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Resistome in gram-negative bacteria from soft cheese in Brazil

Resistoma em bactérias gram-negativas de queijo minas frescal no Brazil

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Abstract

Objective: *evaluation of antibiotic resistance in Gram-negative microbiota from ready-to-eat cheese samples.* ***Methodology:*** *this research applied an adapted methodology to select from a food sample viable Gram-negative microbiota displaying antibiotic resistance. The selected food was a cheese that is commonly consumed without thermal processing, the Minas Frescal cheese. The evaluation was followed by a PCR screening in this resistant microbiota, for genes that provide resistance to antibiotics and also to the quaternary ammonium.* ***Results:*** *all cheese samples harbored a resistant microbiota. In 13.3% of the cheese samples analyzed, the resistance reached all ten different antibiotics tested and, in 80%, 8 to 10 different antibiotics. In antibiotics considered critics as the carbapenems: ertapenem presented resistant microbiota in 86.7% of the samples. In cephalosporins, the resistance reached 100% in the third generation (ceftazidime) and almost half of the samples (46.7%) in the fourth generation (cefepime). In genotypic research, seven different resistance genes were found in 69.2% of the bacterial pools, including the beta-lactamase-producing genes ctx, tem, shv, tetracycline-resistant genes, and a high rate of integrons class 1 and 2.* ***Conclusion:*** *the results indicate phenotypically and genotypically that the Minas Frescal cheese can harbor potential resistant microbiota. Therefore, the methodology used is a viable possibility and with a broader answer about the food microbiota role in resistance. This research corroborates the food area as an important sector to be managed to reduce the process of antibiotic resistance.*
Keywords: *Antibiotic Resistance. Food. Resistome. Gram-negative bacteria. Cheese.*

Resumo

Objetivo: avaliação da resistência a antibióticos em microbiota Gram-negativa de amostras de queijo prontas para consumo**. Metodologia:** esta pesquisa aplicou uma metodologia adaptada para selecionar a microbiota Gram-negativa viável apresentando resistência a antibióticos em uma amostra de alimento. O alimento selecionado foi um queijo frequentemente consumido sem processamento térmico, o queijo Minas Frescal. A avaliação foi seguida de uma triagem por PCR, nesta microbiota resistente, para genes que fornecem resistência aos antibióticos e também ao quaternário de amônio. **Resultados:** todas as amostras de queijo apresentaram microbiota resistente. Em 13,3% dos queijos analisados essa resistência alcançou todos os 10 diferentes antibióticos testados e em 80% entre 8 e 10 antibióticos diferentes. Em antibióticos considerados críticos como os carbapenêmicos: ertapenem apresentou microbiota resistente em 86,7% das amostras. Nas cefalosporinas, a resistência atingiu 100% na terceira geração (ceftazidima) e quase a metade das amostras (46,7%) na quarta geração (cefepime). Na pesquisa genotípica, sete diferentes genes de resistência foram encontrados em 69,2% dos pools bacterianos, incluindo o genes produtores de beta-lactamase, genes de resistência à tetraciclina, ctx, tem, shv e uma alta taxa de integron classe 1 e 2. **Conclusão:** os resultados indicam fenotipicamente e genotipicamente que o queijo Minas Frescal pode apresentar uma potencial microbiota resistente. Portanto, a metodologia utilizada é uma possibilidade viável e com uma resposta mais ampla sobre o papel da microbiota na resistência. Esta pesquisa corrobora a área de alimentos como um setor importante a ser gerenciado para redução no processo de resistência a antibióticos.
Palavras-chave: Resistência a antibióticos. Alimento. Resistoma. Bactéria Gram-negativa. Queijo.

INTRODUCTION

Resistant bacteria can be found virtually everywhere: in the environment, humans, animals, water, and food. The latter, though, is often wrongly neglected (WHO, 2017). The One health approach highlights the need for coordination between human and animal surveillance systems aiming for an integrated strategy to limit the spread of resistance. The use of antibiotics in food-producing animals provides a potentially significant risk factor for the selection and dissemination of antimicrobial-resistant microorganisms to humans, which makes animal-derived food of particular concern (EFSA, 2017).

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In Brazil, cheese, a ready-to-eat dairy product, is widely consumed by the adult population and, above all, by the elderly population (IBGE, 2010). Minas Frescal cheese is a traditional Brazilian white and soft cheese with very high moisture content (≥ 55%) made with pasteurized milk. It has its own rich and diverse microbiota that impacts its quality and safety (BRASIL, 1997). Moreover, they are usually consumed without any further thermal processing, including nosocomial patients (VICENSKI; ALBERTI; AMARAL, 2012).

The research of individual species underestimates the total microbial community and its potential antimicrobial resistance risks (ESCOBAR-ZEPEDA; SANCHEZ-FLORES; QUIRASCO BARUCH, 2016). Since a rapid phenotypic detection of resistance can prevent delays in selecting optimal therapy (AZIMI et al., 2013), in this study, an adapted methodology focused on evaluating the microbiota resistance profile in food rather than the isolation and identification of resistant species.

The World Health Organization recognized the multidrug and extensively drug-resistant Gram-negative bacteria as the most critical microorganisms that researches should focus on (WHO, 2017). In this work, we present an evaluation of the phenotypic resistance and the presence of antibiotic-resistant genes in Gram-negative bacteria population in Minas Frescal cheese available in retail stores in Rio de Janeiro city.

METHODOLOGY

Sampling

Fifteen Minas Frescal cheese samples from 13 different commercial brands were retrieved from fifteen supermarket chains in the city of Rio de Janeiro, Brazil. Only cheese samples in their original intact packaging with the surface temperature at low temperature (less than 8ºC) and containing the Federal or State Inspection Seal (sanitary certifications) were purchased. Samples were transported in a thermal container to the Laboratório de Controle Microbiológico de Alimentos da Escola de Nutrição (LACOMEN) of the Departamento de Ciência de Alimentos of the Universidade Federal do Estado do Rio de Janeiro (UNIRIO) no later than 24 hours after collection.

Pre-selection method

Each cheese sample (25g) was incubated at 35°C ± 0.2°C in 225mL of Gram-negative (GN) broth (Oxoid, Basingstoke, UK) for 24 hours.

After this enrichment stage, aliquots of 50 µL from each cheese (sample) were diluted in 5 mL of tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) containing antibiotic discs (Oxoid, Basingstoke, UK) (CDC, 2008). This procedure was done in triplicate, generating three tubes for each antibiotic tested. The ten antibiotics used in testing were representative of Ambler Classification: Cefepime (30 µg), Ertapenem (10 µg), Gentamicin (10 µg), Ampicillin (10 µg), Ampicillin-sulbactam (10/10 µg), Chloramphenicol (30 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Ceftazidime (30 µg), Trimethoprim-sulfamethoxazole (1.25/23.75 µg).

After 18-24h of incubation, all TSB tubes with turbidity from microbial growth were considered positive and qualified for the next step.

Antibiotic Resistance Selection

An adapted disc diffusion method using Agar MacConkey based on the methodology of the guidelines from the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial disk susceptibility test and the Laboratory Protocol for Detection of Carbapenem-Resistant or Carbapenemase-Producing Klebsiella spp. and Escherichia coli from Rectal Swabs was performed in order to select the Gram-negative resistant microbiota (CDC, 2008; CLSI, 2017). Each positive tube of the triplicate from the previous step was inoculated in an agar plate, conserving the triplicates.

According to breakpoints for Enterobacteriaceae, the bacterial pools that were classified as resistant or intermediate in agreement with the CLSI guidelines for the interpretation of antibiotic susceptibility were collected for DNA extraction (CLSI, 2017).

For the DNA extraction step and consequently for PCR, each positive agar plate from the same triplicate was blended to be considered a unique pool. This procedure does not interfere in the analysis as each pool represents the microbiota from tests with the same antibiotic and the same cheese. Therefore, this strategy means that 15 cheese samples tested with 10 antibiotics generate a maximum number of 150 pools, in case of 100% of resistance.

The reference strains used in this step were E. coli ATCC 25922.

DNA extraction

DNA was extracted from all bacterial pools considered resistant or intermediate by using a NucleoSpin Tissue kit (Macherey-Nagel, Germany, 2016) according to the manufacturer's protocol for DNA extraction from Gram-negative bacteria.

PCR

Molecular characterization of phenotypically screened bacterial pools was performed by PCR by using previously published primers and protocols (Table 1). Primers were synthesized by Eurofins Genomics (Ebersberg, Germany) and Invitrogen Thermo Fisher Scientific (California, USA).

PCR screening for the primers described in table 1 was done in all resistant or intermediate pools except for the ctx-M1, ctx-M2, ctx-M8, ctx-M9, ctx-M15, and ctx-M25 that were done in bacterial pool harboring ctx-M. The oxa-48 gene (involved in resistance to carbapenems) was screened in pools resistant to carbapenems, and tet a and tet b genes screened in pools resistant to tetracycline antibiotics.

Table 1 - *Primers: sequence of nucleotides, amplicon size and references.*

Primer	Primer sequence (5' - 3')	Amplicon (bp[a])	PCR Conditions	Reference
tem (F)[b]	CATTTCCGTGTCGCCCTTATTC	800	10 min at 94°C; 30 cycles of 40s at 94°C, 40 s at 60°C, 60 s at 72°C; and 7 min final extension at 72°C.	(DALLENNE *et al.*, 2010)
tem (R)[b]	CGTTCATCCATAGTTGCCTGAC			
shv (F)[b]	AGCCGCTTGAGCAAATTAAAC	713	10 min at 94°C; 30 cycles of 40s at 94°C, 40 s at 60°C, 60 s at 72°C; and 7 min final extension at 72°C.	(DALLENNE *et al.*, 2010)
shv (R)[b]	ATCCCGCAGATAAATCACCA			
oxa (F)[b]	GACCCCAAGTTTCCTGTAAGTG	564	10 min at 94°C; 30 cycles of 40s at 94°C, 40 s at 60°C, 60 s at 72°C; and 7 min final extension at 72°C.	(DALLENNE *et al.*, 2010)
oxa (R)[b]	GGCACCAGATTCAACTTTCAAG			
int – 1 (F)	CTGCGTTCGGTCAAGGTTCT	882	3 min at 94°C; 35 cycles of 60s at 94°C, 60 s at 68°C, 60 s at 72°C; and 7 min final extension at 72°C.	(LANZ; KUHNERT; BOERLIN, 2003)
int – 1 (R)	GGAATGGCCGAGCAGATCCT			
int - 2 (F)	CACGGATATGCGACAAAAAGG	788	3 min at 94°C; 35 cycles of 60s at 94°C, 60 s at 68°C, 60 s at 72°C; and 7 min final extension at 72°C.	(LANZ; KUHNERT; BOERLIN, 2003)
int – 2 (R)	GTAGCAAACGAGTGACGAAATG			
int – 3 (F)	GCCTCCGGCAGCGACTTTCAG	979	3 min at 94°C; 35 cycles of 60s at 94°C, 60 s at 68°C, 60 s at 72°C; and 7 min final extension at 72°C.	(LANZ; KUHNERT; BOERLIN, 2003)
int - 3 (R)	ACGGATCTGCCAAACCTGACT			
kpc (F)	GTATCGCCGTCTAGTTCTGC	635	5 min at 95°C; 30 cycles of 60 s at 95°C, 60 s at 56°C, 60 s at 72°C; and 5 min final extension at 72°C.	(AZIMI *et al*., 2013)
kpc (R)	GGTCGTGTTTCCCTTTAGCC			
ndm (F)	GGTTTGGCGATCTGGTTTTC	621	10 min at 94°C; 36 cycles of 30 s at 94°C, 40 s at 58°C, 50 s at 72°C; and 5 min final extension at 72°C.	(POIREL *et al.*, 2011)
ndm (R)	CGGAATGGCTCATCACGATC			
tet – a (F)	GGCCTCAATTTCCTGACG	372	1 min at 94°C; 30 cycles of 60 s at 94°C, 60 s at 55°C, 2 min at 72°C; and 10 min final extension at 72°C.	(GUILLAUME *et al.*, 2000)
tet - a (R)	AAGCAGGATGTAGCCTGTGC			
tet - b (F)	GAGACGCAATCGAATTCGG	228	1 min at 94°C; 30 cycles of 60 s at 94°C, 60 s at 56°C, 2 min at 72°C; and 10 min final extension at 72°C	(GUILLAUME *et al.*, 2000)
tet – b (R)	TTTAGTGGCTATTCTTCCTGCC			
ges-1 to 9, 11 (F)[b]	AGTCGGCTAGACCGGAAAG	399	10 min at 94°C; 30 cycles of 40 s at 94°C, 40 s at 60°C, 1 min at 72°C; and 7 min final extension at 72°C	(DALLENNE *et al.*, 2010)
ges-1 to 9, 11 (R)[b]	TTTGTCCGTGCTCAGGAT			
per-1, 3 (F)[b]	GCTCCGATAATGAAAGCGT	520	10 min at 94°C; 30 cycles of 40 s at 94°C, 40 s at 60°C, 1 min at 72°C; and 7 min final extension at 72°C	(DALLENNE *et al.*, 2010)
per-1, 3 (R)[b]	TTCGGCTTGACTCGGCTGA			
veb -1,6 (F)[b]	CATTTCCCGATGCAAAGCGT	648	10 min at 94°C; 30 cycles of 40 s at 94°C, 40 s at 60°C, 1 min at 72°C; and 7 min final extension at 72°C	(DALLENNE *et al.*, 2010)
veb -1,6 (R)[b]	CGAAGTTTCTTTGGACTCTG			

Primer	Primer sequence (5' - 3')	Amplicon (bp[a])	PCR Conditions	Reference
imp (F)	GGAATAGAGTGGCTTAATTCTC	188	5 min at 94°C; 35 cycles of 50 s at 94°C, 60 s at 50°C, 1 min at 72°C; and 8 min final extension at 72°C	(DALLENNE *et al.*, 2010)
imp (R)	CCAAACCACTACGTTATCT			
vim (F)	GATGGTGTTTGGTCGCATA	390	15 min at 95°C; 36 cycles of 30 s at 94°C, 20 s at 57°C, 50 s at 72°C; and 5 min final extension at 72°C.	(KARUNIAWATI; SAHARMAN; LESTARI, 2013)
vim (R)	CGAATGCGCAGCACCAG			
oxa 48 (F)	GCGTGGTTAAGGATGAACAC	438	10 min at 94°C; 36 cycles of 30 s at 94°C, 40 s at 54°C, 50 s at 72°C; and 5 min final extension at 72°C.	(POIREL *et al*., 2011)
oxa 48 (R)	CATCAAGTTCAACCCAACCG			
uni515 (F)	GTGCCAGCMGCCGCGGTA[c]	312	3 min at 95°C; 40 cycles of 15 s at 95°C, 60 s at 67°C, 01 min at 72°C; and 5 min final extension at 72°C.	(BARMAN *et al*., 2008)
ent826 (R)	GCCTCAAGGGCACAACCTCCAAG			
qacEΔ1 (F)	AATCCATCCCTGTCGGTGTT	175	5 min at 94°C; 30 cycles of 60 s at 94°C, 60 s at 56°C, 1 min at 72°C; and 7 min final extension at 72°C.	(GUO *et al*., 2015)
qacEΔ1 (R)	CGCAGCGACTTCCACGATGGGGAT			
ctx - M (F)	ATGTGCAGYACCAGTAARGTKATGGC[c]	593	15 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 60°C, 2 min at 72°C; and 10 min final extension at 72°C.	(LEFLON-GUIBOUT *et al.*, 2004)
ctx - M (R)	TGGGTRAARTARGTSACCAGAAYCAGCGG[c]			
ctx -M15 (F)	ATAAAACCGGCAGCGGTG	483	5 min at 94°C; 30 cycles of 30 sec. at 94°C, 30 sec. at 55°C, 90 sec. at 72°C; and 7 min final extension at 72°C	(LEFLON-GUIBOUT *et al*., 2004)
ctx - M15 (R)	GAATTTTGACGATCGGGG			
ctx - M1 (F)	AAAAATCACTGCGCCAGTTC	415	5 min at 94 °C; 30 cycles of 25 sec at 94 °C; 52 °C at 40 sec.; 72°C at 50 sec; 6 min final extension at 72°C	(LEFLON-GUIBOUT *et al*., 2004)
ctx - M1 (R)	AGCTTATTCATCGCCACGTT			
ctx - M2 (F)	CGACGCTACCCCTGCTATT	552	5 min at 94 °C; 30 cycles of 25 sec at 94 °C; 52 °C at 40 sec.; 72°C at 50 sec; 6 min final extension at 72°C	(LEFLON-GUIBOUT *et al*., 2004)
ctx - M2 (R)	CCAGCGTCAGATTTTTCAGG			
ctx - M8 (F)	TCGCGTTAAGCGGATGATGC	327	5 min at 94 °C; 30 cycles of 25 sec at 94 °C; 52 °C at 40 sec.; 72°C at 50 sec; 6 min final extension at 72°C	(LEFLON-GUIBOUT *et al.*, 2004)
ctx - M8 (R)	AACCCACGATGTGGGTAGC			
ctx - M9 (F)	CAAAGAGAGTGCAACGGATG	205	5 min at 94 °C; 30 cycles of 25 sec at 94 °C; 52 °C at 40 sec.; 72°C at 50 sec; 6 min final extension at 72°C	(LEFLON-GUIBOUT *et al*., 2004)
ctx - M9 (R)	ATTGGAAAGCGTTCATCACC			
ctx - M25 (F)	GCACGATGACATTCGGG	327	5 min at 94 °C; 30 cycles of 25 sec at 94 °C; 52 °C at 40 sec.; 72°C at 50 sec; 6 min final extension at 72°C	(LEFLON-GUIBOUT *et al.*, 2004)
ctx - M25 (R)	AACCCACGATGTGGGTAGC			
mcr-1 (F)	ATTATCCGACTTGGGGCAAGG	309	15 min at 94°C; 25 cycles of 30s at 94°C, 90s at 58°C, 60s at 72°C; 10 min final extension at 72°C.	(CAVACO; MORDHORST; HENDRIKSEN, 2016)
mcr-1 (R)	CGCACGATGTGACATTGCTAA			

a. bp: base pairs;
b. ges, per and veb (multiplex PCR); tem, shv and oxa (multiplex PCR);
c. S = g or c; Y = c or t; K = g or t; M = a or c; R = g or a.

Source: This study.

The PCR products were analyzed by agarose gel electrophoresis in Tris-borate-EDTA buffer. They were stained with Blue-Green Loading Dye I (LGC Biotechnology, São Paulo, Brazil) and visualized under ultraviolet light.

RESULTS

Sample characteristics

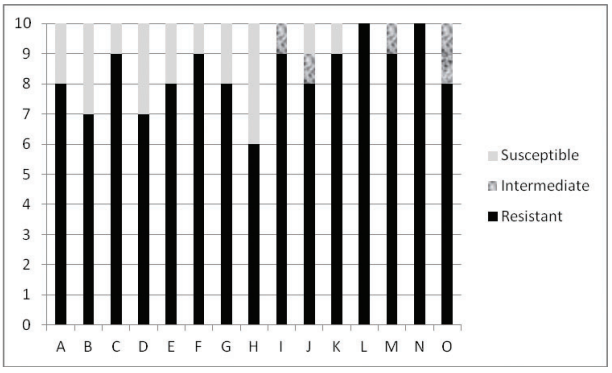
All 15 cheese samples analyzed were within all parameters established by the research for the sampling process, allowing the results to be more representative of the manufacturing process than the storage or trading. The manufacturing process occurred in three different states from Brazil, divided into 67% (10/15) from Minas Gerais, 27% (4/15) from Rio de Janeiro, and 6% (1/15) from Espírito Santo. The cheeses composition was similar: pasteurized milk, sodium chloride, calcium chloride, lactic acid, and rennet, apart from a sample (sample N) added with lactic acid bacteria and without salt. Thirteen different brands were analyzed.

Phenotypic resistance

In the pre-selection phase, 98% (147/150) of pools showed turbidity from microbial growth, indicating a resistant mechanism. Susceptibility 0.2% (3/150) occurred only in ciprofloxacin tests.

Considering the results from pre-selection and disk diffusion steps, the microbiota from all cheese samples displayed a high phenotypic resistance (CLSI, 2017). Resistance proportions varied from 60% to 100% in each cheese. The disc diffusion test revealed that Gram-negative microbiota was resistant to at least eight different antibiotics in 80% (12/15) of the cheese samples and resistant to all tested antibiotics in 13.3% (2/15) (Figure 1).

Figure 1 - *Phenotypic resistance per sample considering the pre-selection and disk diffusion test. In X-axis, cheese samples (A-O). In Y-axis, the number of antibiotics tested.*



Source: This study.

The 20 susceptible results described in figure 1 include three susceptible from the pre-selection step and seventeen from the disk diffusion step. In total, 130 distinct pools (phenotypically resistant and intermediate) were selected for further DNA extraction and PCR investigation.

Considering the results from the antibiotic's perspective, from ten antibiotics tested, 6 of them showed no susceptibility in 100% (15/15) of cheese samples. This alarming resistance profile was verified in the chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and the beta-lactams ceftazidime, ampicillin, and ampicillin-sulbactam. Even in the remaining four antibiotics tested, the resistance reached high values. Samples were resistant to ertapenem in 86.7% (13/15) of cheese, 66.7% (10/15) to gentamicin, 46.7% (7/15) to cefepime (a 4th generation cephalosporin), and 33.3% (5/15) to ciprofloxacin (fluoroquinolone). Intermediate results or SDD (susceptible dose-dependent), which are therefore dependent on concentration and dose frequency, occurred only in the ciprofloxacin in 6.7% (1/15) and cefepime in 26.7% (4/15).

Comparing breakpoint parameters

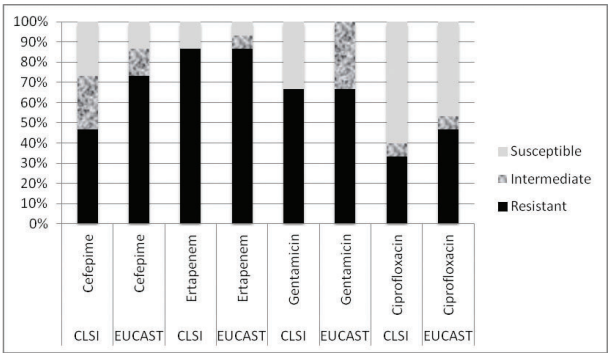
Analyses revealed high rates of resistance according to the CLSI breakpoints. Nevertheless, the resistance indexes would reach even higher rates if the cutoff points of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were adopted (EUCAST, 2018).

In order to perform an accurate comparison between CLSI and EUCAST, results of resistance tests to tetracycline were not used, since EUCAST does not recommend its use for Enterobacteriaceae. Then, exclusive for this comparison, the maximum number of 135 pools (15 cheese tested with 9 antibiotics) will be considered.

Therefore considering EUCAST, the number of susceptible pools would be reduced by half, representing only 7.4% (10/135) compared to the CLSI 14.8% (20/135) susceptible pools in the total. The number of intermediate pools would double, from CLSI 3.7% (5/135) to 8.8% (12/135) using EUCAST, and the resistants would reach 83.7% (113/135) of pools with EUCAST comparing to CLSI 81.5% (110/135) of the pools.

The EUCAST parameter would increase the resistant and intermediate numbers, and decrease the number of susceptible pools to all antibiotics tested. For example, in gentamicin, the 33.3% susceptible samples would turn into zero according to the EUCAST parameter (Figure 2). Although the two cutoff points are different for all antibiotics tested, samples' resistance results were altered only in four antibiotics, less than half. It is related to the fact that there was no measurable inhibition zone formation in 62.2% (84/135) of the pools.

Figure 2 - *Variations in susceptibility ac cording to CLSI and EUCAST*



In Y-axis, the percentage value.

Source: This study.

Table 2 - *Resistance genes quantity in cheese samples and in bacterial pools.*

Gene	Cheese samples	Quantity in pools
int 1	93% (14/15)	48.5% (63/130)
tet b	80% (12/15)	80.0% (12/15)
int 2	73% (11/15)	20.0% (26/130)
Shv	73% (11/15)	33.1% (43/130)
tet a	73% (11/15)	73.3% (11/15)
ctx – M	67% (10/15)	19.2% (25/130)
Tem	67% (10/15)	34.6% (45/130)
ctx- M15	6.7 % (1/15)	12.0 % (3/25)
oxa – 48	6.7% (1/15)	2.4% (1/84)

Source: This study.

Genotypic Resistance

The PCR assay was performed with multi-species DNA samples. In total, a subset of DNA extracted from 130 pools (resistant or intermediate) was tested for the presence of 25 resistance genes.

Seven different resistance genes were found in 69.2% (90/130) pools, totalizing 229 genes (Table 2).

The numbers of PCR analyses on the quantity in pools column vary according to the resistance presented in each pool, as described in the methodology section.

The genes ges, per, veb, imp, vim, kpc, ndm, oxa, oxa-48, ctx-M1, ctx-M2, ctx-M8, ctx-M9, ctx-M25, qacEΔ1, mcr-1 and integron class 3 were not found in the samples.

The most frequently identified gene was the genetic element class 1 integron (int 1), found in 93.3% (14/15) of cheese samples and 48.5% (63/130) of the pools. It was found in resistant pools to all antibiotics tested, including an isolate considered intermediate to the fourth-generation cephalosporin.

All cheeses samples harbored at least one of the resistance genes searched. Notably, 80% (12/15) of cheese presented at least 4 different resistance genes, reaching 7 genes in 20% (3/15) of cheese samples. Table 3 shows the distribution of a total of 229 genes detected in each pool. In only 30.8% (40/130) of the pools, the genes investigated were not found.

Detection of Enterobacteriaceae family

The Enterobacteriaceae family was identified by PCR in all 130 positive pools tested.

Table 3 - *Summary of the genes found in each pool.*

Cheese	Cefepime	Ertapenem	Gentamicin	Ampicillin	Ampicillin-Sulbactam	Chloram-phenicol	Tetracycline	Ciproflox-acin	Ceftazidime	Sulfame-thoxazol Trimetho-prim
A			-	shv, int1, ctx	int 1	int 1	ctx	-		int 1
B	-	-	int1	shv, int1	shv, int1	int1		-		int 1
C			shv, tem, int1, int2	shv, ctx, int2	ctx, int2	shv,int2	shv, tem, tet a, tet b, int1, int2	-	int2	int1
D	-		-	shv	shv, tem		shv, tem, tet a, tet b, int2	-	int1	shv, tem, int1, int2
E			-				tet b	-		
F		int1	-	shv, int1,int2	shv,tem, int1	shv, tem	shv, tem, tet a, tet b, int1, int2			ctx, int1
G	-		ctx, int1	ctx	ctx, int1	int1	ctx, tet a, tet b, int2	-		int1
H	-	-	-	ctx, int1,int2	ctx, int1		tet a	-		ctx, int1
I			shv, tem, int1	shv, tem, int1, int2	shv, tem, int1	shv	shv, tem, tet a, tet b, int1, int2			tem, ctx
J			ctx, int1	shv, int1	ctx	shv, int1	shv, tem, tet a, tet b, int1, int2	Shv		ctx, int1, int2
K		int1	shv, tem, int1, int2	shv, tem, int1, int2	shv, tem, int1, int2	shv, tem, int1, int2	shv, tem, tet b, int1, int2	-		shv, int1, int2
L		int1	tem	tem, int1	tem, int1	tem, int1	tem, tet a, tet b, int1	tem, int1	tem	tem, int1
M				shv, ctx, int2	shv, tem, ctx, int2	shv, tem	tem, tet a, tet b, int2	tem, int1	tem	shv, tem
N	tem	tem, int1	tem, int2	shv, tem, ctx, int1	shv, tem, ctx, int1	shv, tem, int1	shv, tem, ctx, tet a, tet b			ctx, int1
O	int1	int1	int1	shv, tem, ctx-M15, int1	shv, tem, ctx-M15, int1	shv, tem, int1	shv, tem, tet a, tet b, int1			tem, ctx--M15, shv, int1

Blank frames represent resistant pools, in which the genes tested were not found; (-) represents pools that were not considered phenotypically resistant.

Source: This study.

DISCUSSION

Minas Frescal cheese is a ready-to-eat dairy product, not requiring post-purchase thermal processing, which could contribute to a reduction in its microbiota. In Brazil, it is available for the vulnerable population, mostly consumed by the elderly (EFSA, 2017) and also offered to immunosuppressed patients (VICENSKI; ALBERTI; AMARAL, 2012).

Evaluation of antibiotic resistant microbiota, although not usually researched in food, can convey more complete data than those obtained with isolation techniques, corroborated by metagenomics research presenting more than 500 genera in a ripened cheese (ESCOBAR-ZEPEDA; SANCHEZ-FLORES; QUIRASCO BARUCH, 2016).

Phenotypic Resistance

So far, to our knowledge, this study presents the first phenotypic identification of resistance to ertapenem in Minas Frescal cheese in Brazil. The percentage of 86.7% of resistance found in a ready-to-eat food is a high rate, especially considering that Enterobacteriaceae family resistant to carbapenems are ranked as a priority group to development and research of new antibiotics according to the global priority pathogens list (WHO, 2017). Another group established as critic is the Enterobacteriaceae resistant to the third-generation cephalosporin. In this paper, this resistance reached 100% (15/15) of the cheese samples, a high percentage when compared to the 35% found in soft cheese by Omoshaba et al., 2018. It is noteworthy that, even in cephalosporin of fourth-generation resistance was observed in 46.7% of cheese samples.

It is alarming that besides the ceftazidime, five different antibiotics, namely, ampicillin, ampicillin-sulbactam, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole, demonstrated no susceptibility in all different cheese

samples from 13 different brands. Values are higher than those found in multidrug-resistant enterotoxigenic Staphylococcus aureus isolated from milk tanks (PEXARA et al., 2016).

Comparing parameters

The startling growth (of the majority of the pools) to the limit of detection - 6 mm (the disk diameter), allows only the categorization as resistant, irrespective of the cut off applied, which emphasizes the high prevalence of resistance found in cheese microbiota.

Among the pools in which the difference between the two parameters could be evaluated, there was a significant reduction in the number of susceptible pools and an increasing in the resistant and intermediate by applying the EUCAST criterion as it has more stringent breakpoints for susceptibility, though less widespread than the CLSI standards. In other study that difference proved to be less clear, although the EUCAST parameter is slightly more rigid (KASSIM et al., 2016).

Genotypic Resistance

Corroborating the high phenotypic resistance found, 100% of cheese samples harbored at least one gene investigated, reaching up to the co-existence of 7 different genes on the same sample.

Integrons

The integrons are genetic elements that have a key role in the adaptation of bacteria to antibiotic therapy. The cells that contain integrons can select in a pool of genes and fix some genes under intense selective pressure imposed by the use of antibiotics in human medicine, veterinary, and agriculture (GILLINGS, 2014).

The most studied and identified integron in foods, as well as in other areas, is the class 1 integron, having their cassettes more fully described in the literature (GILLINGS, 2014). This ubiquitous characteristic may explain its presence in an SDD pool *to a broad-spectrum antibiotic, the cefepime (fourth-generation cephalosporin).*

In samples analyzed, the class 1 integron was the most prevalent. The values found in this article are even higher than those found in a study with metagenomics approach, where 77% of 35 Minas Frescal cheese presented class 1 integron (DE PAULA et al., 2018).

Despite the recognized importance in the clinical area, the class 2 integrons are still poorly surveyed in the food sector. In our study, it was detected in 73% (11/15) of samples, particularly prevalent in tetracycline-resistant pools, which are commonly associated with int 1 (BELAYNEHE; SHIN; YOO, 2018). An alarming amount of 97% has already been found in a metagenomic study with 35 Minas Frescal cheese samples (DE PAULA et al., 2018).

Research of integron class 3 in food is rare. In our research, none of the cheese samples examined presented class 3 integron likewise in a similar study with cheese samples (DE PAULA et al., 2018)

Though the integrons research is still scarce in the food sector and focused on a small number of bacterial species or class of integron (GILLINGS, 2014),our analysis revealed that more than half (56.2%) of the pools possessed one integron system, and even two systems simultaneously in the same pool which reinforces the importance of investigate these genetic systems to understand its function in food safety.

Tetracycline resistant genes

In our study, 100% of the samples were categorized as phenotypically tetracycline resistant. The high phenotypic resistance and co-existence of tet a and tet b in 66.7% (10/15) of samples may be related to the high rates of integrons found (BELAYNEHE; SHIN; YOO, 2018).

Comparable values were found in Minas Frescal cheese varying from 15% to 100% of prevalence of tet genes (DE PAULA et al., 2018). Lower values were found even in raw milk Egyptian cheese in which it was found in 5% of the isolates (HAMMAD; HASSAN; SHIMAMOTO, 2015).

Study on the use of antibiotics in cattle herds in Minas Gerais State, the State where most of the samples of this study are manufactured, identified that tetracycline and aminoglycosides were the most frequent antibiotics used against mastitis (VIEIRA et al., 2016). This is particularly crucial in the case of cheese, a product of animal origin. Evidence shows that interventions that restrict the use of antibiotics in food-producing animals are associated with a reduction in the presence of antibiotic-resistant bacteria in those animals (TANG et al., 2017).

Beta-lactamase-producing genes

Most extended-spectrum beta-lactamases (ESBLs) evolved from genetic mutations in beta-lactamases tem and shv types and are considered an important mechanism that confers resistance to penicillins and cephalosporins, including the third and fourth generation (DALLENNE et al., 2010).

In our analysis, the most frequent ESBL encoding gene was the shv, present in 73.3% of samples. The shv gene was identified in 6 different antibiotic-resistant pools mainly among the penicillins but either among the non-beta-lactams: tetracycline and chloramphenicol. Multiple drug resistance has been commonly observed in the majority of ESBL-producing, and alarmingly the co-resistance to other antibiotics in common use, such as aminoglycosides, fluoroquinolones, and tetracycline has been frequently reported (XU et al., 2015).

The highest count of tem genes was found in tetracycline's resistant pools, followed by the penicillins. The tem gene rates (67% of the samples) were comparable with a metagenomics study in Minas Frescal cheese, present in 91.4% of the samples but without phenotypic evaluation (DE PAULA et al., 2018).

The ESBLs ctx family that acts in cephalosporins has been identified in Brazil, predominantly in clinical samples, especially in Klebsiella pneumoniae and Escherichia coli (ROCHA; PINTO; BARBOSA, 2016). In our study, the ctx gene was found in 63.3% of samples, mainly among the penicillins-beta-lactams.

Important to highlight that in the 72 pools with ESBLs encoding genes found in this research, 32 presented simultaneously at least two of beta-lactamase (bla) genes described (tem, shv, and ctx). Although the bla genes tem, ctx, and shv genes have already been found in E. coli isolated from cheese and milk, it was restricted to ampicillin-resistant pools and in lower proportions (18.0%, 4.0%, and 1.4% respectively) (OMBARAK et al., 2018). The values found in this paper are superior, even compared to the ESBL-encoding genes researched in E. coli isolated from mastitic milk (EISENBERGER et al., 2018).

The bla genes ges, per, veb, kpc, ndm, imp, vim, oxa, and oxa-48 were not found in samples analyzed. These genes are not commonly investigated in cheese or even dairy products.

Quaternary ammonium resistance

Quaternary ammonium is a class of cationic surfactants present in disinfectants, biocides, and detergents used intensively in hospitals and industry. The resistance genes qac can be carried on class1 integron what increases the co-selection of antibiotic resistance (ZHANG et al., 2015). Although the high prevalence rates of int 1 in this paper, the qacΔE1 gene was not found.

Polymyxin E resistance

The mcr-1 gene that evaluates the genotypic resistance to polymyxin E mediated by plasmid was not found.

CONCLUSION

So far, to our knowledge, this is the first phenotypic identification of a microbiota resistant to ertapenem in Minas Frescal cheese in Brazil.

The amount and diversity of genes found, particularly the integrons, with a methodology that evaluates the microbiota point out the food area as an important sector to be managed to reduce the process of antibiotic resistance.

Data availability

The datasets of the microorganisms analyzed during the current study are available in the Coleção de Cultura de Bactérias Resistentes de Origem Alimentar – CCBROA, available in Sistema de Informação sobre a Biodiversidade Brasileira – SiBBr (https://ipt.sibbr.gov.br/sibbr/resource?r=ccbroa).

A portion of the microorganisms harboring resistance genes identified by PCR in this research had the DNA sequenced and will be published posteriorly.

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