite sequence [21], was applied as described before by Groenewald *et al*., 2008 [20]. The resulting M13-based PCR-fingerprint profiles were compared visually with reference strains.

***"In vitro"* evaluation of the MC probiotic capacity.** An aliquot of MC, was inoculated into acidified MRS broth to pH 2.5, 3 and 4 with HCl (1N) and non-acidified (pH 6.8) and incubated for 3 h at 37˚C.

After incubation, serial dilutions in peptone water were spread onto MRS agar plates. Plates were incubated anaerobically for 48 h at 37˚C.

Percentage of resistance to each analyzed pH was calculated by the equation: %RpH = $[(UFC/ml)_{MRS\ pH...} \times 100]/(UFC/ml)_{MRS\ pH\ 6.5}$ [22].

An aliquot of MC was inoculated into acidified MRS broth with 0.03, 0.05 and 0.1 concentrations of bile salt and within bile salts and incubated for 3 h at 37˚C.

After incubation, serial dilutions in peptone water were spread onto MRS agar plates at 1h and 3 h post incubation. Plates were incubated anaerobically for 48 h at 37˚C.

Percentage of resistance to each analyzed bile salt concentration was calculated by the equation: %RpH = $[(UFC/ml)_{MRS\ pH...} \times 100]/(UFC/ml)_{MRS\ pH6.5}$ [22].

3. Result

3.1. MC Development

In optimum anaerobic culture conditions, the biomass production in creased to 6 ± 0.5 g/l and pH fell to 3.6 ± 0.1 after 24 h culture. Periodic microscopic examination of the MC showed a homogeneous proportion in the quantity of yeast and lactic acid bacteria.

The viability after MC rehydration was 3.2×10^9 lactobacilli plus 1.5×10^6 yeast CFU per gram lyophilized compact biomass.

3.2. Isolation and Biochemical Characterization of the Lactic Acid Bacteria

Most of the random isolates from MC (95%) expressed general characteristics corresponding to lactic acid bacteria (Gram-positive, catalase-negative, non-motile rods).

From each resulting cluster, one to four strains were selected for further analysis so twelve lactobacilli were characterized by fermentation profiles.

Phenotypic characterization of the microbial community indicated the following species and strains: *Lactobacillus helveticus* (ID11609, ID11610, ID11618), *Lactobacillus delbrueckii* (ID11611), *Lactobacillus paracasei* (11614), *Lactobacillus fermentum* (ID11613, ID11616, ID11619), *Lactobacillus* sp (ID11612, ID11615, ID11617), and *Lactobacillus buchneri* (ID11620).

3.3. Molecular Characterization of Lactic Acid Bacteria

The results of the identification using AFLP and 16S and PCR-DGGE are given.

Two colony types were isolated from ID11615: Smooth (t1) and rough (t2) colony. The *Lactobacillus* identificated were: *L. helveticus* (ID11609, ID11610, ID11611 and ID11612), *L. fermentum* (ID11613, ID11615 t1, ID11616), *L. paracasei* ID11614, ID11618), *L. casei* (ID11617), *L. parabuchneri* (ID11620) as are shown in the dendograms of the cluster analyses of AFLP™ profiles (**Figures 1-4**).

For the rough colony and for ID11619, no profile could be obtained. Parcial 16S rDNA sequence analysis was carried out. The percentage 16S rDNA sequence similarity was *Lactobacillus gasseri* DSM 20243^T (100) and *Lactobacillus panis* DSM 6035^T (99.8) respectively.

Similar DGGE results were obtained for both gradients (35% - 60% and 35% - 70%). The results are shown in **Figure 5**.

3.4. Physiological and Molecular Characterization of Yeast

The results obtained are shown in **Table 1**.

3.5. "*In Vitro*" Evaluation of the MC Probiotic Capacity

Obtained percentage of resistance pH and bile salts are shown in **Table 2**.

4. Discussion and Conclusions

The Kefir is a complex probiotic. Its lactobacilli and yeast components were selected over thousands of years towards milk fermentation [23]. In a similar manner, we took from Kefir the most adaptable members to grow in whey. Other lactobacilli and yeast found in our environment, and which improved whey fermentation, were also selected and incorporated to the developing consortium. Based on a consistent fermentation in successive whey baths, determined by pH modification and sensorial examinations like smell and taste, we considered the constituted community as a unit. This consistent fermentation suggested a symbiotic relationship and adaptation capacity of the MC.

This MC is composed by at least seven lactic acid bacteria and two yeast species, comprising agents from separated dominium like Eukaria and Bacteria. The application of molecular biology tools to better understand the integrated process of this complex consortium would allow us, in the near future, to use specific community members, or specifically tailored consortia of members, to target desired interactions, resulting in health benefits and food production improvement [24,25].

Selected lactic acid bacteria and yeast are multispecies and also multistrain assuring high biodiversity. The advantage of multistrain and multispecies probiotics is that a number of favorable characteristics of individual strains are combined in a single preparation [26]. It has been clearly shown that multispecies preparations have advantages when compared to monostrain probiotics or, to a lesser extent, multistrain probiotics [27]. Well-designed

multispecies probiotics can benefit from a certain amount of synergism when the effects of different probiotic species are combined [28,29]. The activity can also be stimulated through simbiosis among strains in the preparation [30-32]. How probiotics, whether monospecies or multistrain/mulitispecies formulations, actually helping the host to attain such greater protection levels is still poorly understood [32,33].

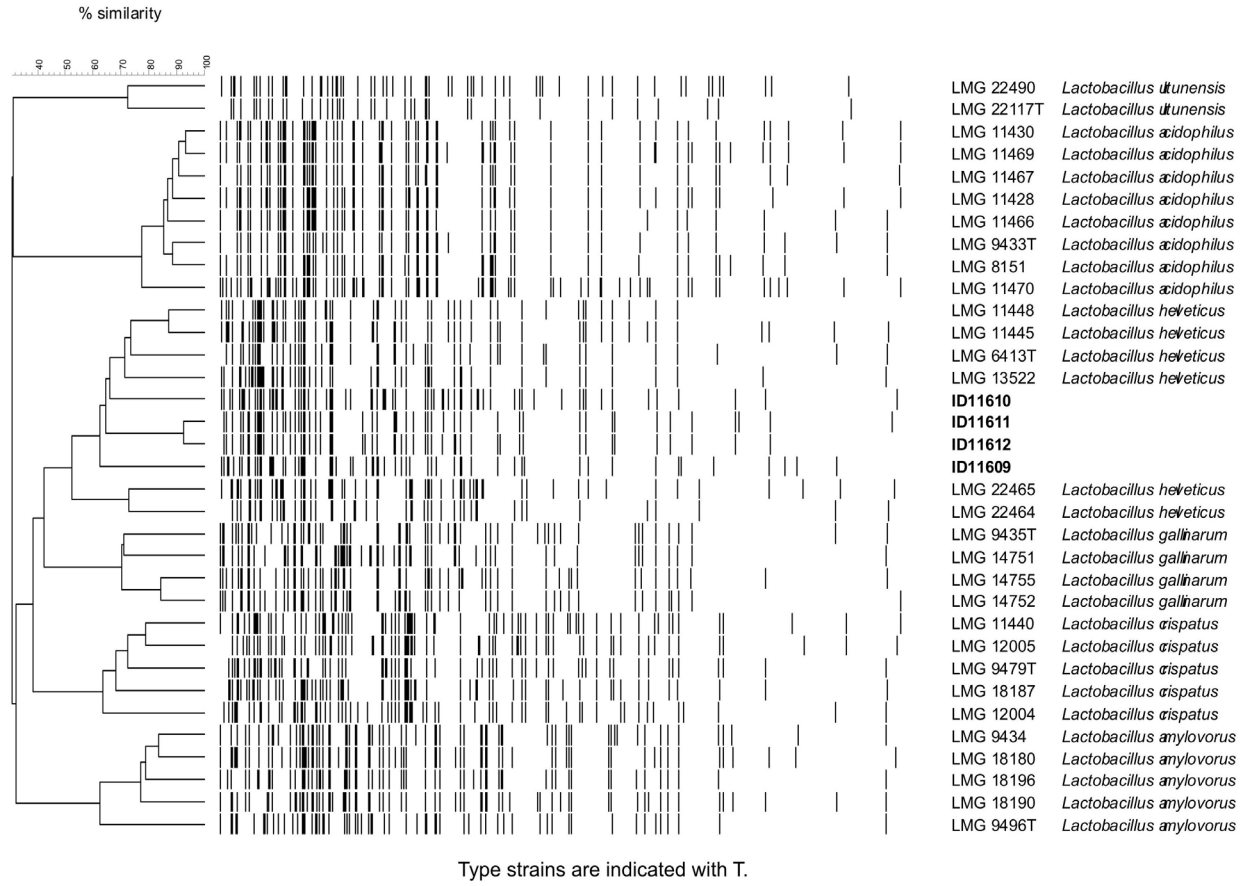


Figure 1. Dendrogram of the cluster analyses of AFLP™ profiles (ID11609-D11612).

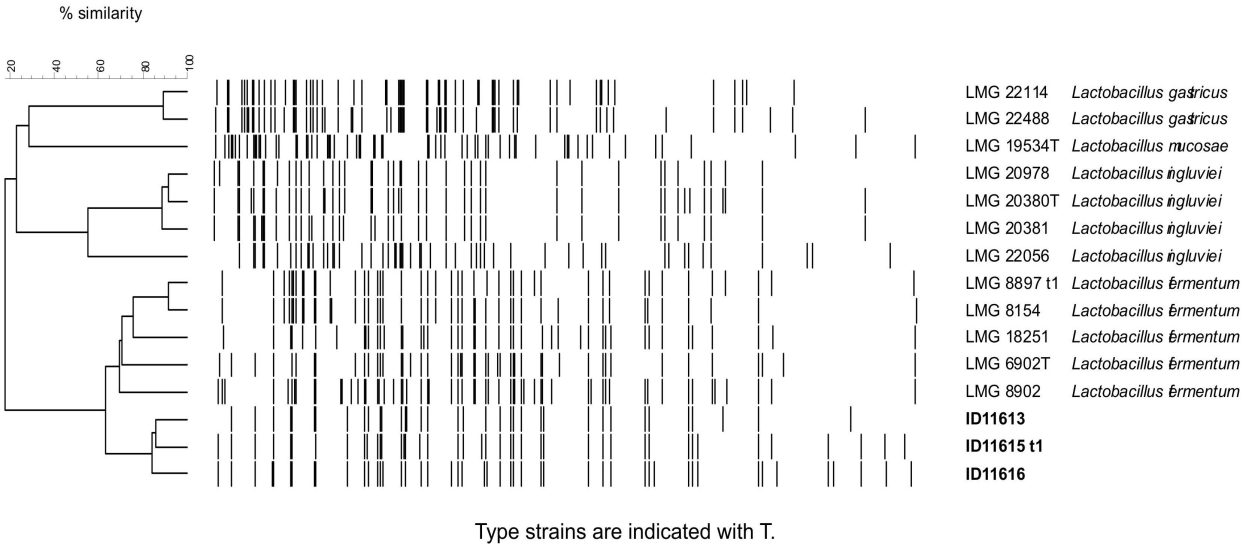
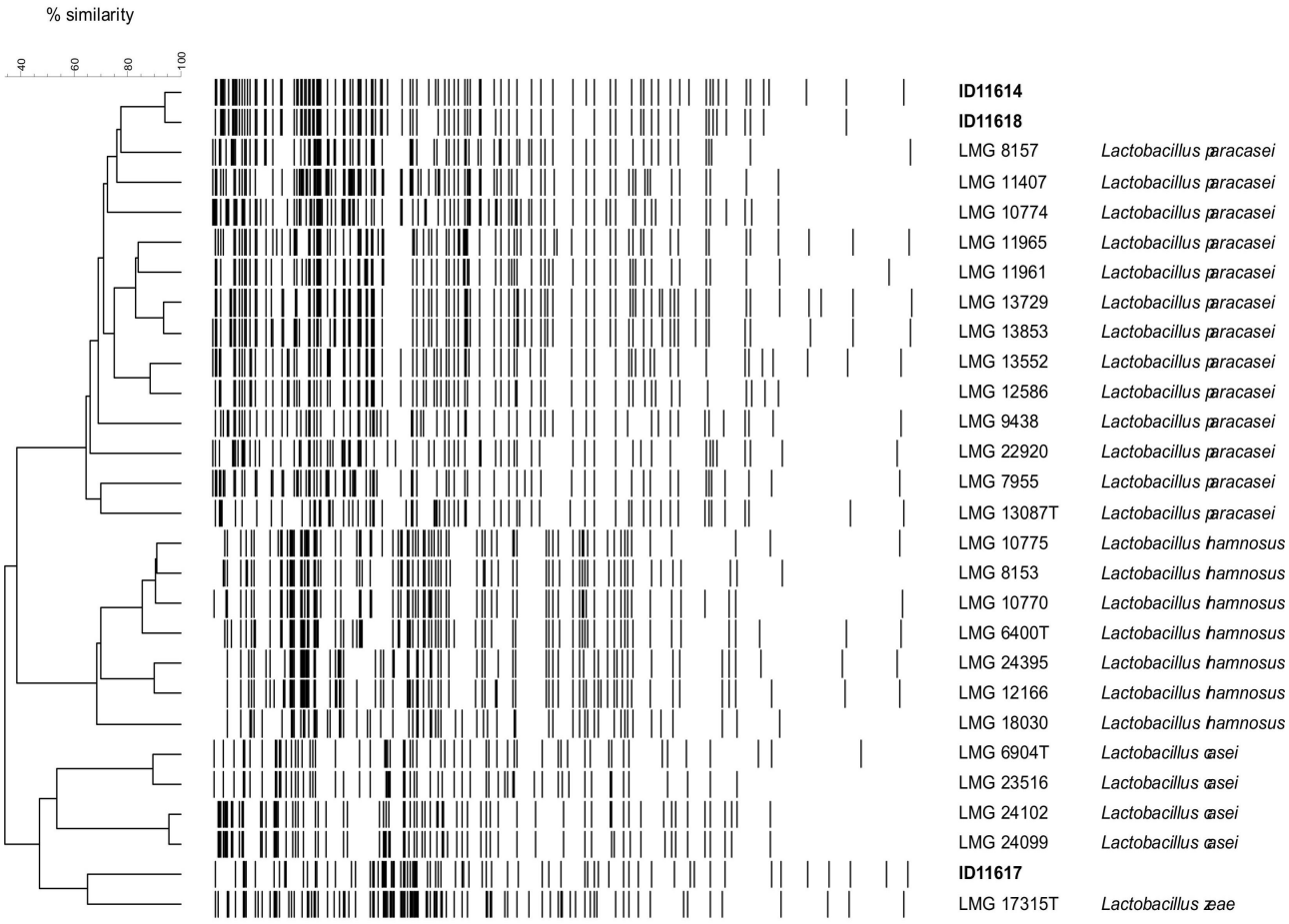
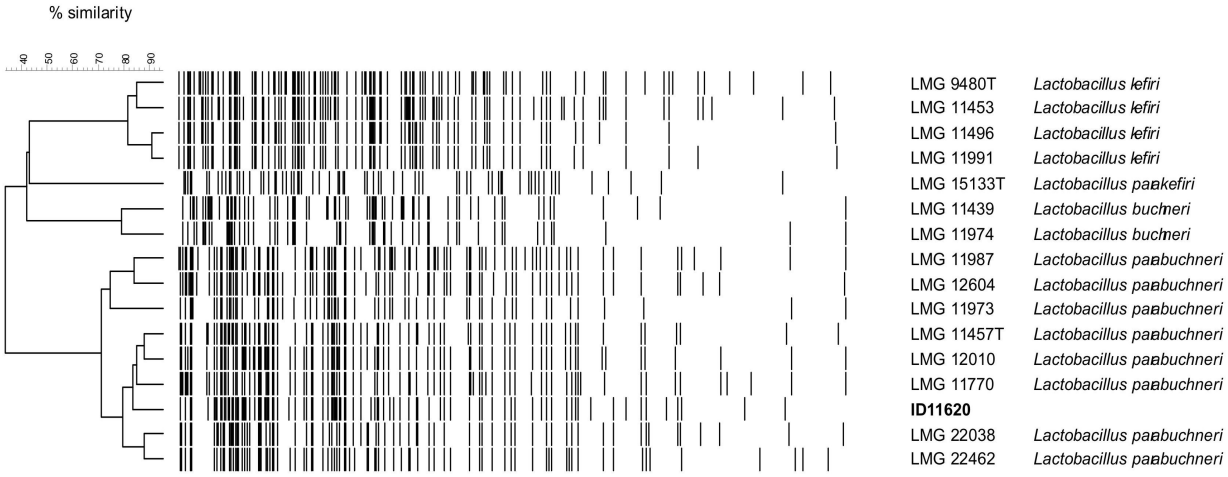


Figure 2. Dendrogram of the cluster analyses of AFLP™ profiles (ID11613, ID11615 t1 and ID11616).



Type strains are indicated with T.
Note: *Lactobacilus zeae* is a later heterotypic synonym of *Lactobacillus casei*.

Figure 3. Dendrogram of the cluster analyses of AFLP™ profiles (ID11614, ID11617 and ID11618).



Type strains are indicated with T.
Please note that the current 'LAB' database does not contain a profile of the type strain of *Lactobacillus buchneri*.

Figure 4. Dendrogram of the cluster analyses of AFLP™ profiles (ID11620).

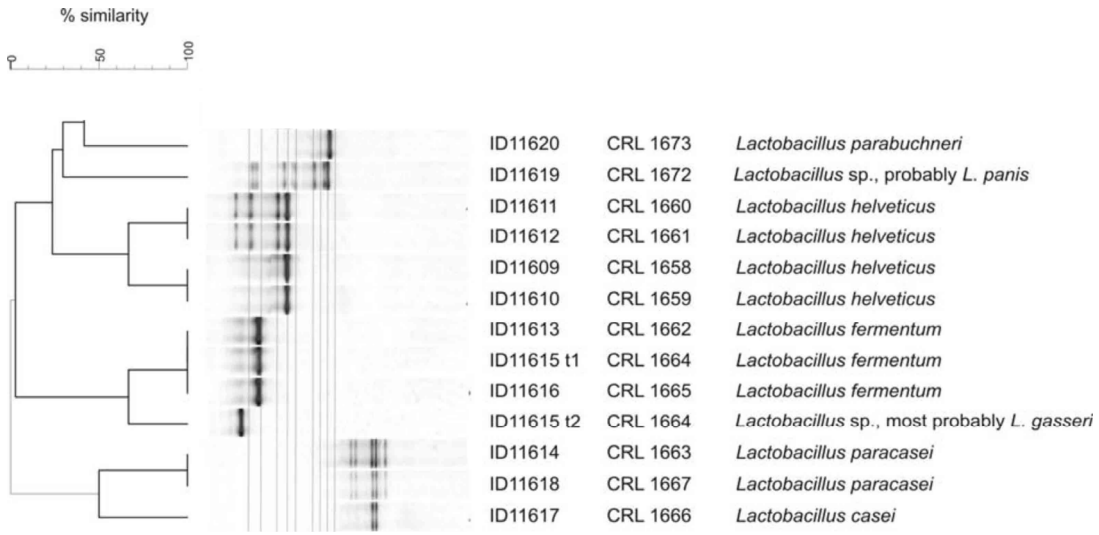


Figure 5. PCR denaturing gradient gel electrophoresis (35% - 70%). Computer out prints of the profiles obtained for the 13 samples.

Table 1. Yeast isolates, colony types and the results morphological and physiological characterization and molecular identification.

Reference	BCCM/MUCL Collection number	Colony type	Morphology and physiological characterization	Molecular identification by D1/D2 large subunit rDNA sequence
DIV/08-182A	MUCL 51663	Surface pulverulent, relief umbonate	*Pichia occidentalis*	*Pichia kudravzevii*
DIV/08-182B	MUCL 51664	Surface cerebriform	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
DIV/08-182C	MUCL 51665	Surface smooth, relief umbonate	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
DIV/08-182D	MUCL 51666	Small colonies, surface smooth	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*

Table 2. Percentage of resistance of CM to 2.5, 3 and 4 pH and to 0.03, 0.05 and 0.1 bile salts concentrations at time different.

Time of incubation (h)	Bile salts concentrations (%)			pH		
	0.03	0.05	0.1	2.5	3	4
1	99.4	15.2	5.9	-	-	-
3	70.3	24.7	2.0	23.3	77.2	85.4

The food transit time through the monogastric animals is about 90 min [34]. The tolerance at different pH and bile salt concentration obtained in this work indicate the possibility to explore the usefulness of this MC as probiotic in animal model. The MC should be tested to detect the possible production of adverse effect and be evaluated if this product could increase the health status of calves through of the stimulation of the immature newborn immune system preventing the diarrhea responsible for high mortality and morbidity in neonates [35-40].

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