RESEARCH ARTICLE

Marcela González[1]
Jorge Olivera[1]
Estefania Amaro[1]
Stella Reginensi[1]

Enterococci isolated from Uruguayan Colonia cheese – occurrence and antimicrobial resistance

Authors' addresses:
[1]Unidad de Tecnología de los Alimentos, Facultad de Agronomía, Garzón 780, Montevideo, Uruguay

Correspondence:
Marcela González,
Unidad de Tecnología de los Alimentos, Facultad de Agronomía,
Garzón 780, Montevideo, Uruguay.
Phone: +59823547991
e-mail: marcelagonzalez@fagro.edu.uy

Article info:
Received:
Accepted:

ABSTRACT

In recent years, there is a growing concern for antibiotic resistance of food-related enterococci. This study aims to provide data for occurrence and antimicrobial resistance of *Enterococcus* spp. isolated from Colonia cheese. The results showed that *Enterococcus* spp. were found in two thirds of the cheese samples (47 out of 70), of which 72% were identified as *E. faecalis*, 16% as *E. faecium*, 6% as *E. mundtii*, 4% as *E. hirae* and 2% as *E. durans*. The antimicrobial suceptibility showed that almost all *Enterococcus* spp. were sensitive to ampicillin and gentamicin. The highest incidence of resistance was displayed against erythromycin, tetracycline, rifampicin and fosfomycin. *E. faecalis* isolates presented high percentages of resistance to high level streptomycin. The multiple antibiotic resistance index was elevated in half of the *Enterococcus* spp., reaching values as high as 0,8, indicating a potential risk for public health. In this research, several *Enterococcus* isolates could be considered as multidrug-resistant, most of them belonging to *E. faecalis*. Results of present study raise concerns about possible role of cheese enterococci as reservoirs of antibiotic resistance.

Key words: *Enterococcus* spp.; cheese; antibiotic resistance

Introduction

Enterococci are included in a group of multiple genera called lactic acid bacteria (LAB) which are widespread in nature, and they can be isolated from soil, water, plant surfaces, the gastrointestinal tract of animals, and food products (Settanni & Moschetti, 2010; Kim et al., 2020). The distribution of enterococci can be due to their ecological adaptability to adverse conditions, such as a broad range of pH, salinity, and high temperaturas (McAuley et al., 2012). Several contradictory properties have been reported for enterococci. They are beneficial in the fermentation and ripening of certain animal products, such as cheeses and contribute to developing of a particular taste and aroma during ripening (Demirci et al., 2021; Rhoades et al., 2021). On the other hand, enterococci can be undesirable as a food contaminant. Since they can survive pasteurization and grow at high NaCl concentration and in wide pH ranges, they could be part of the microbiota of ready-to-eat food. According to literature, enterococci found in food can be an agent promoting the dissemination of antibiotic resistance (Ghosh & Zurek, 2015; Hanchi et al., 2018).

The food chain is considered the main route of transmission of antibiotic-resistant bacteria between the animal and human populations (Witte, 2007). Enterococci are inherently resistant to some antibiotics and can spread resistance to other species and even other bacterial genera through conjugated transposons and plasmids (Perera et al., 2020). The transmission of resistance genes can regularly occur in the gastrointestinal tract of habitats (Guerrero-Ramos et al., 2016). Dissemination of resistance genes plays an essential role in developing multidrug-resistant (MDR) enterococci (Haghi et al., 2019; Monticelli et al., 2018). The *Enterococcus* species, *Enterococcus faecalis* and *Enterococcus faecium* have been routinely investigated due to the high abundance of virulence genes and antibiotic resistance (Heidari et al., 2017; Shridhar & Dhanashree, 2019).

The prevalence of *Enterococcus* spp. in the dairy products has been considered as a result of unhygienic conditions during the milk production and processing. However, enterococci are part of the native microbiota of raw milk (Giraffa, 2003). Its presence in foods has often been shown to be unrelated to direct fecal contamination. Enterococci can enter milk directly from human or animal feaces, or indirectly from contaminated water sources, the exterior of the animal and/or milking equipment and the cold tank (Gelsomino et al., 2002). Different species of *Enterococcus* have been identified in dairy products, but *E. faecalis* and *E. faecium* are the most important, followed by *Enterococcus durans* and *Enterococcus casseliflavus* (Sarantinopoulos et al., 2002; Giraffa, 2003: Jamet et al., 2012). Although differences have been observed in the relevance of different species between countries, which could be attributed to geographical variations or differences in isolation methodologies. A study

carried out in Brazil reported a high proportion of contamination with *Enterococcus* spp. in raw and pasteurized milk, cheese and vegetables, where *E. faecium* was the predominant species, followed by *E. faecalis*, *Enterococcus gallinarum* and *E. casseliflavus* (Gomes et al., 2008). In contrast, *E. durans* was found to be the most abundant species (after *E. faeca*lis and *E. faecium*) in a study of dairy products in Italy (Cariolato et al., 2008). Similar controversies are observed when evaluating the antibiotic resistance of these enterococci. Although *E. faecalis* is generally recognized as the main species carrying virulence genes and greater resistance to antibiotics, isolates of *E. faecium* and *E. durans* have also shown resistance to different antibiotics such as tetracycline, erythromycin, kanamycin and chloramphenicol (Jamet et al., 2012; Terzić-Vidojević et al., 2015; Trivedi et al., 2011).

Based on the above, monitoring this group of bacteria is key to assess the progression, arising and transference of antibiotic resistances. In the present study, a typical Uruguayan dairy product, Colonia cheese, were sampled in order to evaluate the presence, biodiversity and antibiotic susceptibility of *Enterococcus* species.

Materials and Methods

Sample collection and isolation of enterococci from Colonia cheese

A total of 70 Colonia cheese were obtained from local retail stores in Montevideo – Uruguay. The samples were put in insulated boxes with ice bags and were analysed on the day of arrival. Ten grams of each cheese sample was reconstituted in 90 ml of 0.1% sterile peptone water and homogenized using a Stomacher®400 Circulator (Seward Ltd., UK). Microbial counts were performed by plate counting of serial 10-fold dilutions in 0.1% sterile peptone water, on triplicate. *Enterococcus* spp. were aerobically grown in m-Enterococcus Agar (Neogen® Culture Media) at 37 ± 2 ºC for 24-48 h. A total of 165 colonies with typical enterococcal morphology were selected. Presumptive identification of isolates was carried out by Gram staining, cell morphology, catalase test, and growth in the presence of 6.5% (w/v) NaCl. The enterococcal isolates were deposited in the laboratory culture collection with 15% glycerol at −80°C for further analysis.

Isolation of total DNA

Presumptive *Enterococcus* isolates were grown overnight in Tryptic Soy Broth (TSB, Oxoid Ltd., UK) and cells were harvested at 10,000 rpm for 5 min in a Spectrafuge 7M tabletop centrifuge (Labnet International Inc., USA). Cell pellets were suspended in 200 µL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA was purified using a GeneJET Genomic DNA Purification Kit (Thermo Scientific Incorporation, Wilmington, DE, USA) following the manufacturer's instructions. Purified DNA was suspended in 40 µL TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Incorporation, Wilmington, DE, USA).

Isolate identification by multiplex-PCR and 16S rDNA sequence analysis

Enterococcal species were determined by multiplex-PCR using *sod*A species-specific primers. *E. faecalis*, *E. faecium* and *E. durans* species were identified by amplification of 210-, 191- and 264-pb fragments with primer pairs EFS1 (5'-CTGTAGAAGACCTAATTTCA) /EFS2 (5'CAGCTGTTTTGAAAGCAG), EFM1 (5' T(G/T)CAGCAATTGAGAAATAC)/EFM2 (5' CTTCTTTTATTTCTCCTGTA) and ED1 (5'AAACGCAGCTATTGAAAA)/ED2 (5'AAGCGTCCGGCAGCC), respectively. Multiplex- PCR for *sodA* was performed according to Jamet et al., 2012. Multiplex- PCR identified, and other *Enterococcus* isolates yielding negative results by the multiplex PCR, were further speciated by a 16s rDNA sequencing region using universal eubacterial primers fD1 and rD1 (Weisburg et al., 1991). Amplified 16S rDNA fragments were purified and sequenced by Macrogen Sequencing Service, Korea, using an ABI Prism3730XL capillary sequencer (Applied Biosystems, Foster City, CA). The DNA sequences were compared with those of the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify type strains with highest similarity

Genomic fingerprinting analysis using repetitive element palindromic $(GTG)_5$ - PCR

Two most common species identified, *E. faecalis* and *E. faecium*, were subjected to repetitive element palindromic PCR (rep-PCR) genomic fingerprinting analysis using primer $(GTG)_5$ to study intra-species diversity (Versalovic et al., 1994). Analysis was carried out in 25-µL reaction mixtures containing 1X Thermo buffer (Thermo Scientific Incorporation), 1.5 mM $MgCl_2$ 200 µM of each dNTP (Thermo Scientific Incorporation, 1 U of Taq polymerase (Thermo Scientific Incorporation), 2 µM $(GTG)_5$ primer (Gevers et al., 2001), and 20 ng of template DNA. Amplifications were performed in a DNA thermal cycler Corbett CG1–96 with a palmtop computer interface (Corbett Research Ltd.). The PCR cycling parameters included a denaturation step at 95°C for 7 min; 35 cycles each consisting of 94°C for 1 min, 46°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min. Control reaction

mixtures lacking template DNA were included with each analysis. The PCR products were electrophoresed on 1.5% agarose gels using 0.5X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH = 8.0) as running buffer at 10 V/cm for 1 h, stained with GoodView Nucleic Acid Stain 5% (vol/vol; SBS Genetech Co. Ltd.). As a size standard, the Generuler 100-bp DNA Ladder Plus (Thermo Scientific Incorporation) was used. Band profiles were visualized and photographed under UV light using an ultra-compact gel documentation system (Cleaver Scientific Ltd., Warwickshire, UK).

Antibiotic susceptibility testing

Using the disc diffusion method, the *Enterococcus* spp. isolates were assessed for their susceptibility to 11 antibiotics. After incubation at 37ºC for 18 h, the inhibition halos were measured and the strains classified as resistant (R), intermediate resistant (I), or susceptible (S). The assays were conducted and interpreted using the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). The antibiotics tested (Oxoid Ltd., UK) comprised vancomycin (VA-30 µg), ampicillin (AMP-10 µg), tetracycline (TE-30 µg), high level streptomycin (STR-300 µg), high level gentamicin (CN-120 µg), erythromycin (E-15 µg), chloramphenicol (C-30 µg), ciprofloxacin (CIP-5 µg), fosfomycin (FOT-50 µg), rifampicin (RD-5 µg), and nitrofurantoin (F-300 µg). *E. faecalis* ATCC 29212 was used as quality control strain. The multiple antibiotic resistance index (MAR-Index) of each isolate was calculated as a/b (a: number of antibiotics to which the isolates were resistant, b: number of antibiotics against which the isolates were tested) (Krumperman, 1983).

Statistical analysis

All microbial counts were $\log_{10}$-transformed to obtained log-normal distributed data and results were statistical analyzed by the One-way ANOVA. Differences were considered statistically significant at $P < 0.05$. The genomic data $(GTG)_5$-PCR fingerprintings were analyzed by Gel Compar II 6.5 software (Applied Maths, Kortrijk, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation coefficients with the unweighted pair group method with arithmetic mean clustering algorithm (UPGMA) analysis method.

Results

Isolation and identification of Enterococcus spp.

Colonia cheese is a typical dairy product and the most commonly consumed cheese in Uruguay, which does not yet have a Protected Designation of Origin (PDO). Colonia is a cow milk, semi-hard and greasy cheese with holes, with a consistent and elastic texture and smooth taste (Hirigoyen et al., 2018). A total of 70 Colonia cheese were obtained from local retail stores in Montevideo – Uruguay.

The occurrence of enterococci in Colonia cheeses was higher than expected (Figure 1). *Enterococcus* spp. were found in two thirds of the cheese samples (47 out of 70). These occurrence levels are similar to those observed by Jamet et al. (2012) in a study that covered different types of cheeses from France, but lower than those observed in typical cheeses from Egypt or Turkey (Hammad et al., 2015; Sanlibaba & Senturk, 2018). The abundance of the enterococci population varied between 10^2 CFU/g to 10^5 CFU/g. Of the cheeses in which enterococci were present, approximately half of them showed counts greater than 10^4 CFU/g (Figure 1). These count ranges coincide with those observed by Jamet et al (2012), although the maximum values observed in cheeses from France were up to 10^8 CFU/g.

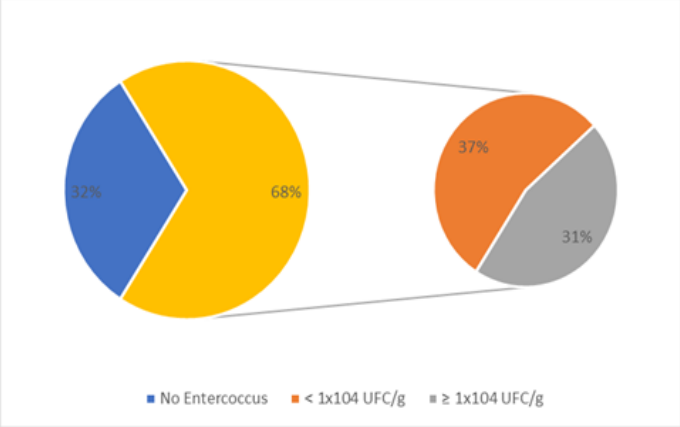

Figure 1. *Occurrence of Enterococcus spp. in Colonia cheese samples.*

From the 70 cheese samples analyzed, 165 suspected *Enterococcus* isolates were obtained. These isolates were characterized by primary biochemical tests such as Gram stain, catalase activity and growth in the presence of 6.5% NaCl. After this primary characterization, a total of 86 isolates presented typical characteristics of the *Enterococcus* genus (cocci, Gram +, catalase -, and growth in 6.5% NaCl), and were identified by 16S rDNA sequencing. The species found were *E. faecalis, E. faecium, E. mundtii, E. hirae and E. durans* in order of relative abundance (Figure 2).

The relative presence of the different species varies between authors, but in general *E. faecalis, E. faecium* and *E. durans* appear as the prevalent species (Sanlibaba & Senturk, 2018; Russo et al., 2018). The study of the genetic diversity present among enterococci isolates was carried out by rep-PCR using the primer $(GTG)_5$, for most prevalent species, *E.*

faecalis and *E. faecium*. For *E. faecalis* isolates, two groups were observed (Figure 3). The same was observed for *E. faecium* isolates, but the divergence between the two groups was greater than in the case of *E. faecalis* (Figure 4).

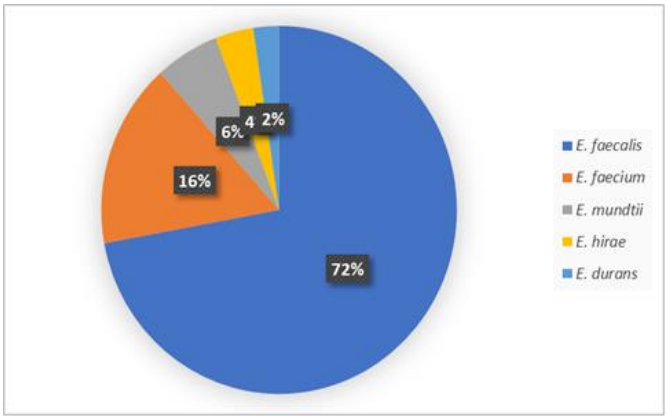

Figure 2. *Relative occurrence of the different Enterococcus species in Colonia cheese samples.*

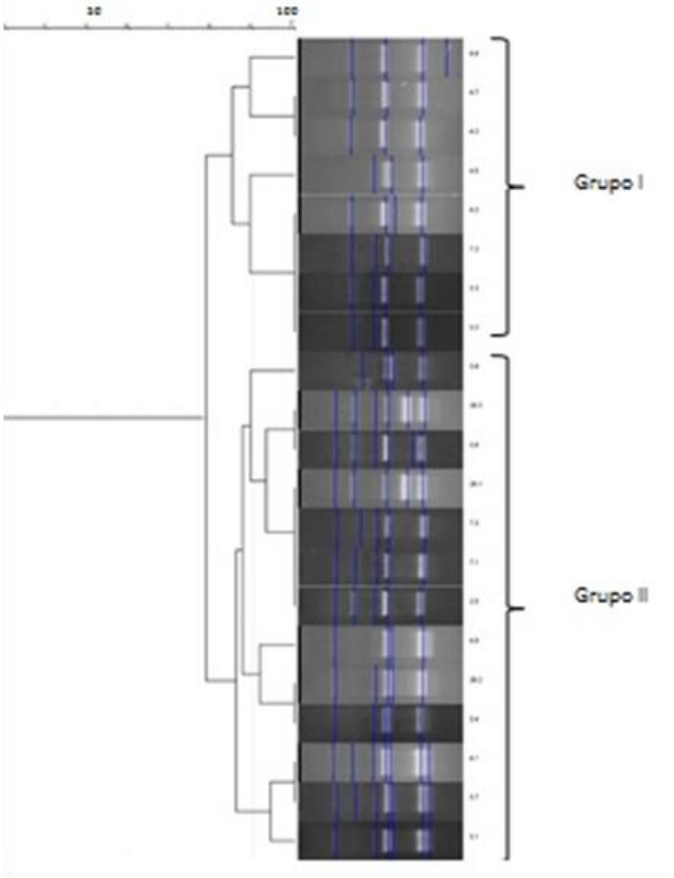

Figure 3. *Genotypic diversity of E. faecalis isolates by rep-PCR using the GTG5 primer.*

Antimicrobial susceptibility testing

The suceptibility of the isolates to different antibiotics showed that 100% of *Enterococcus* spp. isolates were resistant to at least one antimicrobial, Table 1 shows as a percentage, the level of resistance to the 11 antibiotics

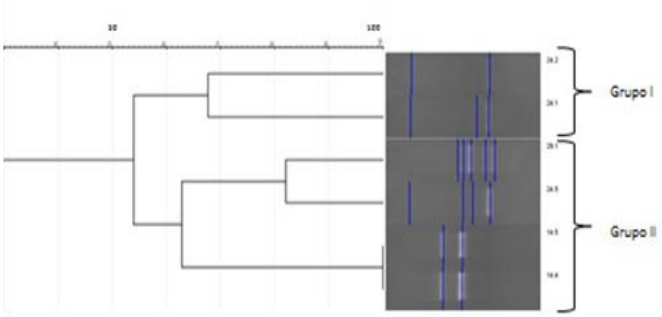

Figure 4. *Genotypic diversity of E. faecium isolates by rep-PCR using the GTG5 primer.*

analyzed. Interpretation of results was based on Clinical Laboratory Standards Institute (CLSI) guidelines and isolates were classified as susceptible (S), intermediate (I) or resistant (R) for each antibiotic. Almost all the isolates studied show sensitivity to ampicillin (100%) and gentamicin (97.6%) at therapeutic concentrations. Half of the isolates were susceptible to streptomycin (53.5%), nitrofurantoin (68.4%) and chloramphenicol (48.9%). On the other hand, more than half of the isolates showed resistance to erythromycin (54.8%), tetracycline (66.7%), rifampicin (76.2%) and fosfomycin (66%). The high occurrence of isolates resistant to these antibiotics has also been observed in Italian cheeses (Russo et al., 2018) and artisanal cheeses from Portugal (Câmara et al., 2020). A high occurrence of tetracycline-resistant isolates was observed. On the base of the results obtained by the disk difussion assay, high percentage of isolates with intermediate resistance was observed for vancomycin, ciprofloxanin and chloramphenicol (Table 1). These isolates should be subjected to the MIC determination by microdilution assay, in order to confirm the classification resulted from the disk difussion assay. For the purposes of calculating MAR-index and the heatmap, these isolates were considered resistant.

The prevalence of antimicrobial resistance with regards to species is shown in Figure 5. The frequency of resistance is expressed as a percentage of the resitant isolates for each species and each antibiotic tested. High levels of susceptibility were observed for ampicillin and high level gentamicin, for all species. These levels of susceptibility were also observed for streptomycin, with the exception of *E. faecalis* isolates of which 56% were resistant to this antibiotic. The highest occurrence of resistant isolates of all species was observed for ciprofloxacin, erythromycin,

Table 1. *Antimicrobial resistance of Enterococcus spp. isolated from cheese (n= 86).*

Antimicrobial agent	S %	I %	R%
Ampicillin (AMP-10 µg)	100	0	0
Chloramphenicol (C-30 µg)	48.9	44	7.1
Ciprofloxacin (CIP-5 µg)	16.7	63	20.2
Erythromycin (E-15 µg)	4.7	40.5	54.8
Fosfomycin (FOT-50 µg)	32	2	66
Gentamicin (CN-120 µg)	97.6	0	2.4
Nitrofurantoin (F-300 µg)	68.4	19	22.6
Rifampicin (RD-5 µg)	4.8	19	76.2
Streptomycin (STR-300 µg)	53.5	4.8	41.7
Tetracycline (TE-30 µg)	26.2	7.1	66.7
Vancomycin (VA-30 µg)	30	49	21

fosfomycin, nitrofurantoin and rifampicin. The ratio of antimicrobial resistance profile to total number of antibiotics tested (multiple antibiotic resistance, MAR-index) was calculated for all *Enteroccoccus* isolates. The MAR-Index of *Enterococcus* isolates ranged from 0 (no resistance to any antibiotic, 1 out of 86) to 0.8 (resistance to 8 different antibiotics, 1 out of 86). About 50% of the isolates had a MAR-index greater or equal to 0.36, which means that these isolates showed resistance to 4-8 of the 11 antibiotics tested. If we take into account the presence of multidrug-resistant isolates per species, *E. feacalis*, *E. feacium* and *E hirae* were the ones that present, on average, the highest multidrug resistance values (Figure 5).

Discussion

Enterococci are present in several fermented foods mainly as a result of their high adaptability to the different food processing and storage conditions. Cheese, as other fermented food, harbours numerous living microorganisms of different genera, among which enterococci are considered part of the microbiota (Giraffa et al., 2003; Jamet et al., 2012).

Despite the globalization and modernization of the dairy industry, many traditional artisanal cheeses are still produced on rural farms, from raw or thermized milk, with or without the use of starter cultures (Domingos-Lopes et al., 2011; Fuka et al., 2017; Montel et al., 2014). In Uruguay, artisanal cheese factories produce about 5.3% of the total milk production and represent 26% of the country's dairy establishments (INALE, 2014). Artisanal cheese is made with raw, pasteurized or thermized milk, produced exclusively on the farm, and is equivalent to 50% of the total cheese consumption in our country (Palau & Mesa, 2007). Colonia cheese is one of the main artisanal cheeses produced in Uruguay.

In this study, enterococci were present in about 70% of the cheeses analyzed. This result was consistent with the results of previous studies, which reported that the percentage of positive samples of enterococci in traditional cheese was 72% in France (Jamet et al., 2012), 100% in Southern Brazil (Furlaneto-Maia et al., 2014), 94.6% in Spain (Nieto-Arribas, et al., 2011), 99% in Turkey (Sanlibaba & Senturk., 2018) and 90% in Egyptian (Hammad et al., 2015). However, despite the high occurrence, the maximum enterococci counts were lower than those reported in French, Mexican or Brazilian artisanal cheeses (Jamet et al., 2012; González-Montiel & Fernández, 2015; Câmara et al., 2020). A collection of 86 enterococci isolates of Colonia cheese origin was characterized phenotypically and genotypically in order

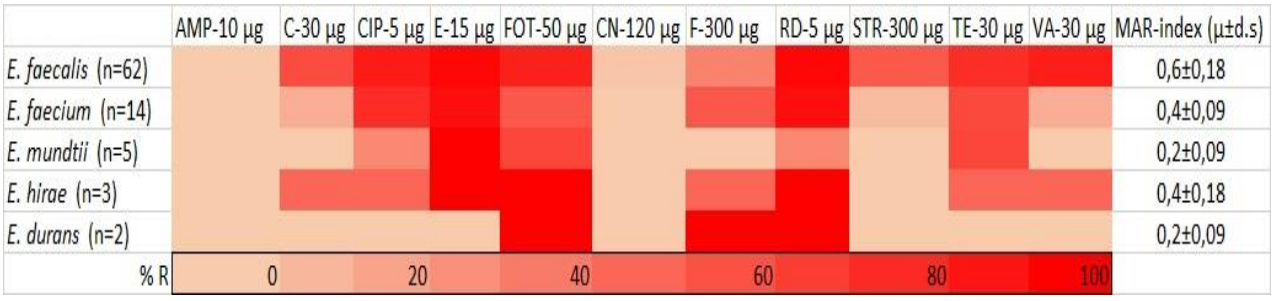

Figure 5. *Antimicrobial resistance heatmap of Enterococcus species. MAR-index for species was caculated as µ±d.s of the individual isolates MAR-index.*

to explore their antimicrobial resistance profile. Overall, the prevalence of *E. faecalis* and *E. faecium* was revealed. Our findings were in agreement with the results reported by several authors, although in some cases the order of prevalence between both species varies (Câmara et al., 2020; Sanlibaba & Senturk, 2018; Nieto-Arribas et al., 2011; Jamet et al., 2012; Hammad et al., 2015). Others such as *E. durans*, *E. hirae*, *E. avium* and *E. casseliflavus* are mentioned by some authors (Nieto-Arribas et al., 2011; Russo et al., 2018). It is worth noting the isolation of *E. mundtii* strains. This species is not frequently found in cheeses, although some bacteriocinogenic strains have been studied as biopreservatives in Brazilian cheeses (Pingitore et al., 2012). The genotypic diversity of the predominant species was evaluated by $(GTG)_5$-PCR. For the isolates belonging to *E. faecalis*, two groups were observed, however, no relationship was observed between the isolates of each group with respect to the origin or type of sample. The same happens with *E. faecium* isolates, but the divergence between the two groups is greater than in the case of *E. faecalis*.

The *Enterococcus* genus generally posses a broad spectrum of natural antimicrobial resistance. *Enterococcus* species are intrinsically resistant to cephalosporins, low levels of most β-lactams, low levels of aminoglycosides, sulfonamides, clindamycin, quinupristin, and dalfopristin. Resistances meanwhile acquired by *Enterococcus* include resistance to chloramphenicol, erythromycin, tetracycline, fluoroquinolones, glycopeptides and high levels of clindamycin, aminoglycosides, and β-lactams (García-Solache & Rice, 2019; Hollenbeck & Rice, 2012). Besides, their innate resistance to antimicrobials, most of the strains included in our study had acquired resistance to at least one of the antimicrobials tested. Our results did not show *Enterococcus* strains resistant to penicillins (ampicillin) and a few were resistant to high level aminoglycoside (gentamicin). However, half of the isolates were resistant to high level streptomycin. In previous studies, resistance to high-level streptomycin has been described to a low extent (5.0%) in isolates from traditional Italian cheese (Gaglio et al., 2016), and recently observed at high percentages in *E. faecium* and *E. faecalis* isolates from clinical samples (Khodabandeh et al., 2018). In this study, only *E. faecalis* isolates presented high percentages of resistance to this antimicrobial. The highest incidence of resistance was displayed against erythromycin, tetracycline, rifampicin and fosfomycin, as previously reported (Jamet et al., 2012; Russo et al., 2018; Dapkevicius et al., 2021). Tetracycline resistance is one of the most acquired antibiotic resistance in *Enterococcus* spp. isolated from food (Kang et al., 2018; Ogier & Serror 2008; Rocha et al., 2022). The high levels of tetracycline resistance are not completely surprising. The overexploitation of these antibiotics in husbandry activities is a possible reason for the high level of resistance frequently found among enterococci (Fuka et al., 2017; Ogier & Serror, 2008; Kürekci et al., 2016). In fact, the widespread prevalence of related resistance genes in the environment and animal facilities has been described (Jamet et al., 2012). For instance, Gaglio et al. (2016) found 17.5% resistance to this antibiotic among enterococci isolated from Italian PDO-cheeses, equipment surfaces, and raw materials used in production. Given that tetracycline, erythromycin, and rifampicin are not the antibiotics of choice in the treatment of enterococci infections, resistance to these antibiotics can be considered trivial (Hammad et al., 2015). However, the spread of enterococcal resistance genes via conjugative plasmids and transposons to other bacterial genera could be a major concern (Haubert et al., 2018). As a result of the genomic plasticity of the enterococcal genome, they have a great capacity to acquire and spread genetic traits, including resistance genes (Ahmed et al., 2018; Bonacina et al., 2017). Enterococci have indeed been found to trade genetic determinants both in vitro and in vivo, not only within their own genus, but also with bacteria belonging to other genera with whom they share habitats, other LAB (lactobacilli, lactococci), streptococci, staphylococci, *Listeria*, and bifidobacteria (Werner et al., 2013; Haubert et al., 2018). Enterococci has also developed effective mechanisms for horizontal gene transfer, and this explains (at least in part) the increasing dissemination of antibiotic-resistance genes within this genus (Chajęcka-Wierzchowska et al., 2019).

It is remarkable to underline that in our investigation high resistance to vancomycin was detected, although half of the isolates were classified as intermediate resistant and should be confirmed by MIC assays. Zooming on antibiotic resistance within both prevalent enterococcal species; with *E. faecalis* confirmed as the most resistant species, in agreement with Dapkevicius et al. (2021). In a more detailed analysis, among the 86 isolates, half of them had a MAR-index greater or equal to 0,36, which means that these isolates showed resistance from 4 to 8 of the 11 antibiotics tested. Multidrug-resistant bacteria has been defined as an acquired non-susceptibility to at least one agent placed in three or more antimicrobial classes with different cellular targets (Magiorakos et al., 2011). In this research, several *Enterococcus* isolates could be considered as multidrug-resistant, most of them belonging to *E. faecalis*. Multidrug-resistant enterococci strain isolates from cheeses were also found in other studies (Câmara et al., 2020; Jamet et al., 2012; Mannu et al., 2003).

The impact of the enterococcal microbiota of cheeses on the health of their consummer is still the object of debate, despite the wealth of knowledge gathered in recent years on their presence, technological properties, potential health benefits and antibiotic resistance (Graham et al., 2020;

Dapkevicius et al. 2021; Centeno & Carballo, 2023). Due to the uncertainty on their safety, enterococci do not possess Qualified Presumption of Safety (QPS) status in the EU and are not Generally Regarded as Safe (GRAS) in the USA. Identifying and typing enterococci isolated from food is key to control, prevent and limit the spread of pathogenic enterococcal strains. In addition, studies on antimicrobial resistance surveillance are very important for the control and reduction of resistance determinants dissemination, as well as for the study of the risk/benefit role of enterococci in fermented foods regarding the QPS status.

Conclusions

This study reports on the presence of *Enterococcus* spp. and its resistance profile to antimicrobial compounds, in Colonia cheese. Results showed that enterococci of dairy origin can be a potential source of dissemination of antimicrobial resistance. The presence of *Enterococcus* spp. in Colonia cheeses is generally not high, although it should be monitored due to the high occurrence of multidrug-resistant isolates. By making use of the increasingly accessible high throughput, culture-independent technologies in conjunction with classical microbiology techniques, one should further investigate enterococcal diversity and population dynamics in these cheeses. A better understanding of their influence upon human health is a must, not only to minimize the risks associated with their ability to disseminate antibiotic-resistance genes, but also to permit a better exploitation of their potential as adjunct, protective, and probiotic cultures.

Acknowledgements

This research was carried out under financial support from “Fondo Vaz Ferreira - 2019” D2C2-MEC (Dirección de Innovación, Ciencia y Tecnología, Ministerio de Educación y Cultura, Uruguay).

References

Ahmed, M.O. & Baptiste, K.E. 2018. Vancomycin-resistant enterococci: A review of antimicrobial resistance mechanisms and perspectives of human and animal health. Microb. Drug Resist. 24: 590–606.

Bonacina, J., Suárez, N., Hormigo, R., Fadda, S., Lechner, M., Saavedra, L. 2017. A genomic view of food-related and probiotic Enterococcus strains. DNA Res. 24: 1–24.

Câmara, S. P. A., Dapkevicius, A., Silva, C. C. G., Malcata, F. X., Enes Dapkevicius, M. L. N. 2020. Artisanal Pico cheese as reservoir of *Enterococcus* species possessing virulence and antibiotic resistance properties: Implications for food safety. Food Biotechnology. 34(1): 25 - 41.

Cariolato, D., Andrighetto, C., Lombardi, A., 2008. Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. Food Control, 19: 386-392.

Centeno, J. A., & Carballo, J. 2023. Current Advances in Cheese Microbiology. Foods, 12(13): 2577.

CLSI (Clinical & Laboratory Standards Institute, US). 2015. Performance standards for antimicrobial susceptibility testing: twenty-fifth informational supplement. Wayne, Clinical and Laboratory Standards Institute. 240 p. (CLSI document M100-S25).

Chajęcka-Wierzchowska, W., Zadernowska, A., Zarzecka, U., Zakrzewski, A., Gajewska, J. 2019. Enterococci from ready-to-eat food-horizontal gene transfer of antibiotic resistance genes and genotypic characterization by PCR melting profile. J. Sci. Food Agric., 99: 1172–1179.

Dapkevicius, M. de L. E., Sgardioli, B., Cˆamara, S. P. A., Poeta, P., & Malcata, F. X. 2021. Current trends of enterococci in dairy products: A comprehensive review of their multiple roles. Foods, 10(4): 821.

Demirci, T., G€oktepe, Ç. K., €Oztürk, H._I., Akın, N., Akyol, _I., & Dertli, E. 2021. Prevalence and fingerprinting of lactic acid bacteria community during 180 days of ripening in traditional Turkish goatskin bag Tulum cheeses produced in the mountainous region of Karaman using culture-dependent and -independent methods. Int. Dairy Journal, 118: 105041.

Domingos-Lopes, M. F. P., Stanton, C., Ross, P. R., Dapkevicius, M. L. E., & Silva, C. C. G. 2017. Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese. Food microbiology, 63: 178-190

Fuka, M.M., Maksimovic, A.Z., Tanuwidjaja, I., Hulak, N., Schloter, M. 2017. Characterization of enterococcal community isolated from an artisan Istrian raw milk cheese: biotechnological and safety aspects. Food Technol. Biotechnol. 55: 368–380.

Furlaneto-Maia, L., Rocha, K. R., Henrique, F. C., Giazzi, A., Furlaneto, M. C. 2014 Antimicrobial Resistance in *Enterococcus* sp. Isolated from Soft Cheese in Southern Brazil. Advanced Microbiology, 4: 18–175.

Gaglio, R., Couto, N., Marques, C., Lopes, M. D. F. S., Moschetti, G., Pomba, C., Settanni, L. 2016. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. International Journal of Food Microbiology. 236: 107 - 114.

García-Solache, M., & Louis B. Rice. 2019. "The *Enterococcus*: a model of adaptability to its environment." Clinical microbiology reviews 32.2: 10-1128.

Gelsomino, R., Vancanneyt, M., Cogan, T.M., Condon, S., Swings, J. 2002. Source of enterococci in a farmhouse raw-milk cheese. Applied and Environmental Microbiology. 68: 3560 - 3565.

Ghosh, A., & Zurek, L. 2015. Antibiotic resistance in enterococci: A food safety perspective. In C. Y. Chen, X. Yan, & C. R. Jackson (Eds.), Antimicrobial resistance and food safety. Methods and techniques (pp. 155–180). New York, USA: Academic Press.

Giraffa, G. 2003. Functionality of enterococci in dairy products. International Journal of Food Microbiology. 88(2-3): 215 – 222

Gomes, B.C., Esteves, C.T., Palazzo, I.C., Darini, A.L., Felis, G.E., Sechi, L.A., Franco, B.D., De Martinis, E.C. 2008. Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. Food Microbiol. 25: 668-675.

González-Montiel, L. & Fernández, F. 2015. Perfil microbiológico del queso de aro consumido en la Cañada Oaxaqueña. Brazilian Journal Food Technology. 18(3): 250 – 257

Graham, K., Stack, H., Rea, R. 2020. Safety, beneficial and technological properties of enterococci for use in functional food applications–a review. Crit. Rev. Food Sci. Nutr. 60: 3836–3861

Guerrero-Ramos, E., Cordero, J., Molina-Gonzalez, D., Poeta, P., Igrejas, G., Alonso- Calleja, C., et al. 2016. Antimicrobial resistance and virulence genes in enterococci from wild game meat in Spain. Food Microbiology, 53: 156-164.

Haghi, F., Lohrasbi, V. & Zeighami, H. 2019. High incidence of virulence determinants, aminoglycoside and vancomycin resistance in enterococci isolated from hospitalized patients in Northwest Iran. BMC Infectious Diseases, 19: 744.

Hammad, A.M., Hassan, H.A., Shimamoto, T. 2015. Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. in Egyptian fresh raw milk cheese. Food Control. 50: 815–820.

Hanchi, H., Mottawea, W., Sebei, K., & Hammami, R. 2018. The genus *Enterococcus*: Between probiotic potential and safety concerns-an update. Frontiers in Microbiology, 9: 1791.

Haubert, L., da Cunha, C. E. P., Lopes, G. V., & da Silva, W. P. 2018. Food isolate *Listeria monocytogenes* harboring *tet*M gene plasmid-mediated exchangeable to *Enterococcus faecalis* on the surface of processed cheese. Food research international, 107: 503-508.

Heidari, H., Hasanpour, S., Ebrahim-Saraie, H. S., & Motamedifar, M. 2017. High incidence of virulence factors among clinical *Enterococcus faecalis* isolates in Southwestern Iran. Infection & Chemotherapy, 49: 51-56.

Hirigoyen, D., De Los Santos, R., Calvo, M. F., González-Revello, A., & Constantin, M. 2018. Chemical composition and seasonal changes in the fatty acid profile of Uruguayan "Colonia" Cheeses. Grasas y Aceites, 69(2): 254-254.

Hollenbeck, B.L. & Rice, L.B. 2012. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence, 3: 421–569.

INALE, 2014. Encuesta lechera INALE 2014: resultados preliminares [En línea]. Montevideo: INALE. Disponible en: http://www.inale.org/innovaportal/file/4086/1/encuesta-lechera-2014--presentacion-resulta- dos-preliminares-foro-a.pdf

Jamet, E., Akary, E., Poisson, M. A., Chamba, J. F., Bertrand, X., Serror, P. 2012. Prevalence and characterization of antibiotic resistant *Enterococcus faecalis* in French cheeses. Food Microbiology. 31(2): 191- 198

Kang, I. B., Kim, D. H., Chon, J. W., & Seo, K. H. 2018. Effect of microbial control measures on farmstead cheesemaking and antimicrobial resistance of *Staphylococcus aureus* and *Enterococcus* spp. isolates. Journal of Food Safety, 38(2): 1–10.

Khodabandeh, M., Mohammadi, M., Abdolsalehi, M. R., Hasannejad-Bibalan, M., Gholami, M., Alvandimanesh, A., Pournajaf, A., & Rajabnia, R. 2018. High-level aminoglycoside resistance in *Enterococcus faecalis* and *Enterococcus faecium*; as a serious threat in hospitals. Infectious Disorders - Drug Targets, 20(2): 223–228.

Kim, S.-H., Kim, D.-H., Lim, H.-W., Seo, K.-H. 2020. High prevalence of non-faecalis and non-faecium *Enterococcus* spp. in farmstead cheesehouse and their applicability as hygiene indicators. LWT Food Sci. Technol., 126: 109271.

Krumperman, P.H. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 46: 165-170.

Kürekci, C., Önen, S. P., Yipel, M., Aslantaş, Ö., & Gündoğdu, A. 2016. Characterisation of phenotypic and genotypic antibiotic resistance profile of enterococci from cheeses in Turkey. Korean Journal for Food Science of Animal Resources, 36(3): 352.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., ... & Monnet, D. L. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection, 18(3): 268-281.

Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Dupr`e, I., & Sechi, L. A. 2003. Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. International Journal of Food Microbiology, 88(2–3): 291–304.

McAuley, C.M., Britz, M.L., Gobius, K.S., Craven, H. 2015. Prevalence, seasonality, and growth of enterococci in raw and pasteurized milk in Victoria, Australia. J. Dairy Sci., 98: 8348–8358.

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. International journal of food microbiology, 177: 136-154.

Monticelli, J., Knezevich, A., Luzzati, R., & Di Bella, S. 2018. Clinical management of non-faecium non-faecalis vancomycin-resistant enterococci infection. Focus on *Enterococcus gallinarum* and *Enterococcus casseliflavus*. Flavescens. Journal of Infection and Chemotherapy, 24: 237-246.

Nieto-Arribas, P., Sesena, S., Poveda, J.M., Chicon, R., Cabezas, L., Palop, L., 2011. *Enterococcus* populations in artisanal Manchego cheese: biodiversity, technological and safety aspects. Food Microbiol. 28: 891-899.

Ogier, J. C., & Serror, P. 2008. Safety assessment of dairy microorganisms: The *Enterococcus* genus. International Journal of Food Microbiology, 126(3): 291–301.

Palau, H. & Mesa Macedo, A. (2007). Cluster de quesería artesanal de San José y Colonia. Montevideo: PACPY- MES

Perera, L. N., Mafiz, A. I., Amarasekara, N. R., Chang, E., Rao, V. B. K., & Zhang, Y. 2020. Antimicrobial-resistant *E. coli* and *Enterococcus* spp. recovered from urban community gardens. Food Control, 108: 106857.

Pingitore, E. V., Todorov, S. D., Sesma, F., & de Melo Franco, B. D. G. 2012. Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. Food Microbiology, 32(1): 38-47.

Rhoades, J., Anastasiou, I., Michailidou, S., Koinidis, A., Doulgerakis, C., Alexa, E. A., et al. 2021. Microbiological analysis of Greek Protected Designation of Origin cheeses and characterisation of the isolated lactic acid bacteria. International Dairy Journal, 123: 105183.

Rocha, P. A. B., Marques, J. M. M., Barreto, A. S., & Semedo-Lemsaddek, T. 2022. *Enterococcus* spp. from Azeitão and Nisa PDO-cheeses: Surveillance for antimicrobial drug resistance. LWT, 154: 112622.

Russo, N., Caggia, C., Pino, A., Coque, T. M., Arioli, S., & Randazzo, C. L. 2018. *Enterococcus* spp. in ragusano PDO and pecorino siciliano cheese types: a snapshot of their antibiotic resistance distribution. Food and Chemical Toxicology, 120: 277-286.

Sanlibaba, P., & Senturk, E. 2018. Prevalence, characterization and antibiotic resistance of enterococci from traditional cheeses in Turkey. International Journal of Food Properties, 21(1): 1955-1963.

Sarantinopoulos, P., Kalantzpoulos, G., Tsakalidou, E. 2002. Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. Int. J. Food Microbiol., 76: 93–105.

Settanni, L., & Moschetti, G. 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food microbiology, 27(6): 691-697.

Shridhar, S., & Dhanashree, B. 2019. Antibiotic susceptibility pattern and biofilm formation in clinical isolates of *Enterococcus* spp. Interdisciplinary Perspectives on Infectious Diseases, Article 7854968.

Terzić-Vidojević, A., Veljović, K., Begović, J., Filipić, B., Popović, D., Tolinački, M., ... & Golić, N. 2015. Diversity and antibiotic susceptibility of autochthonous dairy enterococci isolates: are they safe candidates for autochthonous starter cultures?. Frontiers in Microbiology, 6, 954.

Trivedi, K., Cupakova, S., & Karpiskova, R. 2011. Virulence factors and antibiotic resistance in enterococci isolated from food-stuffs. Veterinarni Medicina, 56(7): 352-357.

Versalovic, J., Schneider, M., De Bruijn, F. J., & Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in molecular and cellular biology, 5(1), 25-40.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of bacteriology, 173(2): 697-703.

Werner, G., Coque, T.M., Franz, C.M., Grohmann, E., Hegstad, K., Jensen, L., van Schaik, W., Weaver, K. 2013. Antibiotic resistant enterococci–tales of a drug resistance gene trafficker. Int. J. Med. Microbiol., 303: 360–379.

Witte, W. 2007. Impact of antibiotic use in animal feeding on resistance of bacterial pathogens in humans. In CIBA foundation symposia (Vol. 207, pp. 61–75). Ciba Found Symp.