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Recovery and Identification of Bovine Colostrum Microflora Using Traditional and Molecular Approaches

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Summary

Colostrum is the first milk produced by the mammary gland during the early postpartum period. In addition to nutrients, antimicrobial and growth factors, bovine colostrum contains valuable microflora, including members of the genera *Lactobacillus* and *Bifidobacterium*, which have been used widely in probiotic food. The aim of this work is to identify and quantify the microflora of colostrum samples from two Holstein dairy cows on the first three postpartum days through culture-dependent 16S rRNA gene sequencing and length heterogeneity polymerase chain reaction (LH-PCR) techniques, using an adapted method to extract the total DNA. Twenty-nine strains isolated in synthetic media were identified by their 16S rRNA gene sequences, revealing two potential probiotic strains (*Lactobacillus casei* and *Bifidobacterium pseudolongum*). Colostrum samples were subjected to LH-PCR analysis to obtain fingerprint profiles of the microflora. *Lactobacillus casei* was the main species present in the samples. The probiotic potential of these bacteria for use in fermented and functional foods remains to be evaluated.

Key words: bovine colostrum, microflora identification, 16S rRNA, LH-PCR, probiotic microorganisms

Introduction

Colostrum is the first milk produced by the mammary gland in the initial 24 to 96 h of the postpartum period. The production varies depending on the animal species. In the cow, colostrum secretion continues until the sixth milking (*1*). In addition to being a rich source of nutrients (protein, fat, lactose, vitamins and minerals), colostrum provides protection to the neonate through antimicrobial substances such as immunoglobulins, lactoferrin, lactoperoxidase, lysozyme and cytokines and also promotes growth through growth factors such as transforming growth factor (TGF) and epidermal growth factor (EGF). In addition, this raw material contains valuable microflora, including members of the genera *Lactobacillus* and *Bifidobacterium*, which have been used widely in functional or probiotic foods (*2*).

PCR-based techniques that have been used to characterise lactic acid bacteria (LAB) populations and other microorganisms include terminal restriction fragment length polymorphism (T-RFLP), tDNA intergenic spacer PCR (tDNA-PCR) and length heterogeneity PCR (LH-PCR) (*3–5*). LH-PCR is a culture-independent method that has

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been used to identify and monitor the evolution of microbial populations of different dairy products (*5,6*), ensiled maize (*7*) and soil (*8*). This technique has proven to be efficient, reliable and reproducible. In addition, microbial composition patterns can be obtained without isolating single components (*6*), and dominant populations can be estimated qualitatively and quantitatively more quickly than by other methods.

The consumption of probiotics is increasing, and the isolation of new probiotics has been explored extensively. Probiotics can be defined as live microorganisms that confer a health effect to the host when consumed in adequate amounts (*2,9*). The natural lactic microflora of bovine colostrum could be a source of new probiotics. In this study, methods will be adapted to better extract and purify the total DNA from bovine colostrum, and the microflora will be detected and identified by culture-dependent 16S rRNA gene sequencing and LH-PCR techniques.

Materials and Methods

Colostrum sampling

Colostrum samples were obtained from the Santa Cecília farm (Piraquara, PR, Brazil) from two Holstein dairy cows (samples 425 and 539) of the same age in second parturition. Samples were collected twice daily (early morning and late afternoon milkings) on the first three days (N=6) postpartum. The cows used in this study exhibited no health problems and were not treated with antibiotics. Following routine udder preparation, which included forestripping, predipping with a 0.5 % iodine-based teat dip and drying the teat ends with a clean cloth towel, all teat ends were scrubbed with an alcohol-soaked gauze pad. Colostrum samples were then collected aseptically in 50-mL sterile tubes, transported to the laboratory and stored at –20 °C.

Bacterial recovery

Serial 10-fold dilutions of colostrum (50 mL) in 50 mM sodium citrate buffer (pH=7.0) were pour plated on agar media. To count and recover the cultivable bacterial population, the following temperature and time conditions were used to incubate the tested media: under aerobic conditions, total plate count on milk plate count agar (MPCA; Oxoid S.p.A, Milan, Italy) at 37 °C for 48 h; under anaerobic conditions, lactobacilli on MRS agar (Oxoid S.p.A.) with 0.05 % cysteine (MRS CYS) at pH= 5.4 and 25 and 37 °C for 72 h, and streptococci on M17 agar (Oxoid S.p.A.) at 25 and 37 °C for 72 h. Counts were performed in triplicate, and the standard deviations (S.D.) of the mean values were calculated.

Strain identification by 16S rRNA gene sequencing

A representative number of colony-forming units (CFU) from the three growth media was selected for each of the samples on the basis of macroscopic morphology and isolated. Twenty-nine strains were purified in MRS CYS or M17 broth, depending on whether the cellular morphology observed after microscopic examination was bacilli or cocci. Genomic DNA from the 29 purified strains was extracted from overnight cultures using Chelex 100 (Sigma-Aldrich Co., St. Louis, MO, USA) as described by Giraffa *et al.* (*10*). The presence of DNA was confirmed on a 1.5 % agarose ethidium bromide gel, and DNA concentration and purity were determined spectrophotometrically at 260 and 280 nm (Jasco V-530 spectrophotometer, Jasco Inc, Tokyo, Japan). DNA amplification and sequencing were performed as previously described by Giraffa *et al.* (*10*). The species were assigned through BLASTn (*11*) alignment of the obtained sequences with the 16S rRNA gene sequences of LAB available from the EMBL database (Cambridge, UK) (*12*).

LH-PCR of the bacterial strains

The V1 and V2 variable regions of the 16S rRNA genes of the bacterial strains were analysed by the LH-PCR technique as previously described (*6*). Using fluorescently labelled forward primer 63F (5′-[6FAM] CAG GCC TAA CAC ATG CAA GTC-3′) and unlabelled reverse primer 355R (5′-GCT GCC TCC CGT AGG AGT-3′), a theoretical amplicon length of 276–327 bp was expected for eubacterial domain A (*13*). The products were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) under denaturing conditions. The peaks of the electropherogram profiles, which corresponded to amplicons of different length, were attributed to bacterial species using a previously obtained LH-PCR database (*5,6*). Amplicon sizes were determined with GeneMapper v. 4.0 software (Applied Biosystems). LH-PCR profiles were analysed in reference to the internal size standard using the Local Southern size calling method and a threshold of 150 fluorescent units.

Total DNA extraction from colostrum and LH-PCR

The method for total DNA extraction from colostrum samples was adapted from that used by Murphy *et al.* (*14*) and Rasolofo *et al.* (*15*). Samples (10 mL) were clarified by the addition of 2 mL of 0.5 M EDTA (ethylenediamine tetraacetic acid) at pH=8.0, followed by centrifugation at 9000 rpm and 4 °C for 30 min. Samples were then incubated at –20 °C for 10 min and the supernatant containing fat and soluble proteins was discarded. The remaining cell pellet was washed twice with 1 mL of sucrose buffer (12 % sucrose, 25 mM Tris-HCl, pH=8.0). The pellet was suspended in 0.4 mL of sucrose buffer containing 800 μg of lysozyme (Sigma-Aldrich Co.). The suspension was incubated for 1 h at 37 °C. Sucrose buffer (78 μL) containing 5 μL of 10 % SDS (sodium dodecyl sulphate), 12 μL of 250 mM EDTA (pH=8.0) and 5 μL of 20 mg/mL of proteinase K was added to the suspension and incubated for 1 h at 55 °C. After this second digestion, 65 μL of sucrose buffer and 135 μL of NaCl (5 M) were added to the suspension. To eliminate the contaminating proteins, the suspension was treated twice with 700 μL of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 18 000×*g* for 15 min at 4 °C. The upper aqueous phase was collected and transferred to a new tube for further extraction with 700 μL of chloroform. The upper aqueous phase was collected again, and 700 μL of cold (–20 °C) 96 % ethanol were added. The suspension was held at –80 °C overnight. The DNA was pelleted by centrifugation at 18 000×*g* for 30

min at 4 °C. The pellet was washed twice with 700 μL of cold (–20 °C) 70 % ethanol, and after draining and air drying, the DNA was suspended in 30 μL of sterile deionised water. The DNA was quantified by measuring the absorbance at 260 nm with a spectrophotometer (Jasco V-530), diluted to 20 ng/μL, and stored at -20 °C until use. The LH-PCR of total colostrum DNA was performed as described above for the bacterial strains.

Results and Discussion

Three representative colostrum samples (0, 24 and 60 h) from two cows were evaluated for bacterial cultivability. Table 1 shows the total cultivable population count on MPCA, the streptococcus count on M17 and lactobacillus count on MRS CYS, pH=5.4. The colostrum samples collected aseptically contained low numbers of bacteria, in agreement with previous results from Rindsig and Bodoh (*16*) and Stewart *et al.* (*17*). The total cultivable microbial count was similar during the evolution of the colostrum over these three days (Table 1).

The level of presumable LAB originating from the colostrum was lower than 3 log CFU/mL (Table 1). The colostrum was generally characterised by mesophilic streptococci (estimated to be 3 log CFU/mL at 25 °C) and thermophilic lactobacilli (estimated to be 2.61 log CFU/mL at 37 °C). The temperatures used were selected to maximise the recovery and isolation of LAB.

The microflora of the colostrum was partially estimated by studying 29 strains isolated in M17 and MRS CYS, pH=5.4, media. Thirteen cultivable species were identified by their 16S-rRNA gene sequences, illustrating the biodiversity present in the colostrum samples (Table 2). The greatest species diversity was recovered from M17 medium (9 species). *Staphylococcus chromogenes* was the most frequently isolated species. Interestingly, two potential probiotic LAB species (*Lactobacillus casei* and *Bifidobacterium pseudolongum*) were recovered in MRS CYS, pH=5.4. Because of the health-promoting effects of probiotics (*2*,*18*), significant efforts have been made to discover and exploit novel strains with scientifically proven probiotic properties from their natural environments, *i.e.* the human gut (*19*). The *L. casei* and *B. pseudolongum* biotypes isolated from the colostrum will be characterised in a future work.

The microbial ecology of colostrum remains incompletely understood, as almost all previous studies have been limited by the use of traditional agar-based and culture-dependent methods. Traditional methods of investigating microbial populations typically reveal the most commonly occurring microorganisms, especially those that

Table 1. Bacterial counts in different media for total cultivable, thermophilic and mesophilic lactic acid bacteria in colostrum samples

Colostrum sample/h	MPCA	M17		MRS CYS (pH=5.4)	
	log CFU/mL	log CFU/mL		log CFU/mL	
	37 °C	25 °C	37 °C	25 °C	37 °C
425/0	5.43±1.36	2.89±0.78	2.61±1.15	1.95±0.63	2.13±1.33
425/24	4.53±2.16	2.89±1.33	2.21±2.02	2.00±1.45	2.61±1.15
425/60	5.00±1.44	3.00±1.45	2.18±1.15	2.18±0.55	2.17±1.10
539/0	5.10±1.80	2.05±0.20	2.52±1.28	2.36±1.19	2.03±0.77
539/24	5.17±1.33	2.55±1.19	3.02±0.42	1.25±1.20	2.35±0.85
539/60	5.89±1.16	2.87±1.24	2.76±1.24	1.97±1.52	2.05±1.36

Table 2. Strains isolated from colostrum samples

Strain	Number of isolates[1]	Origin media	Closest relative strain[2]	Accession number[3]	Match/%
1; 4	2	MRS CYS (pH=5.4)	*Lactobacillus casei*	HM058411	100
2	1	M17	*Staphylococcus pseudintermedius*	FJ858975	98
3; 8; 9; 10; 11; 13; 15; 17; 18; 19; 21; 25	12	M17	*Staphylococcus chromogenes*	AY688044	99
5; 6	2	MRS CYS (pH=5.4)	*Bifidobacterium pseudolongum*	GU361828	99
7;	1	MRS CYS (pH=5.4)	*Propionibacterium acnes*	EF680442	99
12; 16	2	M17	*Brachybacterium* sp.	AB449755	98
14	1	M17	*Streptococcus uberis*	AM946015	99
20	1	M17	*Paenibacillus barcinonensis*	FJ174659	99
22	1	M17	*Bacillus circulans*	EU653002	100
23	1	M17	*Macrococcus caseolyticus*	GU904705	99
24; 26	2	M17	Uncultured bacterial clone	GQ055058	99
27; 29	2	MRS CYS (pH=5.4)	*Paenibacillus graminis*	AB428571	99
28	1	M17	*Cellulosimicrobium funkei*	AY729960	99

[1]quantity of strains isolated from this species, [2]determined by 16S rRNA BLAST, [3]GenBank

are able to grow to a detectable level under the specific culture conditions used (*20*). These methods often underestimate the less abundant components of the microflora, which could be equally important. Microbial isolation from a complex matrix in synthetic media requires optimal conditions to meet the high nutritional demands of the developing strains. The ideal conditions would reproduce the natural ecosystem in the medium (*20*).

To overcome the known limitations of culture-dependent methods, investigate the evolution, and partially quantify the microbial community present in the colostrum, samples were subjected to a PCR-based culture--independent method, LH-PCR. Fig. 1 shows the LH-PCR profile obtained for the colostrum sample 425 at 36 h. The electropherograms of the most representative results for the colostrum sample 539 at 0, 12, 24, 48 and 60 h are shown in Fig. 2.

The different fragment sizes in the LH-PCR profiles were attributed to bacterial species using a previously established database (*5,6*). The peaks detected in the electropherogram of sample 425 at 36 h (Fig. 1) were as follows: (276±1) bp (unattributed), (292±1) bp (unattributed), and (320±1) bp (attributed to *Lactococcus lactis* or *Staphylococcus* sp. or *Streptococcus thermophilus*). None of the species attributed to the (320±1)–bp peak were isolated from the agar plates (Table 2). Representative strains for these species could be quiescent and might be viable but not culturable. Because of its low sensitivity, the LH--PCR technique is only semi-quantitative and requires the microbial count in a system to be higher than 5 log CFU/mL (*6*) for the quantity of template DNA to be sufficient. As expected, the amount of DNA from some of the species in colostrum sample 425 was too small to be

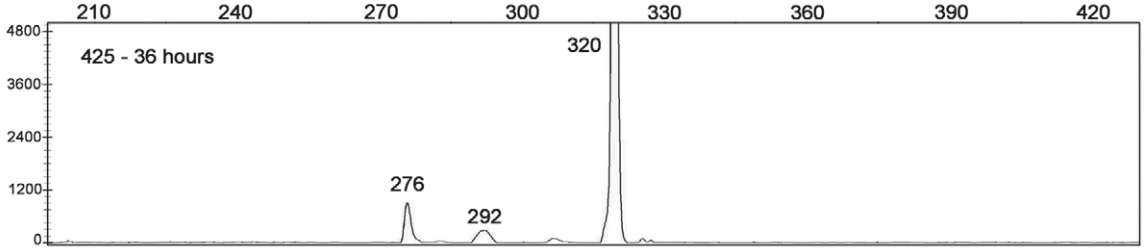

Fig. 1. LH-PCR electropherogram of the microbial community present in the colostrum sample 425 at 36 h. The x-axis shows the peak size in base pairs, and the y-axis shows the peak intensity in relative fluorescence units

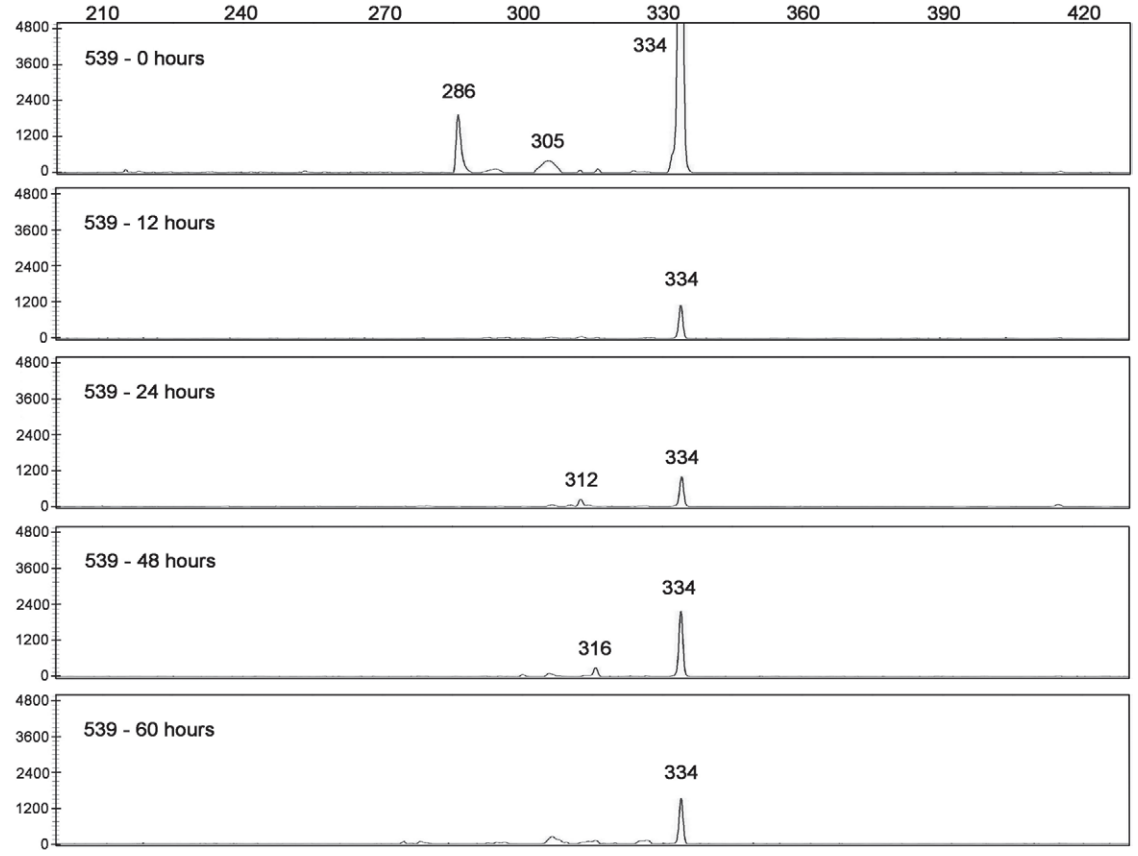

Fig. 2. LH-PCR electropherograms of the microbial communities present in colostrum sample 539 at subsequent stages of maturation. The x-axis shows the peak size in base pairs, and the y-axis shows the peak intensity in relative fluorescence units

amplified, resulting in profiles free from considerable peaks (data not shown).

The following peaks were detected in the electropherograms of colostrum sample 539 (Fig. 2): at 0 h – (286±1) bp (unattributed), (305±1) bp (unattributed), and (334±1) bp (*Lactobacillus helveticus* or *L. casei*); at 12 h – (334±1) bp (*L. helveticus* or *L. casei*); at 24 h – (312±1) bp (*Kocuria kristinae*) and (334±1) bp (*L. helveticus* or *L. casei*); at 48 h – (316±1) bp (unattributed) and (334±1) bp (*L. helveticus* or *L. casei*); and at 60 h – (334±1) bp (*L. helveticus* or *L. casei*). Some of the profiles contained peaks with fluorescence intensities lower than the threshold (150 fluorescence units).

LH-PCR confirmed the presence of some of the species isolated and identified by their 16S rRNA gene sequences (*e.g. L. casei and Staphylococcus* sp.), while other species identified by the peak profiles were not recovered on the culture media used. These strains could be viable but barely cultivable. The area of each peak can provide a measure of the relative proportion of each component in the community (*13*). Using this technique, a fingerprint of the relative quantitative and qualitative compositions of the dominant populations within a microbial community can be estimated. *L. casei* was the main species present in the colostrum samples in this study.

Conclusions

The colostrum contained secondary contaminating agents at levels too low to compromise its microbiological safety. However, LAB and *Bifidobacterium* microbiota were also present and should be assayed for probiotic characteristics. The method for total DNA extraction was adapted to colostrum and was effective for recovering low amounts of DNA from a highly proteinaceous matrix. To our knowledge, this is the first application of the LH-PCR technique to the bovine colostrum ecosystem. This method revealed a general picture of the LAB community in this matrix while avoiding time-consuming culture-dependent techniques.

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