ANTIBIOTIC SENSIBILITY OF *LISTERIA INNOCUA* IN THE PRESENCE OF NATURAL ANTIMICROBIAL AGENTS

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Abstract. *Brazilian agribusiness became increasingly competitive on the global agribusiness stage primarily in the animal production. In the first quarter of 2011 were slaughtered 8.160 million of pigs and 1.306 billion of chickens. To ensure the productivity and competitiveness of the sector, the use of drugs as growth promoters is a common practice. However, the use of substances as antimicrobial growth promoters has been discussed and is subject to a series of rules because the literature has been reporting the presence of bacteria resistant. The concern about the growth of bacterial resistance to antibiotics has stimulated the search for alternatives that will replace these substances as growth promoters. The aim of this study was to evaluate the antibiotic sensibility of Listeria innocua (CECT 910) and Listeria monocytogenes (CECT 4032) in the presence of natural antimicrobial agents. The DMSO was used as dilution agent for obtaining the levels of 0.100 and 0.175 µL/mL for carvacrol and levels of 0.150 and 0.250 µL/mL for citral. Assessing the strains of L. monocytogenes and L. innocua, it was verified that the sensibility to erythromycin was of the 0.250 µg/mL. There was reduction of the minimal inibitory concentration (MIC) of erythromycin in the presence of antimicrobials carvacrol or citral in cultures of L. innocua and L. monocytogenes to 0.094-0.125 µg/mL. In this study the MIC of bacitracin in cultures of L. innocua and L. monocytogenes was of 32 µg/mL. However, there was reduction of MIC in the presence of antimicrobials carvacrol or citral. The MIC of colistin in cultures of L. innocua and L. monocytogenes was reduced in the presence of antimicrobial carvacrol or of citral from 96-128 µg/mL to 4 µg/mL. The results obtained in this study showed that the combined use of antibiotics with natural antimicrobial resulted in a synergistic effect demonstrated by the reduction of the MIC in cultured of L. monocytogenes and L. innocua.*

Keywords . Minimal inibitory concentration, antibiotics, citral, carvacrol, *Listeria*.

Introduction

Brazilian agribusiness became increasingly competitive on the global agribusiness stage primarily in the animal production. In the first quarter of 2011 were slaughtered

8.160 million of pigs and 1.306 billion of chickens (IBGE, 2011). To ensure the productivity and competitiveness of the sector, the use of drugs as growth promoters is a common practice (Regitano & Leal, 2010) that has been adopted since the 50s (Pelicano, *et al.*, 2004, Rocha *et al.*, 2010). However, the use of substances as antimicrobial growth promoters has been discussed and is subject to a series of rules because the literature has been reporting the presence of bacteria resistant to antimicrobial agents by the widespread use of antibiotics in the treatment and prevention of infectious diseases in humans and animals and also the use of antibiotics in animal production (Turnidge, 2004). Some of these antibiotics used in animals are similar to application in humans, which can result in cross-resistance (WHO, 1997, WHO, 2004; BRAZIL, 2004; Regitano & Leal, 2010).

Since 1986, Sweden abolished the use of any antibiotic as growth promoter. Denmark did the same for avoparcin and virginamicin since 1995 and joined the European Union in 1997 for avoparcin and later to four other classes of antibiotics: bacitracin, spiramycin, tylosin and virginamicin (Boerlin *et al.*, 2001; Casewell *et al.*, 2003). In the year 2013 all countries of the European community will no longer use antibiotics as growth promoters, restricting the use only for therapeutic purposes (EC, 2003).

In 2000, the Ministry of Agriculture, Livestock and Supply of Brazil (MAPA) institiuted a Technical Group should review and reassess the use of substances carbadox, olaquindox, bacitracin zinc, spiramycin, virginiamycin and tylosin phosphate as additives in animal feed (BRAZIL, 2003; Spisso, Nóbrega & Marques, 2009). The concern with the growth of bacterial resistance to antibiotics has stimulated the search for alternatives that will replace these substances as growth promoters (Freitas *et al.*, 2001, Rocha *et al.*, 2010).

Material and methods

Listeria innocua (CECT 910) and *Listeria monocytogenes* (CECT 4032) strains were provided by the Spanish Type Culture Collection. The carvacrol and citral was purchased from Sigma Aldrich. The DMSO was used as dilution agent for obtaining the levels of 0.100 and 0.175 µL/mL for carvacrol and levels of 0.150 and 0.250 µL/mL for citral.

It was used Etest® (Biomerieux) that allows the quantitative determination of the minimal inibitory concentration (MIC) of antibiotic agents. Etest® consist of a plastic strip with a gradient of concentrations of the antibiotic.

0.1 mL of an inoculum size approximately of 1-2x10^8 CFU/mL (CLSI, 2010) were pour-plated onto Tryptone Soya Agar (TSA) that was supplemented with 0.6% of yeast extract (TSA-YE). It was added antimicrobial agents (carvacrol and citral) when the agar TSA-YE was in a liquid stated about at 45 °C. It was put on the plastic strips of antibiotics after the agar solidification. The sterile plastic petri plates were incubated upside down at 37 °C for 48 hours.

Thus, *L. innocua* and *L. monocytogenes* were exposed to antimicrobial agents just in the moment of spreading. There was no prior exposure of carvacrol and citral to these bacterial strains. The list of the tested antibiotics and their concentration ranges are listed in Table 1.

Table 1. List of antibiotics tested and their concentrations (µL/mL) by Etest ® (Biomerieux).

Antibiotics	**Range of concentration (µg/mL)**
Bacitracin	0.016-256
Eritromicin	0.016-256
Colistin	0.016-256

Results and discussion

The Gram-positive bacteria are target for antibiotic growth promoters. Records showing the sensibility of *Listeria* spp. by the use of antibiotics combined with natural antimicrobials are inexistent, although there are reports with others bacteria as also there are studies that show the action of natural antimicrobials on gram positive bacteria.

Assessing the strains of *L. monocytogenes* and *L. innocua*, it was verified that the sensibility to erythromycin was of the 0.250 µg/mL. (Table 2 and 3) which supports the reports of breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012) which exhibit sensibility values ≤ 1 mg/L.

There was reduction of the erythromycin MIC in the presence of antimicrobials carvacrol or citral in cultures of *L. innocua* and *L. monocytogenes* to 0.094-0.125 µg/mL (Tables 2 and 3). The carvacrol is composed of two molecules of isoprene being classified in the group of monoterpenes. Citral is a terpenoid that is oxygenated derivative of terpenes, which is compound of a mixture of two isomers. The trans-isomer is known as geranial or citral A. The cis-isomer is known as neral or citral B. Terpenes can be used as absorption enhancers for drugs due to their low toxicity and ability to increase permeability of both hydrophilic and lipophilic molecules (El-Kattan, Asbill & Michniak, 2000). It was know the limitation of drugs for the passage into the biological membrane and cell wall. The effect of terpenes can be attributed to its ability to affect the packing of intercellular lipid.

Erythromycin is an analogue of tylosin (Klare *et al.*, 2003), which is used as an antibiotic growth promoter approved by the MAPA (BRAZIL, 2009). The intensive use of this promoter can result in selective pressure on the resistance genes. In human medicine macrolides have therapeutic application frequent and serve as second choice in cases of allergy to other antimicrobial agents (Jensen, Frimodt-Moller, Aarestrup, 1999). It is well documented macrolide resistance of isolated from humans and animals (Aarestrup *et al.*, 2000; Aarestrup *et al.*, 2001). The bacteria may have intrinsic mechanisms of resistance by low permeability to antibiotics, common to Gram-negative bacteria and acquired by the change of target in the ribosome binding (Fluit, Visser & Schmitz, 2001), the presence of efflux pump (Luna *et al.*, 1999) and degradation of the antibiotic (Leclerq & Courvalin, 1991; Leclercq *et al.*, 1991), common to Gram-positive bacteria.

According to Clinical and Laboratory Standards Institute (CLSI, 2010), the *Listeria monocytogenes* is susceptible to penicillin, ampicillin and gentamicins and intrinsically resistant to cephalosporins.

In this study the bacitracin MIC in cultures of *L. innocua* and *L. monocytogenes* was of 32µg/mL. However, there was reduction of MIC in the presence of antimicrobials carvacrol or citral (Tables 2 and 3). No breakpoint values for *L. monocytogenes* and *L. innocua* with regard to bacitracin. However, according to EUCAST (2012) there are three categories of bacteria sensibility to antibiotics as sensible (= <1 mg/L), intermediate (> 1-8 mg/L) and resistant (> 8 mg/L). Bacitracin is an antibiotic produced by strains of *Bacillus licheniformis*, and is a more stable as the zinc salt being used as animal growth promoter and therapeutically, in topical application on human and veterinary medicine. Bacitracin has the mode of action based on inhibition of bacterial cell wall synthesis. This antibiotic is particularly active on gram-positive bacteria (O'Grady & Greenwood, 1997). It can be used orally and also topically in humans (Bywater *et al.*, 2005; Pavli & Kmetec, 2006).

The MIC of colistin was of 96-128 mg/ml (Tables 2 and 3) for *L. monocytogenes* and *L. innocua*, respectively, indicating resistance to this antibiotic. This resistance occurs because the colistin is an antibiotic for Gram-negative bacteria and do not show activity on gram-positive bacteria. The colistin is produced by *B. colistinus*, also called polymyxin E. It is a bactericidal that interacts with phospholipids and lipopolysaccharides of the cell wall of Gram-negative bacteria and it`s promotes alterations in this structure and the cytoplasmic membrane (Machado Sequeira, 2004). However, the colistin MIC in cultures of *L. innocua* and *L. monocytogenes* was reduced in the presence of antimicrobial carvacrol or of citral from 96-128μg/mL to 4μg/mL (Tables 2 and 3) Therefore, *L. innocua* and *L. monocytogenes* become sensitive to this antibiotic.

In this study also evaluated the combined effect of these antimicrobial agents (citral and carvacrol) and it was observed promising results for the MIC of bacitracin, erythromycin and colistin that was reduced to 0.25-1.0, 0.064 and 0.75 μg/mL, respectively (Tables 2 and 3). The antimicrobial compounds may interact enhancing its activity or reducing it. There are identified three types of interactions between them: addition that occurs when the activity of the mixture of antimicrobial compounds is the sum of individual effects, antagonism that occurs when the activity is less than the sum of the antimicrobial activity individually; synergy that occurs when the compound mixture is greater than the sum of their individual effects (Burt, 2004).

The antimicrobial activity of compounds extracted from plants is directly related to chemical structure, its proportion and interaction that can occur between them (Burt, 2004). Some authors have described additive effects, synergistic or antagonistic combinations between components such as carvacrol and thymol, p-cymene and carvacrol, cinnamaldehyde and eugenol (Burt, 2004; Tajkarimi *et al.*, 2010). The combined use of antibiotics is one strategy that is used to treat infections caused by resistant or multiresistant strains to antibiotics and decreases the occurrence of new mechanisms of resistance (Hemaiswarya *et al.*, 2008). Some studies have shown that mixture the essential oils and their individual components have synergistic effects with antibiotics. For example, has been described that the essential oil of *Pelargonium graveolens* increases the activity of norfloxacin on *B. cereus* and *S. aureus* (Rosato *et al.*, 2007), as also cinnamaldehyde and clindamycin have synergistic effects against *Clostridium difficile* (Shahverdi *et al.*, 2007) and that the essential oil of *Zataria multiflora* improved the vancomycin activity on *S. aureus* (Mahboubi & Ghazian Bidgoli, 2010).

Table 2. Minimum inhibitory concentration of antibiotics in the presence or not of natural antimicrobial (carvacrol and/or citral) in complete culture medium (TSA-YE) for *L. innocua*.

				Listeria innocua					
				Minimum inhibitory concentration					
				Carvacrol (µL/mL)		Citral (µL/mL)		Anitmicrobials mix (µL/mL)	
				0.100	0.175	0.150	0.250	0.100 x 0.150	0.100 x 0.250
Bacitracin (µg/mL)	32.0			4.0	1.5-2.0	24.0	12.0	2.0	0.25-1.0
Erythromycin (µg/mL)		0.250		0.125	0.094-0.125	0.190-0.250	0.250	0.064	0.064
Colistina (µg/mL)			128.0	16.0	4.0	16.0	4.0	1.5	0.75

Table 3. Minimum inhibitory concentration of antibiotics in the presence or not of natural antimicrobial (carvacrol and/or citral) in complete culture medium (TSA-YE) for *L. monocytogenes*.

				Listeria monocytogenes					
				Minimum inhibitory concentration					
				Carvacrol (µL/mL)		Citral (µL/mL)		Anitmicrobials mix (µL/mL)	
				0.100	0.175	0.150	0.250	0.100 x 0.150	0.100 x 0.250
Bacitracina (µg/mL)	32.0			4.0	1.5-2.0	24.0	12.0	2.0	0.250
Erythromycin (µg/mL)		0.250		0.125	0.094-0.125	0.250	0.250	0.094	0.016
Colistina (µg/mL)			96.0	24.0	4.0	16.0	4.0	2.0-3.0	2.0

Conclusions

The results obtained in this study showed that the combined use of antibiotics with natural antimicrobial resulted in a synergistic effect demonstrated by the reduction of the MIC in cultured of *L. monocytogenes* and *L. innocua*.

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