LWT - Food Science and Technology 154 (2022) 112622

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Enterococcus spp. from Azeitão and Nisa PDO-cheeses: Surveillance for antimicrobial drug resistance

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ARTICLE INFO

Keywords:
Enterococcus spp.
PDO-Cheese
Antimicrobial resistance
Food chain
Surveillance

ABSTRACT

Enterococcus spp. were isolated from PDO-cheese of Azeitão and Nisa at six cheesemaking units (Azeitão: A1, A2, A3, A4; and Nisa: N9, N10), over four years (2016–2019). Genomic typing was performed using RAPD and distinct enterococci (n = 145) were identified at the species level by multiplex-PCR and evaluated regarding antimicrobial drug resistance (AMR). Antibiotics from nine distinct classes (aminoglycosides, macrolides, oxazolidinones, chloramphenicol, streptogramins, tetracyclines, glycopeptides, β-lactams, and quinolones) were selected for AMR surveillance and breakpoint criteria defined by EUCAST and CLSI were considered and compared. Regarding species allocation, 78 enterococci were identified as *E. faecium*, 37 confirmed as *E. faecalis* and 30 as *E. durans*. High levels of resistance to quinupristin-dalfopristin, tetracycline and teicoplanin were observed. Some resistances to clinically relevant antimicrobials were also detected, including β-lactams, aminoglycosides, and glycopeptides. Two isolates were considered multidrug-resistant, one according to EUCAST and the other to CLSI breakpoint criteria. Overall, considering the absence of reports regarding enterococcal-related toxinfections or infections resulting from the consumption of PDO-cheeses, traditional foods harbouring these bacteria should be considered safe. However, the possibility of horizontal gene transfer events associated with antibiotic resistance determinants further highlights the importance for AMR surveillance along the food chain.

1. Introduction

The food chain is considered the main route of transmission of antibiotic-resistant bacteria between the animal and human populations (Witte, 2007). Specifically, fermented foods that are not submitted to heat before consumption can provide a vehicle for antibiotic-resistant bacteria, linking the animal microbiota and the human gastrointestinal tract (Mathur & Singh, 2005). Therefore, a great concern with foodborne bacteria is their possible role as reservoirs for antibiotic resistance determinants, which can be problematic if these bacteria act as opportunistic pathogens or if resistance genes are transferred to commensal bacteria and from those to human/animal pathogens, thus impairing antibiotic treatment of common infections (Devirgiliis et al., 2013; Mathur & Singh, 2005). Studying the pathogenic potential and antibiotic susceptibility of food microbiota has become increasingly relevant due to acquired knowledge on horizontal gene transfer (HGT) (Palmer et al., 2010). Indeed, resistance to antibiotics can be acquired and spread horizontally among different bacteria or be intrinsic to a bacterial genus or species, providing the genetic ability to survive in the presence of an antimicrobial agent (Palmer et al., 2010; Paulsen et al., 2003).

Cheese is one of the utmost important fermented milk products produced and consumed by humans. In the European Union, 156.8 million tons of whole milk were processed in 2018, and 10.3 million tons of cheese was produced (Griffin et al., 2020). Of all the milk produced in the EU in 2018, 37.7% was used for cheese production, being the main food obtained from milk. Europe is the second-largest global producer of caprine and ovine milk, Mediterranean countries being the ones which most contribute to this production (Boyazoglu & Morand-Fehr, 2001). The traditional process of fermentation and maturation is globally used

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https://doi.org/10.1016/j.lwt.2021.112622
Received 30 August 2021; Received in revised form 7 October 2021; Accepted 11 October 2021
Available online 15 October 2021

to produce a panoply of cheeses, which involves the exploitation of microorganisms naturally present in raw milk (Macori & Cotter, 2018). As no starter microbial cultures are added, there is much less control on the microorganisms present in the final product. In Portugal, the artisanal production of regional cheeses is an essential part of cultural heritage, and ten traditional cheeses have protected designations of origin (PDO) status (https://europa.eu/rapid/press-release_IP-96-153_en.htm. Consulted: July 10, 2021) (European Comission, 1996). The focus of the present study will be on two PDO cheeses, from Azeitão and Nisa. The production of Azeitão cheese is restricted to counties of Palmela, Sesimbra and Setúbal, whereas Nisa cheese is produced in Nisa, Crato, Castelo de Vide, Marvão, Portalegre, Monforte, Arronches and Alter do Chão. These PDO cheeses are both obtained from raw sheep milk; in Nisa's case the milk comes from a concrete breed of sheep called Merina Branca, while in Azeitão's cheese no breed is specified. The vegetable rennet used in both cheeses is obtained from *Cynara cardunculus* (Queijo de Azeitão PDO, n.d.; Queijo de Nisa PDO, n.d.). In 2018, 38 million euros worth of cheese and cream cheese were exported from Portugal (data from INE, 2018) being Azeitão and Nisa PDO cheeses the second and fifth most produced PDO cheeses in Portugal, respectively (data from Direção-Geral de Agricultura e Desenvolvimento Rural (DGADR)) (Alves et al., 2016).

Enterococci are included in a group of multiple genera called lactic acid bacteria (LAB), which can be found in nutritionally rich environments, ranging from plants to animal raw materials and fermented food products (Settanni & Moschetti, 2010). This vast group has been the target of multiple studies regarding all kinds of fermented foods (Choi & Woo, 2015; Cocolin et al., 2004; Cogan et al., 1997; Delpech et al., 2012; Foulquié Moreno et al., 2006; Franz et al., 1999), and enterococci are the only LAB genus considered as opportunistic pathogens, being a major cause of healthcare-associated infections (Russo et al., 2018). This controversial role is associated with their presence in human and animal microbiota and along the food chain, being accentuated by the known occurrence of intrinsic and acquired resistance to different antibiotics (Bertrand et al., 2000; Giraffa et al., 2000; Peters et al., 2003; Pimentel et al., 2007; Porto et al., 2016; Russo et al., 2018; Teuber et al., 1999; İspirli et al., 2015). Moreover, the use of antibiotics as growth promoters in food animals is known to be one of the most critical factors for reservoirs of transferable antibiotic resistance in this group (Giraffa, 2002). Acquired antibiotic resistance in enterococci has been described, such as resistance to erythromycin, linezolid, chloramphenicol, tetracycline, teicoplanin, vancomycin and ciprofloxacin (Hollenbeck and Rice, 2012). In addition, resistance to tetracycline and erythromycin has been observed in isolates from animal facilities and in foods of animal origin, and resistance to tetracycline has been attributed to the overexploitation of these antibiotics in veterinary practices (Chopra & Roberts, 2001). High levels of enterococci in food products usually result from poor hygienic practices during manufacture. However, it has been proven that they also play a significant role in ripening and aroma development in many cheeses, such as Manchego, Mozzarella, Kefalotyri, Serra da Estrela or Cebreiro (Franz et al., 1999). Studies on raw milk cheese (Foulquié Moreno et al., 2006) showed that this group is a crucial component of the natural cultures involved in fermentation, and contribute to ripening, taste and flavour (Franz et al., 1999; Giraffa, 2003). The persistence of these bacteria during stressful stages, like ripening, can be attributed to their wide range of growth temperatures, high tolerance of heat, salt, and acid (Cogan et al., 1997). In many cheeses, enterococci comprise a major part of the fresh cheese curd microbiota, and, in some cases, they are the predominant microorganisms in the fully ripened product (Giraffa, 2003). As a result, this group of bacteria was not recommended to the qualified presumption of safety (QPS) list (Koutsoumanis et al., 2019) and are not Generally Regarded as Safe (GRAS) in the USA (Dapkevicius et al., 2021). Therefore, their security in foods must be analysed case by case.

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, PDO-cheeses from four Azeitão and two Nisa cheesemaking units (Portugal) were sampled over four years of production to isolate *Enterococcus* spp. After counting the colony forming units (CFUs) present in each cheese sample, a genomic typing of the isolates and identification at the species level was performed. This research aimed to evaluate the antibiotic susceptibility among enterococci isolated from PDO cheese over time, to survey and understand if and how this trend evolved.

2. Materials and methods

2.1. Samples and isolation of enterococci

PDO-cheeses produced in Azeitão (38.5194° N, 9.0138° W) and Nisa (39.5180° N, 7.6484° W) were collected from distinct cheesemaking units, once a year, over four years, four from Azeitão (A1, A2, A3 and A4) and two from Nisa (N9 and N10). Once collected, samples were kept in sterile recipients at −80 °C until characterisation, which was done in the same year of collection for each cheese. The samples were prepared by adding 225 mL of Peptone Water (Scharlau) to 25 g of cheese in a *Stomacher* bag and then processed in a peristaltic blender (Stomacher Lab-Blender 400) for 90 s. Both the rind and the interior filling were part of the total 25 g used to obtain a representative sample of every analysed cheese. The mother solution (10^{-1}) obtained was used to prepare serial dilutions to inoculate 0.1 mL by superficial spread platting in different growth media to quantify cheese microbiota. *Enterococcus* spp. was aerobically grown in Slanetz and Bartley (SBA) growth medium (Scharlau) at 37 °C ± 2 °C for 44h ± 4h in aerobic conditions. Lactic acid bacteria (mostly *Lactobacillus* spp.) were anaerobically grown in Man Rogosa and Sharpe (MRS) medium at 30 °C for 72h ± 4h, and *Lactococcus* spp. was anaerobically grown in M17 growth medium at 30 °C for 72h ± 4h. The CFUs were enumerated, and approximately 20% of the characteristic colonies were randomly selected and purified for further characterisation.

2.2. DNA extraction

The genetic material from purified isolates was extracted using the boiling method (Millar et al., 2000). A colony was suspended in 50 μL of Tris-EDTA buffer with 0.1% (v/v) Tween 20 (Merck) and the bacterial suspension was incubated for 10 min at 100 °C. Immediately after, the samples were put in ice for 5–10 min to induce a thermic shock and centrifuged at 18928×*g* for 2 min in a HermLe® Z233 MK-2 (HermLe, Germany). The supernatant was stored at −20 °C or directly used in PCR reactions.

2.3. Genomic diversity

2.3.1. RAPD-PCR

A reaction mixture with a total volume of 20 μL was prepared, containing 2 μL of Buffer 10x for Taq II Supreme polymerase (NZYtech, Portugal), 1 μL of M13 primer (5′-GAG GGT GGC GGT TCT-3′) at 50 pmol, 1.25 μL of MgCl2 at 50 mM, 0.5 μL of dNTPs at 10 mM, 1U of NZYTaq II DNA Supreme polymerase and 1 μL of DNA (Cocolin et al., 2004; Rossetti & Giraffa, 2005). Amplification was performed using a Doppio thermocycler (VWR, USA) in the following conditions: 94 °C for 5 min, followed by 40 cycles consisting of 94 °C for 1 min, annealing at 40 °C for 2 min, extension at 72 °C for 2 min, and a final step at 72 °C for 10 min. Amplification products were stored at 4 °C until electrophoresis. For electrophoresis (110V for 2 h 15 min), 8 μL of product mixed with 2 μL of GelStar (stock solution 10X, Lonza) fluorochrome was applied to a 1.2% agarose gel with 0,5X TBE buffer. An image of the gel was taken at ChemiDoc XRS+ with the software ImageLab.

2.3.2. Data analysis

The software BioNumerics (version 6.6.5, Applied Maths, Belgium)

was used to analyse the different profiles obtained. All images were normalised, Pearson correlation coefficient was calculated, and dendrograms were created through unweighted pair group method with arithmetic mean (UPGMA). Those dendrograms were used to choose genomically distinct representative isolates for subsequent analysis.

2.4. Identification of enterococcal isolates by PCR multiplex

In order to confirm the genus and identify the species of the selected isolates, a multiplex polymerase chain reaction (PCR) was performed (Ke et al., 1999). A set of primers was used to confirm genus allocation. As to species identification, three sets of primers were used to identify the three most common species found in traditional cheeses, as shown in Table 1 (Arias et al., 2006; Jurkovič et al., 2006). The reaction mixture had a total volume of 20 μL, containing 4 μL of Buffer 5x for Taq II polymerase (NZYtech, Portugal), 0.2 μL of primer Ent1 and Ent2 at 50 pmol, 0.3 μL of each of the other primers at 50 pmol, 0.8 μL of MgCl2 at 50 mM, 0.3 μL of dNTPs at 10 mM, 1U of NZYTaq II DNA polymerase and 1 μL of DNA.

2.5. Antibiotic susceptibility testing

The antibiotic susceptibility to thirteen agents (amoxicillin-clavulanic acid 30 μg (AMC), ampicillin 10 μg (AMP), chloramphenicol 30 μg (C), ciprofloxacin 5 μg (CIP), gentamicin 120 μg (CN), erythromycin 15 μg (E), levofloxacin 5 μg (LEV), linezolid 30 μg (LZD), quinupristin-dalfopristin 15 μg (QD), streptomycin 300 μg (S), tetracycline 30 μg (TE), teicoplanin 30 μg (TEC), vancomycin 30 μg (VA); Oxoid, UK), and was evaluated among the representatives of *Enterococcus* spp.. The assessment of antibiotic susceptibility was performed using the Kirby-Bauer disc diffusion method. Each bacterial culture was aerobically grown overnight on BHI and suspended on sterile Ringer solution (Oxoid, UK) to a concentration of 0.5 in the McFarland scale (approximately 10^8 CFU/mL). Bacterial suspensions were spread out on a squared petri dish with a sterile swab, and a disc of each antibiotic was placed with equidistant space between each other. After incubation at 37 °C for 24 h, the resulting halo diameters were measured and interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2016) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) breakpoints.

2.6. Statistical analysis

Using Python3, chi-square statistics with respective p-values were calculated to evaluate if there were significant differences between the number of resistances observed in different years, cheesemaking units and species allocation. The significance level (α) was set at 5%. Graphics were plotted using Seaborn library.

Table 1
PCR amplification details for enterococcal species and genus identification.

Target bacteria	Primer	Sequence (5′ to 3′)	Product (bp)
E. faecalis	ddlE1	ATCAAGTACAGTTAGTCTT	941
	ddlE2	ACGATTCAAAGCTAACTG	
E. faecium	ddlF1	GCAAGGCTTCTTAGAGA	550
	ddlF2	CATCGTGTAAGCTAACTTC	
E. durans	mur2edF	AACAGCTTACTTGACTGGACGC	177
	mur2edR	GTATTGGCGCTACTACCCGTATC	
Enterococcus spp.	Ent1	TACTGACAAACCATTCATGATG	112
	Ent2	AACTTCGTCACCAACGCGAAC	

3. Results and discussion

3.1. Isolation and enumeration of bacteria

Cheeses from six distinct cheesemaking units (A1, A2, A3, A4, N9 and N10) were collected over four years of production (2016–2019) and bacterial enumeration (CFU/gr) was determined, per sample, for each group of isolated bacteria (plotted on Fig. 1). The lactic acid bacteria and *Lactococcus* spp. presented similar CFU counts. However, lactococci were the predominant microbial group, representing 38% of the cheese isolates and the highest CFU count of $2.77x10^{12}$ in unit N9 in 2017. Regarding enterococci, the lowest count observed was $1.14x10^4$ for N9 in 2018 and the highest $2.95x10^8$ for A4 in 2019. Enterococcal CFU counting results were consistent with previous studies that analysed Terrincho, Manchego, Cebreiro, La Serena, White-brined, Kefalotyri, Teleme and bryndza cheeses in which CFU/g varied between 10^4—10^7 (Franz et al., 1999; Jurkovič et al., 2006; Pintado et al., 2008). In addition, studies with Manchego, Armada, Cebreiro, Picante, Majoero, Feta, Telemem Mozzarella, Monte Veronese, Fontina, Caprino, Serra, Venaco and Comté cheeses demonstrate that *Enterococcus* spp. are predominant in the fully ripened product (Giraffa, 2003). However, we clearly saw that this was not the case for Azeitão and Nisa cheeses, since enterococci had consistently lower counts than the other bacteria. In a study with Serra da Estrela cheeses (Macedo et al., 2004) it was observed that lactobacilli and lactococci caused a reduction in enterococci counts. This reduction was due to the importance of pH value for the survival of enterococci in cheeses, lactobacilli and lactococci in high counts cause a drop in pH that allows a natural control of enterococcal growth. In the present study, the lowest dilution presenting a countable number (up to 150 colonies) of enterococci was used to randomly select 20% of characteristic colonies from the isolation plates, for further analysis.

3.2. Diversity assessment and identification of Enterococcus spp.

After bacterial purification achieved by subsequent streaking of individual colonies in selective medium, the DNA was obtained from each isolate and the genetic material used in RAPD-PCR amplification, using primer M13. Amplification patterns were analysed with Bionumerics software, aiming to assess microbial diversity and select genomically distinct enterococci for further characterisation (data not shown).

Overall, a total of 145 isolates were selected and submitted to genus and species allocation (details in Table 2). Seventy eighty enterococci (53.8%) were identified as *E. faecium*, whereas 37 (25.5%) were confirmed as *E. faecalis* and 30 as *E. durans* (20.7%). This result is not in line with other studies, in which *E. faecalis* was the most frequent species in cheese (Suzzi et al., 2000; Çitak et al., 2004). However, Jorkovic et al. (2006) also identified *E. faecium* as the predominant species in bryndza cheese (Jurkovič et al., 2006). To a lesser extent, *E. durans* is expected to be present in artisanal cheeses (Dapkevicius et al., 2021; Giraffa, 2002).

3.3. Antibiotic susceptibility assay

Antibiotic susceptibility was evaluated on 145 genomically distinct isolates, using thirteen antibiotics belonging to nine different classes (aminoglycosides, macrolides, oxazolidinones, chloramphenicol, streptogramins, tetracyclines, glycopeptides, β-lactams and quinolones) and three distinct cellular targets (protein synthesis inhibition, cell wall synthesis inhibition and DNA synthesis inhibition) were tested. Resistance to the chosen antibiotics had been previously studied for different foods and cheeses from Portugal, Germany, Italy, Turkey and other parts of Europe (Bertrand et al., 2000; Delpech et al., 2012; Giraffa et al., 2000; Elal Mus et al., 2017; Peters et al., 2003; Pimentel et al., 2007; Porto et al., 2016; Russo et al., 2018; Teuber et al., 1999).

The breakpoints considered were established by EUCAST (EUCAST, 2021) and CLSI (CLSI, 2016) classifications for susceptibility. There are breakpoint discrepancies between the two criteria and breakpoints to

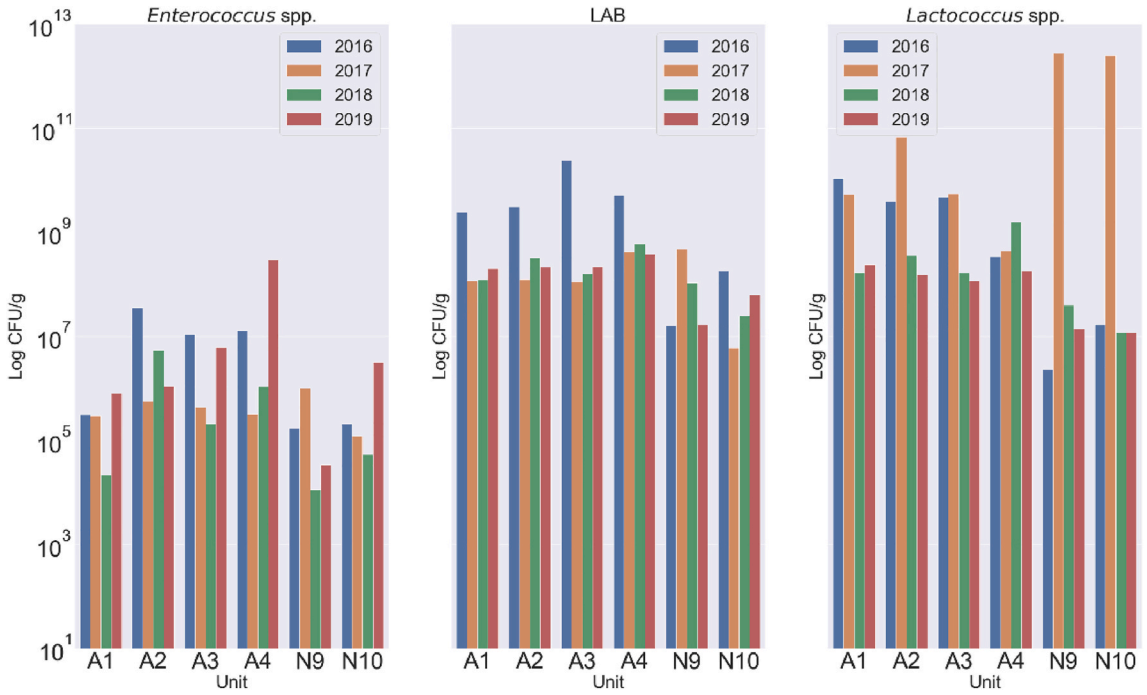

Fig. 1. Enumeration of CFUs/g in *Enterococcus* spp., LAB (mostly *Lactobacillus* spp.) and *Lactococcus* spp., by year of production and cheesemaking unit.

Table 2
Frequency of each enterococcal species (*E. faecium*, *E. faecalis*, *E. durans*) by year and cheesemaking unit.

Variables		*E. faecium* (n = 78)	*E. faecalis* (n = 37)	*E. durans* (n = 30)
Year, n (%)	2016	9(30.0)	5(16.7)	5(16.7)
	2017	5(17.9)	8(28.6)	8(28.6)
	2018	3(6.5)	13(28.3)	13(28.3)
	2019	20(48.8)	4(9.8)	4(9.8)
Unit, n (%)	A1	10(30.3)	11(33.3)	11(33.3)
	A2	6(23.1)	4(15.4)	4(15.4)
	A3	8(32.0)	6(24.0)	6(24.0)
	A4	3(11.1)	3(11.1)	3(11.1)
	N9	4(30.8)	1(7.7)	1(7.7)
	N10	6(28.6)	5(23.8)	5(23.8)

some of the chosen antibiotics have not been defined by EUCAST. Nevertheless, both criteria were used, not only to compare the obtained results, but also to assess if resistances were found using both classifications. However, it is important to emphasize that CLSI may be over-represented in the results. In addition, it must be highlighted that enterococci from A4 cheesemaking unit (2016) were not tested for erythromycin, this antibiotic not being represented in the statistical analysis regarding those isolates. Moreover, only *E. faecium* isolates were considered for statistical tests for quinupristin-dalfopristin according to EUCAST, which influences its representativity.

Considering the total of susceptible and resistant enterococci, 9% of the isolates showed resistant phenotype by following EUCAST criteria, while according to CLSI only 6.9% were classified as resistant. In Table 3 the frequency of resistances is shown, following both the EUCAST and CLSI breakpoint criteria reported by year, including information regarding cheesemaking unit and species allocation. In 2016, the percentage of resistances reported was 3.8% according to EUCAST and 8.5% considering CLSI. In 2017, there was a significant decrease to 3.8% resistance according to EUCAST and 4.7% regarding CLSI, the lowest percentage of resistance in both criteria. As for 2018, 5.6% of the isolates were resistant according to EUCAST and 4.8% to CLSI. Finally, in 2019 the highest resistance rates for both criteria were found: 13.7%

Table 3
Frequency of resistances reported and chi-square analysis of resistant enterococcal isolates throughout the years (2016–2019), considering cheesemaking units and species allocation, and following EUCAST and CLSI breakpoint criteria. Percentages were calculated considering the total of resistant and susceptibility results.

Variables	Frequency of resistance, n(%)		p-value (df)	
Year	**EUCAST (n=948)**	**CLSI (n=1881)**	**EUCAST**	**CLSI**
2016	26(13.3)	33(8.5)	7.65e-05* (3)	0.002* (3)
2017	7(3.8)	17(4.7)		
2018	17(5.6)	29(4.8)		
2019	36(13.7)	52(9.8)		
Unit				
A1	18(8.6)	31(7.2)	0.236(5)	0.001*(5)
A2	16(9.3)	22(6.5)		
A3	18(11.2)	22(6.8)		
A4	19(10.4)	18(5.2)		
N9	10(11.6)	25(14.8)		
N10	5(3.7)	13(4.8)		
Species allocation				
E. durans	2(1.1)	71(7.0)	2.57e-05* (2)	0.031 (2)
E. faecalis	17(7.7)	43(8.9)		
E. faecium	67(12.3)	17(4.4)		

*Significant values for $\alpha = 0.05$.

according to EUCAST and 9.8% for CLSI. These results were further compared using statistical analysis (Table 3), which confirmed significant differences in the frequency of resistances throughout the four years under study, according to both EUCAST ($\chi^2 = 21.667$, $p < 0.001$) and CLSI ($\chi^2 = 14.992$, $p = 0.002$) criteria. Similar values of resistance percentage were observed in 2017 and 2018, and in 2016 and 2019, for both classifications. Significant differences in resistant isolates were found between cheesemaking units according to CLSI values (χ^2=19.893, $p = 0.001$). Regarding this classification, the highest percentage of resistance among the six cheesemaking units was reported in N9 unit, while N10 presented the lowest resistance rates. Unit N10 showed the lowest resistance percentage according to both classifications, which is a promising result in terms of food safety. In addition, there were significant differences in the number of resistant isolates

between different species (*E. faecium*, *E. faecalis* and *E. durans*), according to both EUCAST (χ^2=21.214, p < 0.001) and CLSI (χ^2=6.942, p = 0.031) breakpoints, *E. faecalis* being the species showing higher resistance percentage using CLSI classification. As quinupristin-dalfopristin resistance is only considered by EUCAST's guidelines for *E. faecium*, this species presented higher resistance percentage according to this criterium.

A representation of the antibiotic resistance among the representative isolates of *Enterococcus* spp. is shown in Fig. 2: clustered by year (Fig. 2A), cheesemaking units (Fig. 2B), and species allocation (Fig. 2C) according to EUCAST standards.

According to EUCAST criteria, quinupristin-dalfopristin was the antibiotic to which most of the isolates were resistant in all years considered, with 2016 (87%) and 2019 (94%) (Fig. 2A) showing the higher rates, especially regarding A3 and N9 units from Azeitão and Nisa, respectively (Fig. 2B). However, it is important to highlight that according to EUCAST guidelines, quinupristin-dalfopristin resistance only applies to *E. faecium*, which represents 55.2% of our isolates, and to which 61% were resistant (Fig. 2C). Although in 2017 and 2018 the resistance diminished, suggesting a progressive decrease, in 2019 the rates augmented, presenting levels similar to 2016, compromising this apparent trend. Resistance to quinupristin-dalfopristin is considered intrinsic in enterococci and is common among isolates from food animals, but rare in *E. faecium* isolated from humans (Hershberger et al., 2004), suggesting that the spread of this species may be associated with the production facilities and not due to human contacts. Few studies evaluated enterococcal susceptibility to quinupristin-dalfopristin, but none found resistance percentages as high. Gaglio et al. (2016) reported 20.0% resistance to quinupristin-dalfopristin among enterococci isolated along the production chain of three traditional Italian cheeses (Gaglio et al., 2016). A recent 13-year study on Italian raw milk cheeses reported low rates of resistance (14,35%) to quinupristin-dalfopristin, far below the levels here described (Silvetti et al., 2019).

A considerable percentage of resistance to teicoplanin was observed in enterococcal isolates from all years under study, except for those recovered from N10 cheesemaking unit, with none teicoplanin resistant enterococci. As resistance to teicoplanin is considered extrinsic and acquired by horizontal gene transfer in *Enterococcus* spp., this result is especially relevant since this antibiotic is currently a therapeutic option against *E. faecium* clinical infections (de Nadaï et al., 2019; Escolà-Vergé et al., 2019). A prior study on enterococci isolated from artisanal cheeses found resistance in 9,44% of the tested isolates, according to CLSI guidelines (Porto et al., 2016), below the resistance percentages found in our work, except in 2018, which presented only 4% of resistant enterococci. Despite an apparent trend to lower resistance percentages from 2016 to 2018, 2019 registered the highest resistance percentage

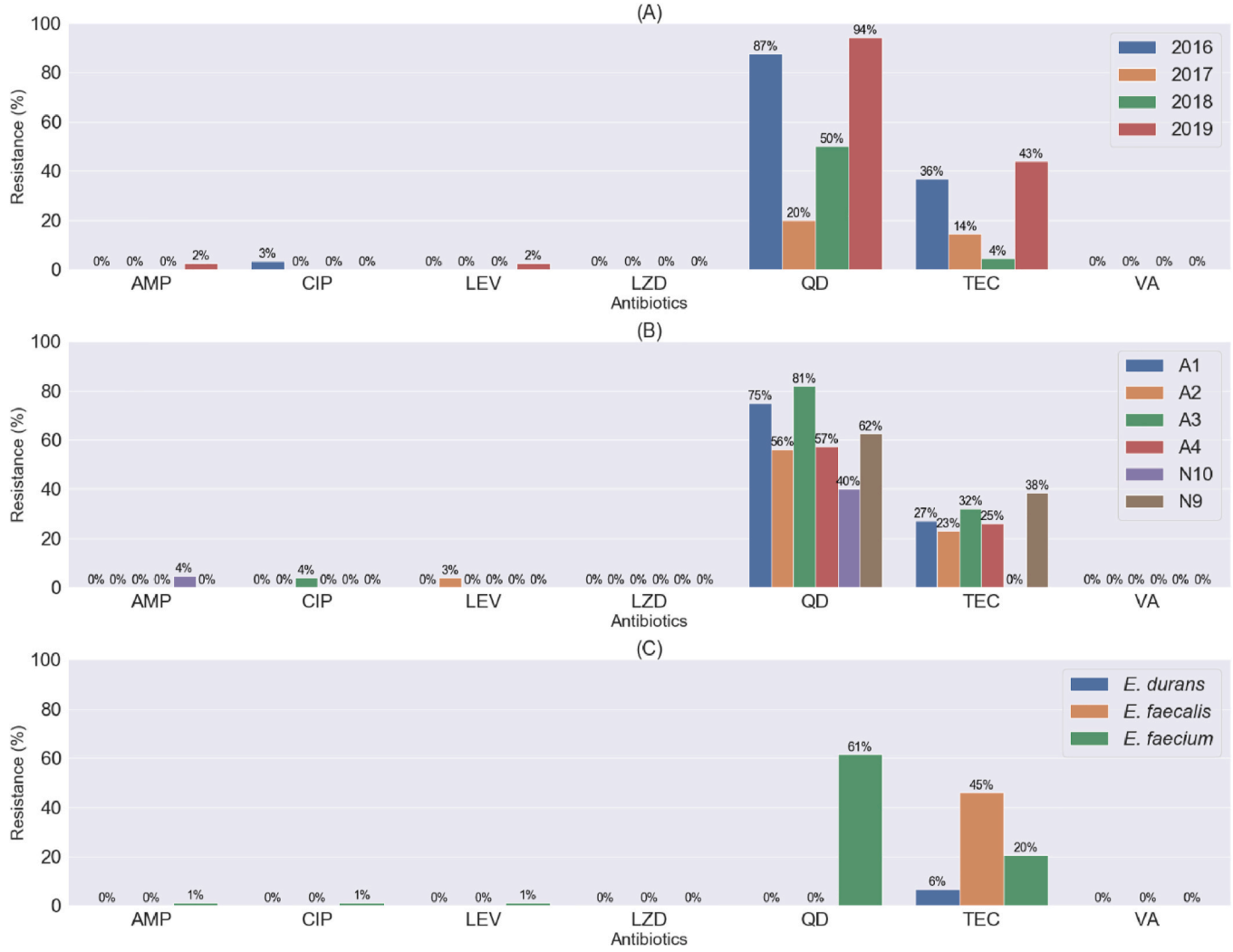

Fig. 2. Antibiotic resistance of cheese *Enterococcus* spp. according to **EUCAST**, **(A)** clustered per year; **(B)** clustered per cheesemaking unit and **(C)** clustered per species allocation. Antibiotics: AMP – ampicillin; CIP – ciprofloxacin; LEV – levofloxacin; LZD – linezolid; QD – quinupristin-dalfopristin; TEC – teicoplanin; VA – vancomycin. Quinupristin-dalfopristin antibiotic susceptibility testing was only considered for *E. faecium*.

(43%). In fact, in 2019 the appearance of novel antibiotic resistances was observed, such as resistance to ampicillin and levofloxacin found in isolates belonging to N10 and A2 units, respectively. As the percentage of resistance to ampicillin was only 2%, evaluating the resistance rates in subsequent years would be important. As for ciprofloxacin, 3% of resistance to this antibiotic was found in 2016 and only in isolates of A3 unit, but after that year no other resistance to this antibiotic was detected.

Fig. 3 plots the antibiotic resistance frequency of the representative isolates of *Enterococcus* spp., clustered by year (Fig. 3A), cheesemaking units (Fig. 3B) and species allocation (Fig. 3C) considering CLSI standards. Vancomycin-resistant enterococci were not found according to the criteria established by EUCAST, but some resistance to this antibiotic was found when following CLSI breakpoint values, although in low extent (Fig. 3A) and particularly in A1 and A2 cheesemaking units (Fig. 3B). The susceptibility to this antibiotic suggests that the acquired resistance may be still limited to hospital environments, although Çitak et al., 2004 found high levels of vancomycin resistance in traditional cheeses isolates (Çitak et al., 2004).

The combined vancomycin-teicoplanin resistance in *E. faecium* isolates has been previously described in hospital settings (Qu et al., 2009; Santona et al., 2018), environmental samples and in food (Messi et al., 2006). As intragenus horizontal transfer of resistance genes can occur between human and food enterococci, it is crucial to survey the antimicrobial resistance to these glycopeptides among enterococci along the food chain. However, none of our isolates reported the combined resistance to the vancomycin and teicoplanin, according to neither EUCAST nor CLSI, although resistance to both antibiotics occurred. As Çitak et al., 2004, our results show that *E. faecalis* isolates were found to be more resistant to vancomycin and teicoplanin than *E. faecium* (Fig. 3C), which emphasises its pathogenic potential, as *E. faecalis* is known to be associated with most human infections caused by enterococci (Çitak et al., 2004). Interestingly, this species is also the one with higher resistance percentages to quinupristin-dalfopristin.

Regarding resistance to quinupristin-dalfopristin, the breakpoints defined by CLSI led to lower resistance levels among the cheese enterococci, by comparison with EUCAST criteria. Quinupristin-dalfopristin and tetracycline resistances were observed in isolates from all years of production (Fig. 3A) and among all cheesemaking units (Fig. 3B). In the case of quinupristin-dalfopristin, the resistance increased drastically from 2018 to 2019, from 23% to 46%. In contrast, tetracycline had higher resistance percentages in 2016 (93%) but decreased considerably in 2017 and 2018. The high level of resistance found in the cheesemaking unit N9 from Nisa should be highlighted since 92% of the isolates from this production were resistant to tetracycline. The species with more resistance level to this antibiotic was *E. faecalis* (53%), which has a wide dispersion of antibiotic resistance genetic determinants on isolates from traditional cheeses (Dapkevicius et al., 2021). As this is one of the most acquired antibiotic resistance in *Enterococcus* spp. isolated from food (Kang et al., 2018; Ogier & Serror, 2008), the high levels of tetracycline resistance are not completely surprising. The wide use of tetracycline in husbandry activities is a possible reason for the high level of resistance frequently found among enterococci (Barbosa et al., 2009; Busani et al., 2004). In fact, the

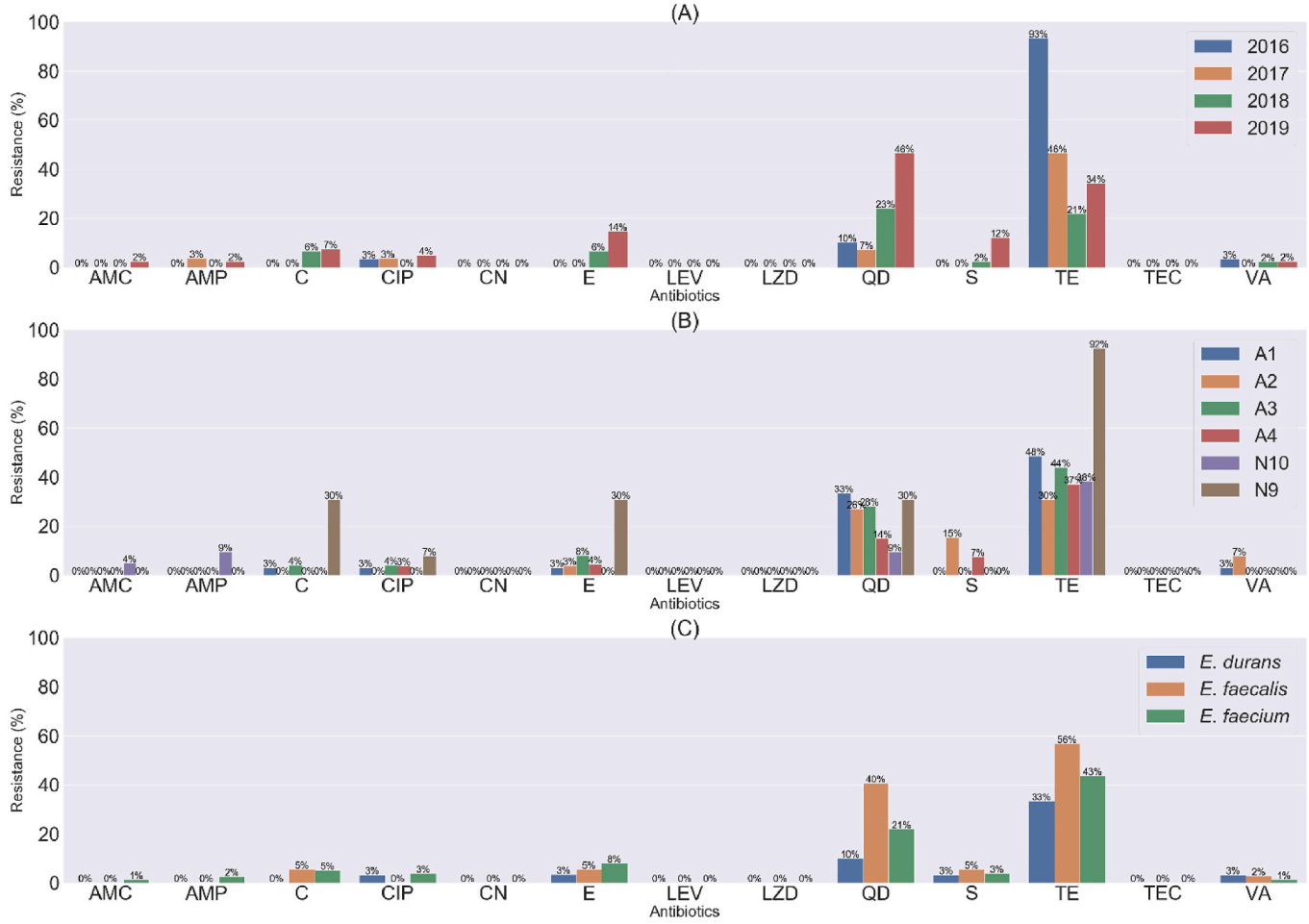

Fig. 3. Antibiotic resistance of cheese *Enterococcus* spp. according to **CLSI**, **(A)** clustered per year; **(B)** clustered per cheesemaking unit and **(C)** clustered per species allocation. Antibiotics: AMC – amoxicillin-clavulanic acid; AMP – ampicillin; C – chloramphenicol; CIP – ciprofloxacin; CN – gentamicin; E – erythromycin; LEV – levofloxacin; LZD – linezolid; QD – quinupristin-dalfopristin; S – streptomycin; TE – tetracycline; TEC – teicoplanin; VA – vancomycin.

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. In addition, most tetracycline-resistant isolates presented co-resistance to additional antibiotics, suggesting that this trait might represent a molecular basis for selecting resistance to other antibiotics (Kang et al., 2018).

Tetracycline and erythromycin resistance combination has been reported (Templer & Baumgartner, 2007), as the resistance genes to both these antibiotics are described to be widespread in the environment (Franz et al., 2001; Mathur & Singh, 2005; Ogier & Serror, 2008) and in animal facilities (Diarra et al., 2010; Jamet et al., 2012; Stine et al., 2007). It can be pointed out that, in the cheese-enterococci, resistance to erythromycin, streptomycin and chloramphenicol was reported for the first time in 2018, being also present in 2019 and in higher percentages. Streptomycin resistance was only present in isolates from two cheese units from Azeitão, A2 and A4. As enterococci are known to be intrinsically resistant to aminoglycosides and monotherapy with these antibiotics is ineffective, gentamicin and streptomycin are only tested for high-level resistance. 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). Furthermore, the last study reported that susceptibility to high-level streptomycin was related to vancomycin and multidrug resistance, highlighting the importance of monitoring resistance to these antibiotics, since they are already present in clinical environments. In our study, however, resistance to streptomycin and vancomycin was only found in the A2 cheesemaking unit, but not on the same isolate. Regarding chloramphenicol resistance, its putative emergence in enterococci has been previously reported (Barbosa et al., 2009; Low et al., 2001), being also found in our work in enterococci recovered in 2018 (6%) and 2019 (7%); and is clearly more present in the N9 cheesemaking unit from Nisa. The fact that chloramphenicol usage was banned from animal husbandry in Europe since 1994 may have influenced the low resistance extent found in the cheese isolates (Barbosa et al., 2009; Peters et al., 2003). Although the N10 unit showed the lowest resistance percentage to β-lactams, resistance to ampicillin and amoxicillin-clavulanic acid was only observed in this cheesemaking unit in 2017 (only for ampicillin) and 2019. Moreover, low frequencies of ciprofloxacin resistance were observed, according to both guidelines. In general, following CLSI guidelines, 2019 was marked by an increase in resistance in streptomycin, chloramphenicol, quinupristin-dalfopristin, ciprofloxacin and erythromycin, comparing to prior years. Therefore, surveillance should continue to confirm a possible trend among enterococci isolated from traditional PDO-cheeses in the forthcoming years.

In a more detailed analysis, among the 145 isolates, 76 (52.4%) reported no resistance to the tested antibiotics according to EUCAST, and 58 (40%) according to CLSI. Accordingly, 69 (47.6%) of the enterococci under analysis were resistant to one or more antibiotics when following EUCAST guidelines, whereas according to CLSI criteria, 60% were resistant to one or more antibiotics. For instance, only one isolate (0.7%) was resistant to half or more of the assayed antibiotics ($n \geq 6$), when considering EUCAST ($n \geq 3$), and none when following CLSI criteria. Multidrug-resistant (MDR) bacteria has been defined by scientists from CLSI, EUCAST and the United States Food and Drug Administration (FDA) commissions 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). Nowadays, multidrug-resistant enterococci constitute a leading cause of nosocomial infections, bringing even more significant challenges regarding treatment (Dapkevicius et al., 2021). In this research, one isolate from A2 cheesemaking unit was considered MDR following EUCAST classification, and one isolate from A1 unit according to CLSI. Multidrug-resistance occurred in only 0.7% of the isolates, when considering each criterion. Although these isolates belong to distinct units, both were isolated in 2019. Since no MDR isolates were found prior to 2019, further sampling and antibiotic resistance evaluation is needed to confirm if there is a trend to MDR emergence. 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).

Enterococci are members of the normal human microbiota and, more than being predominantly found in numerous fermented dairy foods, they play an important role in cheese ripening and in the organoleptic properties of the final product (Foulquié Moreno et al., 2006). In addition, enterococci are used in cheese manufacture as autochthonous starter cultures and even as probiotics for both humans and farm animals (Bertrand et al., 2000; Giraffa, 2003; Ogier & Serror, 2008). However, widespread antibiotic resistance in *Enterococcus* spp. raises a concern on the cheese ecosystem as a potential reservoir of antibiotic resistance (Kang et al., 2018), as cheeses represent an additional vehicle for acquiring antibiotic-resistant bacteria (Bertrand et al., 2000). The treatment of farm animals with antibiotics is also a growing concern. Not only constitutes half of the world's antibiotic output, but its usage as growth promoters or prophylaxis treatments contributes drastically to the selection of resistance bacteria, which may contaminate food and ultimately influence the efficacy of antibiotic therapy in humans (Bertrand et al., 2000). 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 qualified presumption of safety (QPS) assessment (Gaglio et al., 2016). Despite the antibiotic resistances found in the present research, to our knowledge, no enterococcal foodborne infections have been reported, nor infections resulting from the consumption of traditional PDO-cheeses (Dapkevicius et al., 2021), which are considered safe food products. Moreover, both the traditional PDO cheeses and their producers are extremely valued, as they contribute to both the cultural heritage and economy of the country.

4. Conclusions

The present work aimed to monitor the antimicrobial resistance among enterococcal isolates recovered from Portuguese traditional cheeses. Overall, the isolates showed some resistance to clinically relevant antimicrobials, including β-lactams (amoxicillin-clavulanic acid, ampicillin), aminoglycosides (erythromycin) and glycopeptides (teicoplanin and vancomycin). The percentage of resistant enterococci diminished from 2016 to 2018 but increased in 2019 cheeses, which can be a warning sign to possible fluctuations from one year to the next or suggest an increasing trend, to be further confirmed. Lastly, two MDR isolates were found in cheeses sampled in 2019, one classified according to EUCAST and the other according to CLSI guidelines. Moreover, it is important to emphasize that there are no known cases of human toxinfections caused by *Enterococcus* spp. from Portuguese PDO-cheeses, such as the ones analysed in this research. These cheeses are not only considered safe for consumption, but also delicacies which contribute to both the cultural heritage and economy of the country. Our results show that when sampled, traditional cheeses can provide an accurate overview of how AMR is evolving through the food chain, from the farm to the fork, allowing to reduce and/or prevent the potential transmission of resistance determinants.

Funding

This research was funded by FCT—Fundação para a Ciência e Tecnologia I.P., grants PTDC/OCE-ETA/1785/2020 and UIDB/00276/

2020. Teresa Semedo-Lemsaddek was financially supported by national funds through FCT—Fundação para a Ciência e Tecnologia I.P., under the Transitional Standard—DL57/2016/CP1438/CT0004.

CRediT authorship contribution statement

Patrícia A. Bastião Rocha: Methodology, Writing – original draft, Formal analysis, Investigation. **Joana M. Monteiro Marques:** Methodology, Formal analysis, Investigation, Writing – original draft. **António Salvador Barreto:** Writing – review & editing, Project administration. **Teresa Semedo-Lemsaddek:** Conceptualization, Formal analysis, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgments

The authors would like to acknowledge the technical support of Esther Batista, Margarida Baptista, Francisca Eusébio and Pedro Cotovio.

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