

ORIGINAL ARTICLE

Bacteria and yeasts associated to Colonial cheese production chain and assessment of their hydrolytic potential

Bactérias e leveduras associadas às etapas de produção de queijo Colonial e avaliação de seu potencial enzimático

Priscilla Vieira de Souza¹, Cristina B. Zaffari Grecellé², Fabiano Barreto³,
Maurício Ramírez-Castrillon⁴, Patrícia Valente⁵, Marisa da Costa^{5*} 

¹Universidade Federal do Rio Grande do Sul (UFRGS), Programa de Pós-graduação em Microbiologia Agrícola e do Ambiente, Porto Alegre/RS - Brasil

²Universidade Luterana do Brasil (ULBRA), Faculdade de Veterinária, Canoas/RS - Brasil

³Ministério da Agricultura Pecuária e Abastecimento, Laboratório Federal de Defesa Agropecuária/RS, Porto Alegre/RS - Brasil

⁴Universidad Santiago de Cali, Facultad de Ciencias Básicas, Research Group in Mycology, Cali - Colombia

⁵Universidade Federal do Rio Grande do Sul (UFRGS), Instituto de Ciências Básicas da Saúde (ICBS), Departamento de Microbiologia, Imunologia e Parasitologia, Porto Alegre/RS - Brasil

*Corresponding Author: Marisa da Costa, Universidade Federal do Rio Grande do Sul (UFRGS), Instituto de Ciências Básicas da Saúde (ICBS), Departamento de Microbiologia, Imunologia e Parasitologia, Rua Sarmento Leite, 500, Sala 216, Prédio ICBS, Campus Reitoria, CEP: 90050-170, Porto Alegre/RS - Brasil, e-mail: mdcosta@ufrgs.br

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Abstract

Different types of microorganisms are important in cheese-making because of the contributions their metabolism offers during the process. Few microorganisms present in Colonial cheese are known, in addition to the ones that are introduced to kick-start the processes or the ones that are associated with infections or poisonings. This study aimed to identify, by MALDI-TOF and/or DNA sequencing, the bacteria and yeasts isolated from samples collected in the main stages of Colonial cheese production, i.e., a type of cheese produced in the southern region of Brazil. The lytic capacity of these microorganisms at 5 °C and 30 °C was also evaluated. The 58 bacterial strains were distributed in 10 species among the genera *Bacillus*, *Citrobacter*, *Klebsiella*, *Lactococcus*, *Paenibacillus*, *Staphylococcus* and *Raoutella*. From the 13 yeasts strains analyzed, three species were identified as following: *Candida pararugosa*; *Meyerozyma guilliermondii*; and *Rhodotorula mucilaginosa*. In three yeasts isolates it was possible to identify only the genus *Candida* sp. and *Trichosporon* sp. The species *L. lactis* (48%) and *M. guilliermondii* (46%) were, respectively, the predominant bacteria and yeasts species isolated. The highest microbial lytic activity observed was at 30 °C. Lipase activity on isolates was proportionally more observed with yeasts and proteolytic activity with bacteria. Lower caseinase and lipase activity was observed at 5 °C, demonstrating the importance of refrigeration in controlling microbial activity. This research highlighted the cultivation of some microorganisms that are part of the Colonial cheese microbiota as well as that several of them can hydrolyze various compounds present



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in milk and that could be associated with its maturation or, in uncontrolled circumstances, could be the cause of product deterioration.

Keywords: Microbiota; Enzymes; Proteolytic activity; Lipolytic activity; Agro-industries; MALDI-TOF.

Resumo

Durante o processo de produção do queijo, é necessária a presença de diversos microrganismos que contribuem na sua elaboração pela ação de seu metabolismo. Pouco é conhecido sobre os microrganismos presentes no queijo Colonial, além daqueles inseridos para iniciar o processo ou daqueles indesejados, potenciais causadores de infecção ou intoxicação. Este trabalho teve como objetivo principal identificar, através de MALDI-TOF e/ou sequenciamento de DNA, bactérias e leveduras isoladas de amostras coletadas nas principais etapas de produção de queijo Colonial, um tipo de queijo produzido na Região Sul do Brasil. Também foi verificada a capacidade lítica destes microrganismos a 5 °C e 30 °C. Foram analisados 58 isolados de bactérias, sendo identificadas 10 espécies de bactérias distribuídas nos gêneros *Bacillus*, *Lactococcus*, *Paenibacillus*, *Staphylococcus*, *Citrobacter*, *Klebsiella* e *Raoutella*. Dos 13 isolados de leveduras, foram identificadas três espécies: *Candida pararugosa*, *Meyerozyma guilliermondii* e *Rhodotorula mucilaginosa*. Em três isolados de leveduras, foi possível a identificação somente dos gêneros *Candida* sp. e *Trichosporon* sp. As espécies *Lactococcus lactis* (48%) e *M. guilliermondii* (46%) foram os isolados predominantes de bactéria e levedura, respectivamente. A maior atividade lítica dos microrganismos identificados foi na temperatura de 30 °C. Proporcionalmente, foi verificado maior número de isolados com atividade de lipase pelas leveduras e maior atividade proteolítica pelas bactérias. Menor atividade de caseínase e lipase foi observada a 5 °C, demonstrando a importância da refrigeração no controle da atividade microbiana. Este trabalho mostrou, através de cultivo, alguns dos microrganismos que fazem parte da microbiota do queijo Colonial e que vários deles possuem capacidade de hidrolisar vários compostos presentes no leite. Mostrou também que podem estar associados com a maturação do mesmo ou, em circunstâncias não controladas, que poderiam ser a causa de deterioração do produto.

Palavras-chave: Microbiota; Enzimas; Atividade proteolítica; Atividade lipolítica; Agroindústrias; MALDI-TOF.

1 Introduction

Colonial cheese is a variety of cheese produced by family cheese-making agro-industries in southern Brazil. This cheese is produced with raw or pasteurized bovine milk added to commercial cultures, curd salting and a maturation period of at least 20 days. The microbiota in cheeses is largely variable, and depends on seasonality, animal feeding, environment, milk microbiota and processing (heat treatment, type of cheese, equipment, and hygiene) (Tilocca et al., 2020). Microbial diversity in cheeses is necessary to obtain the sensory characteristics and desirable textures specific to each variety (Montel et al., 2014). Those sensory characteristics are the result of many factors, depending on the milk quality, which is influenced by the animal's breed, breeding conditions, kind of feed, soil composition, microbial load, etc.; and by the activity of milk enzymes and microbial metabolism (Milani et al., 2019). It was also observed that some cheese bacteria can transiently colonize the human gut in cheese consumers, providing a benefit through their enzymatic activities (Robinson et al., 2021; Milani et al., 2019; Zheng et al., 2015). Some of the benefits observed for occasional milk or cheese consumers were the increase in lipid excretion and the decrease in blood cholesterol levels; however, it has not yet been established whether this is correlated to a specific microorganism (Robinson et al., 2021; Zheng et al., 2015). Although the microbial composition of cheeses can influence a multitude of factors, almost nothing is known about the microorganisms present in Colonial cheese, except for its main contributors (such as Lactic Acid Bacteria (LAB)) or its potential pathogens (Funck et al., 2015; Pereira et al., 2017; Jonnala et al., 2018; Ausani et al., 2019; Carvalho et al., 2019; Pegoraro et al., 2020). Further information can assist in identifying the product regarding probiotic characteristics and potential, and determining the geographic origin and/or the qualification of a regional cheese.

This research aimed to identify a total of 71 strains of bacteria and yeast isolated throughout Colonial cheese manufacturing process as well as characterize their abilities to grow and produce specific enzymes at 30 °C and 5 °C.

2 Material and methods

All strains (71) were isolated in a previous study (Grecellé et al., 2020) in which the microbial quality of two Colonial cheese family agro-industries were evaluated. Both agro-industries were inspected by a regional official agency and produced an average of 370 kg of cheese per day. These cheeses are made from pasteurized milk with an added commercial starter (LAB). All Colonial cheese samples had the expected standard characteristics, of texture and flavor, at the time of analysis. The isolates were obtained from counting of aerobic mesophilic bacteria and molds from raw milk, curd, ripening cheese, and ready-to-eat cheese. Each different colony morphotype of bacteria and yeasts were selected and stored in Brain Heart Infusion (BHI) broth with 30% glycerol at -20 °C. To perform the identification, all isolates were restored first in BHI broth at 30 °C for 24h and were later isolated on solid media (Plating counting agar for bacterial strains and/or GYP agar [glucose 2%, peptone 1%, yeast extract 0.5%, agar 2%] for yeast), twice to check purity, before identification procedures.

All isolates were tested for cell characteristics (Gram stain) and colonial morphology before using Matrix-Assisted Laser Desorption/Ionization - Time-of-Flight Mass Spectrometry (MALDI-TOF MS) procedure and/or DNA sequencing. For the MALDI-TOF MS identification, protein extraction was performed according to previous studies (Bruker, 2015) and the cutoff used for characterization was proposed by Wieser et al. (2012), where scores values above 2.0 were considered for species level identification and values between 2.0 and 1.7 were reliable for genus level. Isolates not identified by MALDI-TOF MS were thus identified by sequencing.

Bacterial DNA extraction was performed after thermal extraction exactly as described by Riyaz-Ul-Hassan et al. (2008). Yeast DNA extraction was performed after pure colonies of each strain were grown in Glucose-Yeast-Peptone (GYP) broth at 30 °C for 18 hours. After centrifugation and washing with distilled water, the biomass of each culture was re-suspended in 400 µL of lysis buffer (0.5 M NaCl, 10 mM EDTA, 2% SDS, 50 mM Tris-HCl, pH 8) and incubated for 60 min at 65 °C. The other steps were done as described in Osorio-Cadavid et al. (2009).

Bacterial DNA amplification and sequencing were performed using 515F and 806R primers for the hypervariable gene V3-V4 of 16SRNA region specific for prokaryotic cells (Bates et al., 2011). The amplification conditions followed were those described by Franco et al. (2016). For yeast DNA amplification, the ITS1-5.8S-ITS2 region was amplified and sequenced with ITS1 and ITS4 primers (White et al. 1990). Amplification conditions were as follows: one initial cycle at 94 °C for 5 min; 35 cycles at 94 °C for 15 s; 55 °C for 45 s; 72 °C for 90 s; and a final extension cycle at 72 °C for 6 min.

All Polymerase Chain Reaction (PCR) products were examined by electrophoresis on a 1.5% of agarose gel at 100 V for 45 min and stained with GelRed and visualized under UV light. Digital image capturing was done using the Geni2 gelDoc System (Syngene, Cambridge, UK) (Ramírez-Castrillón et al., 2014). Before sequencing, PCR products were purified using the PCR Clean-Up System purification kit (Promega Corporation, Madison, USA) and then quantified on the spectrophotometer (NanoDrop Lite Thermo Fischer Spectrophotometer). Finally, Sanger sequencing of the PCR products were outsourced at the *Unidade de Análises Moleculares e de Proteínas* (UAMP), from *Hospital de Clínicas de Porto Alegre*, edited, assembled, and compared with the sequences of strain types catalogued in the GenBank database. A similarity cutoff of 99% was used to identify the isolates according to taxonomic level.

Bacteria and yeasts were also cultivated at ±5 °C to detect psychrotrophic strains and went through testing to observe their lipolytic and proteolytic activities at 30 °C and ±5 °C, performed within 48 h (Ben-Gigirey et al., 2000). DNase (Himedia), lactose tryptone broth (Himedia), and Litmus Milk (Fluka) tests were also performed at 30 °C and ±5 °C. These essays were repeated once when there was no growth or dubious results were observed on the first essay. Results were evaluated qualitatively as positive or negative.

3 Results and discussion

A total of 58 bacterial (53 gram-positives and five gram-negatives), and 13 yeasts were isolated and identified after freezing recover (Table 1). A total of seven gram-positive and three gram-negative species were identified. Yeasts isolates were distributed in four genera and three species. Most isolates were identified by the MALDI-TOF MS, excluding one species of *L. lactis* and the *Rhodotorula mucilaginosa* isolate, which were identified by DNA sequencing. Three yeast isolates were identified only at genus level using MALDI-TOF. Their species definition could not be realized by biochemical identification because of their viability being lost in the testing process.

Table 1. Bacteria and yeasts isolates identified from samples of the main production stages of Colonial cheese.

Identification ^a	Milk	Curd	Ripening cheese	Read-to-eat cheese
Gram-positive				
<i>Bacillus cereus</i>	1	-	-	-
<i>Bacillus mycoides</i>	4	1	-	-
<i>Lactococcus lactis</i>	5	10	5	8
<i>Paenibacillus humicus</i>	-	-	3	2
<i>Staphylococcus xylosus</i>	-	1	-	-
<i>Staphylococcus saprophyticus</i>	1	2	1	3
<i>Staphylococcus warneri</i>	1	2	3	-
Gram-negative				
<i>Citrobacter freundii</i>	-	-	1	-
<i>Klebsiella variicola</i>	-	-	-	1
<i>Raoutella ornithinolytica</i>	3	-	-	-
Total bacteria	15	16	13	14
Yeasts				
<i>Meyerozyma guilliermondii</i>	4	-	-	2
<i>Candida pararugosa</i>	-	-	-	3
<i>Candida</i> sp.	1	-	-	-
<i>Rhodotorula mucilaginosa</i>	1	-	-	-
<i>Trichosporon</i> sp.	2	-	-	-
Total yeasts	8	0	0	5
Total microorganisms	23	16	13	19

^aIdentification using MALDI-TOF MS for all isolates except *L. lactis* and *R. mucilaginosa* isolates that were identified by DNA sequencing.

More than 50% of the bacteria isolates were identified as *L. lactis* and they were isolated in all stages of the cheese manufacturing. This is unsurprising, because this species is commonly seen in the dairy industry environment; it is the starter bacteria used in the manufacture of various cheeses, as well as in Colonial cheeses, where it is the predominant species (Camargo et al., 2021; Kamimura et al., 2019; Peláez & Requena, 2005; Leroy & De Vuyst, 2004; El-Baradei et al., 2007; Ercolini et al., 2008). Kamimura et al. (2019) were also able to demonstrate that *L. lactis* was the most abundant species in various Brazilian cheeses using metagenomics. This species is important because it also prevents the growth of spoiling bacteria by acidifying the environment with lactose breakdown and by producing bacteriocins (Ávila et al., 2020; Bukvicki et al., 2020; Yang et al., 2019). Indeed, they are particularly needed for providing specific aromatic compounds. The majority of *L. lactis* isolates demonstrated a great ability to hydrolyze proteins and it was observed that approximately half of them had the ability to hydrolyze lactose at 30 °C, which is essential to the coagulation step (Table 2). A second species that was observed in all stages of cheese-making was *S. saprophyticus*, a common bacterium found in the microbiota of bovines, humans, and other warm-blooded animals, as well as in their environment and in the cheese agro-industries (Schleifer, 2009; Grecellé et al., 2020). Approximately half of the *S. saprophyticus* and *S. warneri* isolates demonstrated the ability to hydrolyze proteins and lipids, and depending on its load, could facilitate cheese maturation or even hinder it. Three spore forming bacteria

were isolated as following: two of them that had protease activity, *Bacillus cereus* and *B. mycoides*, were found on milk and curd; and the third species *P. humicus* was isolated from the cheese sample and had the ability to hydrolyze proteins and lactose. Of the three species, *B. cereus* is toxigenic and can cause food born intoxication depending on its cellular load. Whereas *P. humicus* is an environmental species that was described as capable of causing a blood infection (Berthold-Pluta et al., 2019; Sáez-Nieto et al., 2017). All the identified gram-negative species belonged to the *Enterobacteriaceae* family, a bacterial family frequently observed in the local environment, as well as in some dairy facilities, and could indicate poor hygiene conditions (Pinto et al., 2015; Rodríguez-Medina et al., 2019; Hajjar et al., 2020; Jonnala et al., 2018). The *Citrobacter* sp., *Klebsiella* sp. and *Raoutella* sp., in addition to being opportunistic pathogens, are considered spoilage bacteria (Camargo et al., 2021; Teider Júnior et al., 2019; Jonnala et al., 2018). Although few isolates of gram-negative bacteria were identified, almost all demonstrated having the ability to hydrolyze proteins, lipids, and lactose. In Entre Serra and Minas Frescal cheeses, other regional Brazilian cheeses, *C. freundii*, *L. lactis* and *Kelbsiella* sp. were also isolated (Camargo et al., 2021; Teider Júnior et al., 2019).

Table 2. Enzymatic activity of bacteria and yeasts isolated from samples of the main production stages of Colonial cheese.

Genus /Species	N ^{o.a}	Number of isolates with enzymatic activities							
		Proteinase		Lipase		Litmus Milk		Lactose Ferm. ^c	
		30 °C ^b	5 °C ^b	30 °C	5 °C	30 °C	5 °C	30 °C	5 °C
Gram-positive									
<i>Bacillus mycoides</i>	5	5	1	3	- ^d	3	-	-	-
<i>Bacillus cereus</i>	1	1	-	-	-	-	-	-	-
<i>Lactococcus lactis</i>	28	24	3	11	5	10	-	5	-
<i>Paenibacillus humicus</i>	5	2	-	-	1	3	-	2	-
<i>Staphylococcus saprophyticus</i>	7	3	1	5	4	6	-	-	-
<i>Staphylococcus warneri</i>	6	4	1	5	2	4	-	-	-
<i>Staphylococcus xylosus</i>	1	1	-	-	-	-	-	-	-
Gram-negative									
<i>Citrobacter freundii</i>	1	1	-	-	-	-	-	1	-
<i>Klebsiella variicola</i>	1	1	1	1	-	1	-	-	-
<i>Raoutella ornithinolytica</i>	3	-	-	2	1	1	-	2	-
Yeasts									
<i>Meyerozyma guilliermondii</i>	6	1	-	3	-	1	-	2	-
<i>Candida pararugosa</i>	3	-	-	2	-	2	-	-	-
<i>Candida</i> sp.	1	-	-	1	-	1	-	-	-
<i>Rhodotorula mucilaginosa</i>	1	-	-	1	-	1	-	-	-
<i>Trichosporon</i> sp.	2	-	-	2	-	1	-	1	-
Total microorganisms	71	43	6	36	13	34	0	11	0

^aNumber of total isolates for each genus and/or species. ^bTemperature used. ^cLactose fermentation. ^dNo isolate positive for this test, at this temperature.

Most microbiological research involving Colonial cheese has been directed towards the detection of pathogens, the study of lactic acid or coliform bacterial activity (Carvalho et al, 2019; Pegoraro et al., 2020; Grecellé et al., 2020; Pereira et al., 2017; Zaffari et al., 2007). Some bacterial species have already been

identified in ready-to-eat Colonial cheese in these studies, such as *Staphylococcus* spp. (*S. aureus*, *S. epidermidis*, *S. equorum*, *S. intermedius*, *S. haemolyticus*, *S. saprophyticus*, *S. sciuri*, *S. warneri*, *S. xylosus*), *Listeria* spp. (*L. grayi*, *L. welshimeri*, *L. monocytogenes*) and *Escherichia coli*. The first study for identifying the microbial composition of Colonial cheese was carried out by Kamimura et al. (2019) using metagenomics, where they observed the predominance of the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc*. Of the 10 bacterial species identified in the present study, four of them had not yet been found in Colonial cheese microbiota (*B. cereus*, *P. humicus*, *K. variicola* and *R. ornithinolytica*).

None of the yeast genera identified had been previously reported in Colonial cheese. The species *M. guilliermondii* was the most isolated one (Table 1). Yeasts are often part of the cheese microbiota due to their ability to survive in unfavorable conditions and to metabolize milk constituents. These microorganisms play a significant role in the ripening of some varieties of cheese and, under poor hygiene conditions, in the spoiling of products (Irlinger & Mounier, 2009). Some *Candida* sp., *Trichosporon* sp. and *R. mucilaginosa* strains had also been isolated in fresh cheese and involved in spoilage (Garnier et al., 2017). However, they have also been observed to be part of the cheese microbiota unrelated to loss of quality (Togay et al., 2020; Cardoso et al., 2015). Among the positive effects of yeasts in cheese production, the development of cheese flavor and texture is well recognized. These effects are related to the ability to ferment lactose, produce aromatic compounds and to the proteolytic and lipolytic capacity (Agarbati et al., 2021; Biagiotti et al., 2018). Most of the identified isolates hydrolyzed lipids at mesophile temperature, and no activity was observed at refrigerated conditions (Table 2). Some yeast species are being studied to be used as starters, as potential probiotic activity in the human intestine, or to limit the growth of undesired microorganisms (Agarbati et al., 2021; Togay et al., 2020; Helmy et al., 2019).

At 30 °C, 69% of the yeast isolates demonstrated lipolytic activity (Table 2). Proteolytic activity was observed in only one of the strains. Lactose fermentation was identified in 23% of the strains. As it has been observed that yeast present in cheese could help lower the fat and cholesterol levels in people consuming them, perhaps the lipolytic activity observed in many isolates (bacteria and yeast) could have the same effect (Robinson et al., 2021; Zheng et al., 2015). Other authors observed lipolytic activity in several cheese isolated yeasts, such as: *Candida* sp.; *Debaryomyces* sp., *Galactomyces* sp., *Kluyveromyces* sp., *Kodamaea* sp., *Meyerozyma* sp., *Pichia* sp., *Saccharomyces* sp., *Torulasporea* sp., *Trichosporon* sp. and *Yarrowia* sp. (Atanassova et al., 2016; Cardoso et al., 2015; Golić et al., 2013; Roostita & Fleet, 1996; Ferreira & Viljoen 2003; Spanamberg et al., 2004; Fadda et al., 2004). However, some authors could not find proteolytic and lipolytic activity of the identified yeasts depending on the type of cheese (Togay et al., 2020).

The highest lytic activity for bacteria and yeasts occurred at a temperature of 30 °C. This is not surprising as these isolates were obtained from total mesophilic counting. Cheese metatranscriptomic studies confirmed the direct relationship between the increase in temperature and the increase of bacteria expression of protein and lipids enzymes (De Filippis et al., 2016). Bacteria had higher proteolytic activity than yeasts. The bacterial lipolysis and lactose fermentation were lower than those observed for yeasts (46% and 17% respectively). Unlike yeasts, the bacteria showed proteolytic and lipolytic activity at 5 °C (12% and 22% respectively), although in lower percentages than at 30 °C. This lower activity at 5 °C is essential for avoiding product changes during storage. This understanding of the lytic activity of microorganisms can be used to select promising functional starters, for example, as was done by Agarbati et al. (2021) with yeasts strains isolated from dairy environment and Pecorino cheese. These authors obtained 75% of isolates able to hydrolyze at least one substrate (lactose, proteins, and lipids).

No microorganism demonstrated any activity in the Litmus Milk at 5 °C. The species *L. lactis* (nine isolates) and *S. saprophyticus* (six isolates) had the highest activity in Litmus Milk at 30°. All yeasts presented at least one strain with clot formation in this test. This medium has three components that could be metabolized by the microorganisms: glucose, lactose, and casein (MacFaddin, 2000). When casein is precipitated by microorganisms, the milk coagulates to form a curd, and this was the reaction observed in 33

of the 34 microorganisms positive in this test. *L. lactis* is specifically used for this purpose in cheese production. This species was also the microorganism with the highest number of isolates that fermented lactose (test tube). This result was also expected, since the fermentation of lactose by these bacteria used in starter cultures is desired to start the curdling process in the first step in making Colonial cheese (Ávila et al., 2020; Bukvicki et al., 2020; Yang et al., 2019).

Another three isolated species were also able to ferment lactose and break down the casein, and therefore may be responsible for the same deterioration process during maturation depending on their cellular load (*C. freundii*, *P. humicus*, *M. guilliermondii*). Therefore, further studies should be done to assess whether all of these microorganisms affect the organoleptic characteristics of Colonial cheese.

4 Conclusion

This was the first report on culture-based microbiota present in Colonial cheese that was not related to hygiene indicators. This study identified some microorganisms likely involved in the maturation of Colonial cheese using their lytic activity. But some of the species associated with poor hygiene were also found and could cause damage if environmental conditions favor their growth. More research is needed on the species involved in Colonial cheese maturation and their influence on it. This study explored a tiny part of the isolated microbiota in Colonial cheese and undoubtedly metagenomics associated to culturomics could increase the number of species identified and assist dairy industries in producing standard organoleptic characteristics and quality in dairy products, and perhaps even improve them.

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