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Massive sequencing of artisan cheeses from raw sheep's milk

Abstract

Lactic acid bacteria (LAB) are used in the food industry to confer aromatic characteristics and their antibacterial capacity. In this study the native flora of LAB that participates in the traditional fermentation of semi-hard cheeses made with raw sheep's milk from the region of Andalusia, Spain was analyzed. Three samples of four different commercial cheeses were taken. Massive sequencing was carried out to identify the lactic and accompanying flora. Predominant lactic flora was *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus paracasei* and *Lactococcus raffinolactis*, and to a lesser extent other species of the genera *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconestoc*. The accompanying flora was composed of species of the genera *Mycoplasma*, *Pseudomonas*, *Acinetobacter*, *Chryseobacterium*, *Mannheimia*, *Trueperella*, *Enterococcus*, *Vibrio*, *Serratia*, *Macrococcus*, *Staphylococcus*, *Massilia*, *Flavobacterium*, *Yersinia*, *Gallaecimonas*, *Hafnia*, *Leclercia*, *Obesumbacterium*, *Morganella* and *Kluyvera*. These results show that modern molecular techniques are very good tools to identify natural LABs of artisanal dairy products. The characterization of the native flora of the artisanal cheese allows us to evaluate the microbiological diversity of the natural population of LAB and the symbiosis with another type of flora.

Keywords: artisan cheese, metagenomic, bacteria, pasteurized milk

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Abbreviations: LAB, lactic acid bacteria; DNA, Deoxyribonucleic acid

Introduction

Hundreds of cheese varieties are produced in the world based on the differences both in the type of milk used and in the method of production. However, only a small number of these varieties have commercial importance and most of them are produced and consumed locally. The production of cheese is essentially achieved by joining four ingredients: milk, rennet, microorganisms and salt. The production of lactic acid from lactose is the main biochemical change in the production of cheese, responsible for lactic acid bacteria (LAB). These starter bacteria reduce the pH, form the dough and allow the rejection of water. The demand for cheese in the early 1900s generated large-scale production and therefore the use of pasteurized milk as a criterion of hygiene. This process involves the elimination of the natural flora of LAB contained in milk and the possible generation of a variety of flavors and aromas. Consequently, to make cheese from pasteurized milk, an external source of LAB initiators is needed.

The production of raw milk cheeses has long been practiced in Europe, particularly in Spain, France, Italy and Switzerland. Raw milk cheeses are often characterized by richer and stronger flavour intensity than cheeses made from pasteurized milk,[1] and they are considered more natural. Andalusia has a great variety of excellent traditional cheeses quality and linked to the territory from which they come. Each of these cheeses is characterized by various quality attributes such as a certain race native farming, grazing-based food, as well as knowledge of the master cheese passed down generation after generation, among others.[2] The current demand for fresh and easy to prepare products has brought an increase in the market of minimally processed products. This trend responds to the generalized idea that these foods are healthier. However, it must be borne in mind that it is raw food, which requires extreme good handling conditions and other techniques that allow some microbial inactivation. Minimally processed foods are fresh and, therefore, raw. This implies that the health risks may be higher than those of foods that have been treated with any technological process. Therefore, more common pathogenic microorganisms can be transmitted, among them, *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Salmonella*, among others.

The strains that make up the microflora in raw milk and therefore in the cheese made with it have relevant functions in terms of conferring attributes of protection against pathogenic bacteria, generation of flavors and flavor or modification of texture.[3] Genomic identification of promising species of LAB for development of novel starter culture candidates was the prime aim of the present study.

Materials and methods

Cheese sampling

Three samples of four semi-hard commercial cheeses of raw sheep's milk cheese were taken: from the center, the middle and the crust. A total of 12 samples were processing for massive sequencing.

DNA extraction from cheese samples

DNA extraction was performed with the Power Food Microbial DNA Isolation Kit (MO BIO, ref 21000-50). 1.8 ml of microbial food culture was pelleted by centrifugation and resuspended in lysis buffer. The supernatant was transferred to a bead beating tube containing beads designed for small cell (microbial) lysis and vortex mixed using a specially designed MO BIO Vortex Adapter. After the protein and

inhibitor removal steps, total genomic DNA was captured on the MO BIO Laboratories flat bottom silica spin column. The bound DNA was washed and eluted from the spin column membrane.

Preparation of the amplicons and library

Metagenomic kit Ion 16S (pooles primers V2-48 y V3-7, 7-9) was used (Thermo Fisher, ref A26216). This allowed the rapid and complete analysis of mixed microbial populations through the sequencing flow of Ion Torrent TM semiconductors.

The Seuser Ion Plus fragments library Kit was used, an integral component of the Personal Genome Machine TM (Thermo Fisher, ref 4471252). This kit allowed to design libraries of high-quality DNA through several workflows with an input of 100 ng of DNA.

Library enrichment and chip loading

The PGM HiQ View Chef Ion Kit (Thermo Fisher, ref A29902) was used. The templates were prepared, the loading of chips and the sequencing of libraries of 200 bp in the Ion Chef™ system and in the Ion PGM™ system.

Sequencing and analysis

We use the Ion PGM equipment, chip 318. Kit Ion PGM HiQ View Sequencing Kit (Thermo Fisher, ref A30044). The 200 and 400 base pair libraries were sequenced using the Ion OneTouch™ 2 system combined with the Ion PGM™ system. The analysis of the Ion PGM™ system data was performed with the Ion Reporter™ software.

Results

Information related to samples (Table 1).

Sequence analysis with Ion Reporter

Ion Reporter uses the QIIME open bioinformatics source to produce diversity analyzes and visualizations. The results of alpha diversity describe the diversity within a single sample at the Species, Gender and Family levels. The beta diversity results describe the diversity among multiple samples at the species, genus and family level (Table 2).

Table 1 Information related to samples

Barcode Name	Sample	Bases	≥ Q20	Reads	Mean Read Length
NO barcode	none	4,21,54,975	3,85,11,027	2,02,922	208 bp
IonXpress_001	A1	4,65,80,044	4,45,06,413	1,85,693	251 bp
IonXpress_002	A2	5,92,05,232	5,66,35,881	2,35,520	249 bp
IonXpress_003	A3	12,10,97,845	11,59,23,675	4,86,327	250 bp
IonXpress_004	B1	4,80,13,228	4,60,23,378	1,91,976	252 bp
IonXpress_005	B2	8,74,19,483	8,36,46,502	3,47,482	247 bp
IonXpress_006	B3	5,66,69,596	5,40,91,466	2,28,998	250 bp
IonXpress_007	C1	7,32,73,652	7,00,92,992	2,92,978	247 bp
IonXpress_008	C2	8,16,73,881	7,79,72,444	3,30,729	251 bp
IonXpress_009	C3	6,76,27,167	6,45,90,193	2,68,960	241 bp
IonXpress_0010	D1	8,72,95,056	8,29,84,652	3,61,688	541 bp
IonXpress_0011	D2	7,49,10,425	7,14,86,030	3,07,600	244 bp
IonXpress_0012	D3	4,08,03,151	3,89,42,102	1,69,602	241 bp

Table 2 Predominant flora in cheese samples

Sample	Flora	Center (%)	Meddle (%)
Cheese A	*Chryseobacterium haifense*	<0.05	<0.05
	Lactobacillus casei	<0.05	<0.05
	Lactobacillus paracasei	0.13	0.57
	Lactococcus lactis	7.76	4.61
	Lactococcus raffinolactis	0.47	0.55
	Streptococcus thermophilus	32.08	29.37
	Acinetobacter ursingii	<0.05	<0.05
	Pseudomona vranovensis	0.2	<0.05
	Mycoplasma bovis	<0.05	<0.05

Citation: Julia R, Analía E, Nora P, et al. Massive sequencing of artisan cheeses from raw sheep's milk. *J Appl Biotechnol Bioeng.* 2019; 6(5): 236–240. DOI: 10.15406/jabb.2019.06.00198

Table Continued...

Cheese B	*Trueparella pyogenes*	<0.05	<0.05	<0.05
	Corynobacterium	<0.05	<0.05	<0.05
	Macrococcus caseolyticus	<0.05	<0.05	<0.05
	Enterococcus faecalis	0.13	0.15	0.17
	Lactobacillus	0.07	0.11	0.12
	Lactobacillus brevi	<0.05	<0.05	<0.05
	Lactobacillus paracasei	3.03	3.27	2.13
	Lactobacillus sp.	<0.05	0.06	<0.05
	Lactococcus	0.16	0.17	0.21
	Lactococcus lactis	6.69	6.89	9.7
	Lactococcus raffinolactis	0.15	0.34	0.52
	Streptococcus	0.13	0.16	0.21
	Streptococcus salivarius	<0.05	<0.05	<0.05
	Streptococcus thermophilus	32.19	35.1	27.1
	Streptococcus uberis	<0.05	<0.05	<0.05
	Serratia	<0.05	<0.05	<0.05
Cheese C	*Lactobacillus*	0.15	0.31	0.08
	Lactobacillus diolivorans	0.31	0.13	0.38
	Lactobacillus paracasei	2.97	2.42	0.69
	Lactobacillus sp.	<0.05	<0.05	<0.05
	Lactobacillus zeae	0.13	<0.05	0.11
	Pediococcus	<0.05	<0.05	<0.05
	Pediococcus pentosaceus	0.12	0.08	<0.05
	Lactococcus	0.11	0.14	0.13
	Lactococcus lactis	4.13	3.85	2.4
	Lactococcus raffinolactis	0.27	0.24	0.26
	Streptococcus	0.12	0.18	0.12
	Streptococcus thermophilus	40.52	39.29	46.31
	Streptococcus uberis	<0.05	<0.05	<0.05
	Manheimia varigena	<0.05	<0.05	<0.05
	Mycoplasma agalactiae	<0.05	<0.05	<0.05
	Mycoplasma bovis	1.04	0.81	0.81
Cheese D	*Enterococcus*	<0.05	<0.05	<0.05
	Lactobacillus	<0.05	<0.05	<0.05
	Lactobacillus futsaii	<0.05	<0.05	<0.05
	Lactobacillus paracasei	0.09	0.16	0.22
	Lactobacillus sakei	<0.05	<0.05	<0.05
	Leuconestoc	<0.05	0.06	0.01
	Leuconestoc mesenteroides	0.58	0.38	0.82
	Leuconestoc pseudomesenteroides	<0.05	0.08	0.08
	Lactococcus	<0.05	0.22	0.11
	Lactococcus lactis	6.73	8.08	4.22
	Lactococccus raffinolactis	19.38	21.58	16.19

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Table Continued...

Lactococcus sp.	0.14	0.15	0.09
Streptococcus parauberis	0.06	0.11	<0.05
Hafnia alvei	<0.05	<0.05	<0.05
Hafnia paralvei	0.81	0.79	1.1
Kluyvera	<0.05	<0.05	<0.05
Obesumbacterium proteus	1.73	1.64	2.17
Providencia	<0.05	<0.05	<0.05
Serratia glossinae	0.27	0.21	0.14
Serratia grimesii	0.6	0.33	0.76
Serratia liquefaciens	0.17	0.14	0.13
Serratia quinovorans	0.56	0.31	0.61
Yersinia intermedia	<0.05	<0.05	<0.05
Pseudomonas	0.21	0.38	0.66
Gallaecimonas xiamennensis	<0.05	<0.05	0.06

Discussion

Artisan cheeses are characterized by a rich source of various LAB with interesting functional properties. In recent years, many researchers have focused on the isolation of native LAB from artisanal cheeses made with raw milk without the addition of starters cultures. As in this study, other researchers have isolated LAB from cheeses made with raw sheep's milk. Avnİ Kırmacı[4] in raw sheep milk cheeses from Turkey, Ramírez-López[5] in raw goat milk cheeses in Mexico, Vernile et al.,[6] in the classic Pecorinos cheese from Italy, among others. The preliminary identification of isolates from dairy products generally shows the presence of four dominant LAB genera: *Lactococcus* spp., *Enterococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp. and *Streptococcus* spp. Terzic-Vidojevic et al.[7] In another study, the percentage distribution of isolated LABs from highest to lowest has been: *Enterococcus* spp., *Lactococcus* spp., *Lactobacillus* spp., *Streptococcus* spp. and *Leuconostoc* spp.[4]

These strains generally play an important role in the development of cheese flavor. Specifically, the *Enterococci* play an important role in the development of the typical flavor of the cheese and could be good components of cheese starter cultures.[8] Identification at the species level is an important issue since it can help verify the presence of multiple isolates and distinguish groups of strains or unique strains with peculiar technological properties.

Taxonomic identification often cannot determine up to species level. The importance of knowing what type of bacteria colonizes a given environment provides information to be able to associate microbial activities with certain populations. For this reason, in this study, massive sequencing was used as a metagenomic method.[9] A broad panorama of the constituent flora of the cheese was obtained. *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus paracasei* and *Lactococcus raffinolactis* were detected as dominant lactic flora, and to a lesser extent (0.01-0.99) *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus diolivorans*, *Lactobacillus futsaii*, *Lactobacillus paracasei*, *Lactobacillus sakei*, *Lactobacillus* sp., *Lactobacillus zeae*, *Lactococcus*, *Lactococcus raffinolactis*, *Lactococcus* sp., *Streptococcus parauberis*, *Streptococcus salivarius*, *Streptococcus uberis*, *Pediococcus pentosaceus*, *Leuconestoc mesenteroides* and *Leuconestoc pseudomesenteroides*. The accompanying flora was composed of species of the genera *Mycoplasma*, *Pseudomonas*, *Acinetobacter*, *Chryseobacterium*, *Mannheimia*, *Trueperella*, *Enterococcus*, *Vibrio*, *Serratia*, *Macrococcus*, *Staphylococcus*, *Massilia*, *Flavobacterium*, *Yersinia*, *Gallaecimonas*, *Hafnia*, *Leclercia*, *Obesumbacterium*, *Morganella* and *Kluyvera*. In a similar metagenomic study performed by López[10] the genera *Lactobacillus*, *Weissella* and *Leuconostoc* were identified as dominant and more than 500 non-dominant genera.

With this information it is possible to determine the symbiotic relationships between the communities that participate in the cheese fermentation and maturation process. The use of a metagenomic analysis allowed to perform a taxonomic and functional characterization of the microbiome at a highly detailed level. This would explain the sensory and safety characteristics of semi-hard cheeses of raw sheep's milk. In this context, it is important to emphasize that the native LAB microflora that characterizes artisan cheeses generally consists of microorganisms adapted to the product, to specific environmental conditions and production technology. Therefore, the availability of valid molecular methods for a reliable allocation of species and a specific differentiation at the strain level is of great importance.[11–13]

Conclusion

The isolation and characterization of LAB strains from artisanal products is of great importance since it allows to increase the knowledge about the potential and the application of native strains to be used as starter cultures. The improvement and optimization of the control of the fermentation processes, allows the development of products with defined and constant properties but with characteristics such as artisan products.

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Conflicts of interest

The author declares there are no conflicts of interest.

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