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Patterns of resistance to antibiotics in *Enterococcus spp* isolated from raw milk tank of dairy farms; a risk for animal and public health?

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Abstract

Antimicrobial resistant microorganisms are responsible of emerging infections in both, humans and animals which represent a health challenge, especially in public health due to the possible spreading of this resistance from animals to humans.

According to this idea, a study was carried out on raw milk tank from twenty four dairy herds with the objective to detect the prevalence of *Enterococcus spp*, a bacteria inhabitant of the animal and human environment. Antibiotic resistance patterns and phenotypes of the isolated strains were studied in order to evaluate their potential role in the transmission of the antimicrobial resistance to humans and also to other animals from milk production environment. *E. faecium* was the most prevalent microorganism while *E. faecalis* was the second one. The level of *Enterococcus spp* strains with resistance to the antibiotics used in veterinary medicine were medium-high while the level of resistance to some reference antibiotics in human medicine (glicopeptides) was low but in most of the strains the value was in the limit of the cut-off point. Multidrug resistance (more than three classes of antibiotics tested) was detected in the majority of the isolated strains. While no resistant phenotypes were identified according to the MIC studies and *vanA* phenotype was not detected, some strains presented MIC values near this *vanA* phenotype.

These results suggested that implementation of antimicrobial resistant microorganisms surveillance programs in raw milk tank of dairy herds could contribute to define future animal treatments at farm level and to identify the animal and public health risks of antimicrobial resistance transmission associated to milk production.

Key words: Milk, Antibiotic, Resistance; *Enterococcus spp, Public Health*.

Introduction

Emerging infectious diseases are the third cause of death in the world, a challenge for human health services (Heymann, 2006; EASAC, 2007). The microorganisms responsible of emerging diseases may be pathogenic to both humans and animals, but these microorganisms also can be a part of the environment where people and animals live and where they interact (Bywater, 2004; Molbak, 2004).

Antimicrobial-resistance in both, pathogen and non-pathogen microorganisms, become a part of these emerging infectious diseases. While initially it was identified as a problem linked to hospitals, it has been extended into the community and now it is possible to isolate resistant bacteria to the commonly used antibiotics. Nosocomial infections associated to methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *S aureus* (VRSA) and *Enterococcus faecium* (VREF), *Streptococcus pneumoniae* penicillin resistance (PRSP) or *Escherichia coli* Extended Spectrum Beta-Lactamases producers (ESBL) are major examples of this health problem (Woodforde, 1998; Bywater, 2004; Weese *et al*, 2005; Alanis, 2005; EMEA, 2009).

As the antibiotics use were increasing, bacteria developed new mechanisms of resistance which resulted in the emergence of microorganisms with simultaneous resistance to several antibiotic classes, the origin of multidrug resistance (MDR), bacteria or “superbug” (Alanis, 2005).

The development of resistance at hospital or community level in humans and animals means that a permanent elimination of antibiotic residues and resistant bacteria are sent to the environment, which suggests the later importance of environment in the potential in resistance dissemination and transmission (Martinez, 2010).

Enterococcus spp are inhabitants of humans and animals usually as commensal of the gastrointestinal tract but also as occasional pathogens in the case of some *Enterococcus* species which are associated to high environmental isolation frequency (Bonten *et al*, 2001). While they are not considered primary pathogens, its ability to acquire antibiotic resistance forced to consider *Enterococci* genus as an emergent pathogen worldwide (Kühn, *et al* 2005). These microorganisms are able to acquire or develop resistance to antibiotics by direct mutation or by the acquisition of genetic information from other bacteria. *Enterococcus spp* might play the role of resistance reservoirs transferring genes to another microorganisms (Van denBogaard & Stobbering, 2000; EASAC, 2007), which finally could be the main way of transferring antibiotic resistance from animals and environment to humans (Molbak, 2004; Van *et al*, 2007; SAGAM, 2008; EFSA, 2009).

At this level, transmission of resistant *Enterococci* between humans or from animals to humans can happen by direct contact with infected or colonized specimens or by indirect contact by the way of environmental components

(Kühn *et al*, 2005; Tacconelli & Cataldo, 2008). In addition to that, some microorganisms are able to generate biofilms in animal products, in milk for instance, or in environmental that allows the bacteria to persist and on which the necessary Minimum Inhibitory Concentrations (MIC) are higher than those necessary for free bacteria (Melchior *et al*, 2006).

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Making it worse, the systematic use of antibiotics in animal production as treatment or prevention strategy, encourages the selection of the resistant microorganisms strains (Casal *et al*, 2006, Landers *et al*, 2012) as it has been observed in MRSA or *E coli* strains producing extended-spectrum β-lactamases (ESBLs) that could be related with the use of 3rd and 4th generation cephalosporins (Cavaco *et al*, 2008) or *Enterococcus faecium* resistant to glycoppetides as a consequence of the avoparcin use (.Kühn *et al*, 2005).

Antibiotic resistance in microorganisms of farm animals have been detected in species such as swine, horse, poultry and also in dairy herds (Van Duijkeren *et al*, 2008; Denis *et al*, 2009; Spohr *et al*, 2011; Landers *et al*, 2012). At this level, vancomycin resistant *Enterococcus spp* species have been detected not only in animals but also in feces of the farms, soil or in animal origin meat (Devriese *et al*, 1996). Also, some relationship between *Enterococci spp* resistant strains isolated in animals and related persons have been described (Stobberingh *et al*, 1999, Willems *et al*, 2001).

Not so much specific information exists about the role of *Enterococcus spp* resistant to vancomycin in dairy farms, being it usually found in the environment of these farms and in the animal origin products like milk (Landers *et al*, 2012). Their ability to acquire and transfering resistance genes, suggests that antibiotic resistant strains suppose a risk to the public health, raising the need to implement antibiotic resistance surveillance programs in farms and to evaluate the risk of resistance transmission associated to antibiotics use in preventive medicine programs such as mastitis treatments.

In anyway, it should be considered that resistance to antibiotics is a phenomenon not always acquired, also it can be a natural resistance (intrinsic resistance) on which the ecological pressure caused by the presence of the antibiotic does not play any role as it happens in the case of acquired resistance, the most classical case of resistance, on which the selective pressure caused by the presence of the antibiotic is the stimulus for the bacterial adaptation (Alanis, 2005, Hollenbeck & Rice, 2012).

According to that, we carried out a preliminary study at bull tank milk level, as a part of a general *Enterococcus spp* surveillance program in the animals, persons and environment of dairy farms, with two related objectives: the first one was to detect the prevalence of *Enterococcus spp* genus and its species distribution by farms, and the second one was to define the antibiotic resistance patterns and the resistance phenotype of the isolated strains in order to evaluate the public health risk of antibiotic resistance transmission,

taking special account to the role of *E. faecium* and *E. faecalis* and human medicine reference antibiotics such as vancomycin.

Material and methods

The study was carried out in 24 dairy farms in the province of Zaragoza (Spain) during the year 2014. All the farms have a size ranged from 50 to 200 cows in milk production system. Every farm was sampled two times in the year (spring and autumn).

Milk samples were taken from milk tank at the end of the milking process. Two samples containing 10 ml of milk were taken in every farm. Samples were shipped in sterile recipients and were sent to the Veterinary Faculty of the University of Zaragoza. Microbial analyses were initiated within 24 hours of their collection.

Samples were heated, agitated and added to enrichment culture media, Brain Hearth Infusion (BHI) with 10% NaCl for *Enterococcus spp* at a 1/10 final dilution of the sample. After incubation at 37ºC during 24 hours, a sample of the BHI media was cultured in Esculin Bile Agar (BEA), for specific *Enterococcus spp* isolation. Cultures were analyzed at 24 and 48 hours after incubation at 37ºC.

All isolated strains were identified using staining and biochemical tests to detect *Enterococcus* genus. Finally, *Enterococcus* species were identified using RapID STR system-REMEL System (OXOID).

Enterococcus spp isolated strains were screened for antibiotic susceptibility using both tests, Kirby Bauer disk diffusion test (KB) (Bauer *et al*, 1966) and E-test (MICEvaluator) (OXOID) (Torres & Cercenado, 2010).

KB was made in Mueller Hilton Agar at a bacteria dilution of $1.5x10^8$cfu/ml (0.5 McFarland scale). Incubation was made at 35-37 °C during 18 to 20 hours (24 hours for vancomycin) (CLSI, 2015).

A panel of 19 antibiotics was tested by KB tests. The groups of tested antibiotics and their concentration ranges were as follows: penicillins (amoxicillin-clavulanate [30 µg/ml], ampicillin [10 µg/ml], penicillin [6 µg/ml], oxacillin [1 µg/ml]), cephallosporins (cefoxitin [30 µg/ml], ceftiofur [30 µg/ml], cephalexin [30 µg/ml]), quinolones (ciprofloxacin [5 µg/ml], enrofloxacin [5 µg/ml], marbofloxacin [5 µg/ml]), macrolides (erythromycin [15 µg/ml]), aminoglycosides (streptomicyn [10 µg/ml]), glycopeptides (teicoplanin [30 µg/ml], vancomycin [30 µg/ml], valnevulin [30 µg/ml]), lincosamides (clindamycin [2 µg/ml]), oxazolidones (linezolid [30 µg/ml]), carbapenems (imipenem [10 µg/ml]), phenicols (florphenicol [30 µg/ml]).

Special attention was put on to vancomycin and teicoplanin as reference antibiotics in human medicine for *E. faecium* and *E. faecalis* associated

infections treatment. In these two antibiotics the Minimal Inhibitory Concentrations (MIC) was studied using the MICEvaluator in order to identify their *Van* phenotype (Torres & Cercenado, 2010). MICE were made in Mueller Hilton culture media at the same bacterial concentration and culture conditions than KB test. Antibiotics concentration in the MICE tests ranged from 0. 015µg/ml to 256µg/ml.

Isolated *Enterococcus spp* were categorized as resistant (intermediate was classified as resistant) or susceptible in the KB tests according to CLSI standards (CLSI, 2012; CLSI, 2015). MIC for phenotype characterization of the isolated strains was read according to the criteria presented in table 1 (Leclercq & Courvalin 1997; Torres & Cercenado, 2010). In that case, strains were considered as resistant, intermediate (values 8-16µg/ml) or susceptible.

Table 1- *Enterococcus spp:* phenotypes, type of resistance, transmission and their classification criteria.

Phenotype	Vancomycin MIC (µgr/ml)	Teicoplanin MIC (µgr/ml)	Type of resistance	Transmission
vanA	≥ 16 **(R)**	≥16 **(R)**	Acquired	Yes
vanB	4 to1024 **(I/R)**	≤0,5 **(S)**	Acquired	Yes
vanC	2 to 32 **(S/I/R)**	≤0,5-2 **(S)**	Intrinsic	No
VanD	16-128 **(I/R)**	≤4 **(S)**	Acquired	No
None	**(S)**	**(S)**	---------	-------

MIC Minimal inhibitory Concentration value
(R- resistant, I- intermediate, S- susceptible)

The results of the isolations and antibiotic susceptibility tests were included in a data base and analyzed using EPIINFO 2007 software (http://cdc.gov). The distribution of the isolated strains and the proportions of resistance to the tested antibiotics were calculated. According to the resistance patterns and phenotypes of the *Enterococcus spp* isolated strains, a qualitative approximation to the risk for public health was made using the OIE standards for risk analysis for animal movements and animal origin products (http://www.oie.int/en/international-standard-setting/terrestrial-code/access-online/, access April 2016).

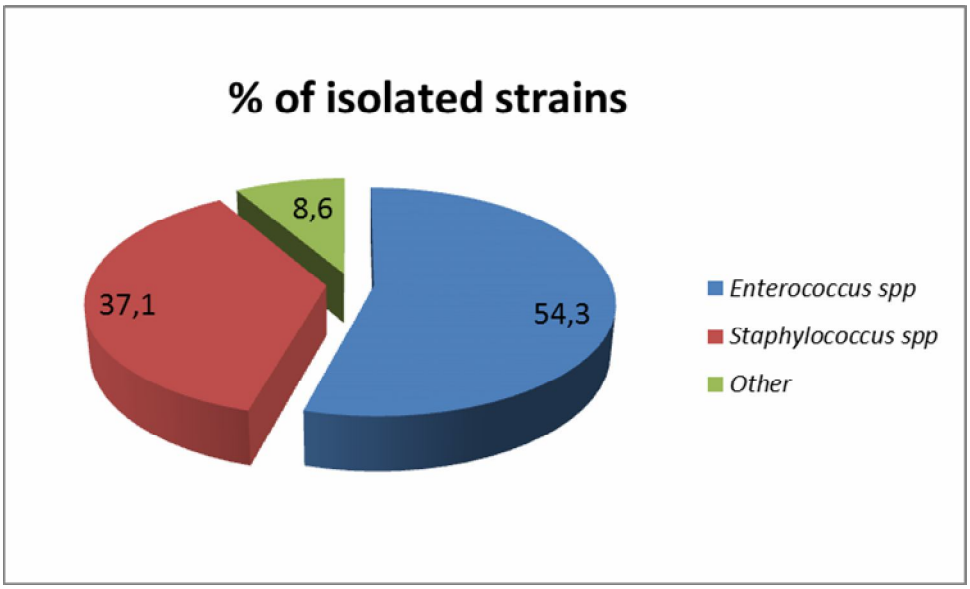
Results and discussion

Prevalence of *Enterococcus genus*, in bull milk tank, species and their importance

Off the 24 farms studied, the *Enterococcus* spp was isolated from 18 raw milk tanks (75%). A total number of 35 different bacteria were isolated on which *Enterococcus spp* genus were the most prevalent isolated bacteria (19/35 isolated strains; 54.3%) (Graphic1).Two different strains of *E. faecium* that presented different results in both, the identification tests and the

susceptibility to the antibiotics, were isolated in a farm, consequently, they were considered as different strains in our study. In the rest of the cases, when more than one strain of *Enterococcus spp* was isolated in the same farm and the biochemical and susceptibility tests were similar, they were considered as the same strain

Graphic 1- Distribution of the bacterial genus in the isolated strains



In 29.2% (7/24) of the sampled farms, two or more different genus were detected at the same time in the raw milk tank and in all the cases, at least one of them was an *Enterococcus spp* .

The most prevalent *Enterococcus spp* were *E. faecium* (63.1% [12/19]) and *E. faecalis* (21.1% [4/19]), both of them secondary pathogens in animals but microorganism able to produce nosocomial pathologies in humans.

Patterns of antibiotic resistance of the *Enterococcus spp* isolated strains
Results of the resistance to the tested antibiotics by the KB test are presented in table 2.

A high proportion of *Enterococcus spp* resistant strains were observed. Values upper 50% have been detected with antibiotics of the different studied families such as, quinolones, macrolides lincosamides or oxazolidones, reaching to 100% of resistant strains for streptomycin (intrinsic resistance). Only penicillins and glycopeptides presented a proportion of resistant strains lower than 50%, while resistance to carbapenems was 0%.

Resistance to cephallosporins was higher 70%, In some cases, it has been described that *Enterococcus spp* genus has intrinsic resistance to this group of antibiotics (Johnston & Jaykus, 2004), as it also happens to lincosamides which reached to 84.2% of resistant strains to clindamycin in our study. Studies carried out in Europe have demonstrated a general development of

cephallosporins resistant microorganisms (SAGAM, 2008). The intrinsic resistance is not always detected *in vitro*, but the antibiotics are not effective *in vivo* (CLSI, 2015), in that case bacteria should not be reported as susceptible.

Table 2: Proportion of resistance to the tested antibiotic of the *Enterococcus spp* isolated strains

Antibiotic family	Antibiotic	Resistant strains (%)
Penicillin	Oxacillin	52.6
	Amoxicillin- Clav	0
	Ampicillin	0
	G- Penicillin	15.8
Glycopeptides	Vancomycin	36.8
	Teicoplanin	21.1
	Valnevulin	73.3
Cephallosporin	Cefoxitin	73.7
	Ceftiofur	52.6
	Cefalexine	94.7
Lincosamides	Clindamycin	84.2
Aminoglycosides	Streptomycin	100
Quinolones	Ciprofloxacin	68.4
	Marbofloxacin	73.7
	Enrofloxacin	57.9
Macrolids	Erithromycin	73.7
Oxazolidones	Linezolid	94.7
Carbapenems	Imipenem	0
Phenicols	Florphenicol	21.1

All of the isolated *Enterococcus spp* strains were resistant to at least three antibiotics of the tested families which suggest that multidrug resistance could be an increasing phenomenon in dairy farms.

We should focus special attention in the interpretation of glycopeptides (vancomycin, teicoplanin and valnevulin) resistance. The observed proportions of resistant strains using the KB test suggest a low or median resistance level in the case of vancomicycin and teicoplanin (36,8% and 21,1% for both antibiotics), but a high proportion in the case of valnevulin, antibiotic used, sometimes ago, as growth promoter in food production animals.

In spite of the fact that vancomycin and teicoplanin showed low or median proportion of resistant strains, it has been observed that the inhibition diameters in the KB test for both antibiotics were in the limits of the cut-off points, pointing out the difficulties for a final resistance interpretation, being it a critical point for public health because vancomycin is a reference antibiotic in human medicine for the treatment of some nosocomial infections.

Patterns of resistance to antibiotics in *E. faecalis* and *E. faecium* isolated strains

Table 3 presents the distribution of resistance to antibiotics in the isolated *E. faecium* and *E. faecalis* species. In a general point of view, the proportions of resistant strains were similar in both species. It has been observed low-medium level of resistance to important antibiotics as penicillins and none resistant strain to carbapenems.

The rest of tested antibiotics have presented medium-high level of resistance in both *Enterococcus spp* species. Only in the cases of glycopeptides and quinolones, some differences have been observed between the two analyzed *Enterococcus spp* species.

However, the number of *E. faecalis* isolated strains was small, which means the significance of these differences must be considered with caution.

We have seen previously that glycopeptides like vancomycin are reference antibiotics in the treatment of infections associated to these microorganisms (or other important microorganisms as MRSA) in human medicine, which increases the importance of the resistance detected in the isolated strains. This is of particular importance for *E. faecalis* on which 75% of the strains were resistant to vancomycin in the KB test. Environment of the dairy farms should be considered a reservoir for potential transmission of these resistant microorganisms or their genes to both animals and humans.

An important link between human and animal isolated *Enterococcus spp* strains has been suggested in some European countries on which avoparcin (glycopeptide) has been used in the past as food additive. In these countries, the prevalence of vancomycin resistant *Enterococcus spp* strains in urban and hospital sewage samples and pig manure, was higher than countries where it was not used (Künhn *et al*, 2005). Most of the isolated strains in those cases were *vanA* phenotype.

However, some authors consider unlikely that selective pressure associated to antibiotics use could be alone the origin of all the resistant *Enterococci* strains, their hypothesis is that horizontal transfer of genes must be the main way on which prevalence of resistant strains is increasing (Bonten *et al*, 2001).

Surveillance programs developed in some countries with similar production characteristics that the studied herds, have shown an increase in vancomycin resistant strains of *E. faecium* in slaughterhouse samples coming from bovine production systems while the same program demonstrated that this prevalence was falling in another production species such as pig or poultry (AFSSA, 2006). In our study, *E. faecium* presented similar proportions of vancomycin resistant strains than the observed in these surveillance programs, but also we saw that these proportions were lower than the observed in the rest of the isolated *Enterococcus spp* species.

In these surveillance programs, the antibiotics with higher proportion of resistant *E. faecium* strains were erythromycin (equivalent to 50%) and tetracyclin (40%). The rest of tested antibiotics presented resistant proportions under 15%, while in our study only penicillins presented values lower than it (AFSSA, 2006).

Different situation it has been observed to teicoplanin, another important glycopeptide in human medicine, antibiotic on which the resistant strains were 33% in *E facium* and 0% in *E. faecalis*. It again must put attention to the results of the KB tests on which these antibiotics presented diameters in the limit of the cut off points.

Table 3- Distribution of the resistance to antibiotics in the isolated strains of *E. faecium* and *E. faecalis* species

Antibiotic family	Antibiotic	*E. faecium* (%)	*E. faecalis* (%)
Penicillin	Oxacillin	58,3	50
	Amoxicillin-Clav	0	0
	Ampicillin	0	0
	G- Penicillin	25	0
Glycopeptides	Vancomycin	25	75
	Teicoplanin	33,3	0
	Valnevulin	66,7	100
Cephallosporin	Cefoxitin	66,7	100
	Ceftiofur	66,7	25
	Cefalexine	75	50
Lincosamides	Clindamycin	75	100
Aminoglycosides	Streptomycin	100	100
Quinolones	Ciprofloxacin	88,3	25
	Marbofloxacin	66,7	75
	Enrofloxacin	75	50
Macrolids	Erithromycin	75	75
Oxazolidones	Linezolid	100	75
Carbapenems	Imipenem	0	0
Phenicols	Florphenicol	25	25

%- Proportion of isolated resistant strains

Resistance phenotypes of the isolated strains of *Enterococcus spp.*

Glycopeptides like vancomycin are antibiotics usually applied in human medicine to the treatment of nosocomial infections caused by *E. faecium*, *E. faecalis* or MRSA. In these bacteria, the development of resistant strains is increasing, especially associated to the presence of phenotype *vanA*,it is the case of the prevalence of human *Enteroccus spp* strains resistant to vancomycin, most of them included in the *E. faecium* specie (Torres &

Cercenado, 2010; Lopez *et al*, 2013) and identified as *vanA* resistance phenotype (Johnston & Jaykus, 2004).

While the results of our study suggests that, at present, none *vanA* resistant phenotype strain was identified in the raw milk tank of the farms because none strain presented MIC for vancomycin and teicoplanin higher 16µg/ml (according to the criteria defined in methodology, Leclercq & Courvaln, 1997, torres & Cercenado, 2010), it has been observed that two strains presented values in the limits of these cut off points. These two strains showed MIC for both antibiotics at an intermediate resistance level, patterns near the *VanA* resistant phenotype.

The rest of the isolated strains presented MIC values of susceptibility to the antibiotics suggesting that none resistance phenotype is present in them, but values near the limit of susceptible/intermediate result for these two antibiotics were observed in some of these last strains. It must be considered that these strains could be near phenotypes *vanB, vanC* or *vanD* or could evolve in the future to some of these phenotypes. In order to clarify this result, complementary genetic studies must be done to be able to identify the genotype of these strains.

Also in animal populations, both production and companion animals, and their environment such as feces of dogs, pigs and horses *E. faecium vanA* phenotype were the most prevalent isolated strain (Devriese *et al*, 1991). Later, studies developed in urban sewage and pig farms found again that the *vanA* phenotype of the vancomycin resistant *Enterococcus spp* strains was predominant (Kühn *et al*, 2005).

Although *vanA* phenotype seems to be predominant in human and animal infections, an increase in the prevalence of *vanB* phenotypes has been observed in Europe in the last years, including no clinical human, animal and environment isolations (EARS 2008 in http://www.rivm.nl/earss/result/; Torres & Cercenado, 2010).

Risk considerations for Public Health

The *Enterococcus spp* strains isolated in raw milk tank of the studied farms presented a ranged medium to high levels of resistance to the tested antibiotics. Taking account that the majority of the tested antibiotics are usually applied in animal production treatments as curative or preventive strategies, it is expected a future increase in the levels of resistance which means an increase in the risk of transmission to humans in contact with these animals or their products or by the environmental route as it has been observed in human resistant *Enterococcus spp* isolates (Tacconelli & Cataldo, 2008).

Nevertheless, in some cases the resistance to antibiotics is intrinsic (Hollenbeck & Rice, 2012) as it has been described for cephallosporins,

aminoglycosides, lincosamides, Trimethoprim-sulfamethoxazol and Fusidic Acid in the *Enterococci* genus. This resistance seems to be not transmissible (Leclercq & Courvalin, 1997), but in the rest of the cases it is an acquired resistance which must be considered linked to the possibility of its transmission to other pathogens.

In the other side, the important proportion of multidrug resistant isolated strains, most of them *E. faecium* and *E. faecalis*, could be an additional risk factor for public health because of the possibility for the bacteria transmission or their resistance genes directly or by the way of milk products. Genetic and virulence studies carried out in human *E. faecium* isolated at hospital level demonstrated that all isolated strains were multi drug resistant to some used antibiotics such as ampicillin, criprofloxacin or erythromycin (Lopez *et al*, 2013).

In our study, the level of *Enterococcus spp* strains resistant to reference antibiotics in human medicine such as vancomiycin or teicoplanin, could be considered low, but a high proportion of these strains showed results in the borders of resistance (cut off points in the tests), which must be considered as another critical point to increase the risk of resistance transmission.

While the phenotype of the isolated strains could not be clearly identified, the MIC study suggests that none *vanA* phenotype, the most prevalent phenotype detected in human medicine (Lopez *et al*, 2013) and in animal environments (Kühn *et al*, 2005), is present in the milk tank of the farms.

Taking in account these results, it should be recommended to stablish an antibiotic resistant microorganisms surveillance program in dairy herds, including milk, animals, environment and humans, in order to identify antibiotics resistant patterns of the microorganisms, their spreading directions and the risk associated to management in the farms and the animal origin products as reservoirs of resistant microorganisms such as *Enterococcus spp*.

At this last level, we propose to use a surveillance strategy based on the identification of *Enterococcus spp* resistance patterns in bull tank milk as reference to know the general antibiotic resistance status in the farm and to identify the risk of its transmission in the public health context. This surveillance must be completed with educational programs based on hygiene and biosafety directed to the people working with animals in the farm as it has been made in human hospitals to fight the nosocomial *Enterococcus spp* infection and colonization (Tacconelli & Cataldo, 2008).

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