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MICROBIOLOGY

Methods for the evaluation of antibiotic resistance in *Lactobacillus* isolated from fermented sausages

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ABSTRACT: *The present study aimed to assess the antibiotic resistance in 54 indigenous Lactobacillus plantarum isolated from artisanal fermented sausages. The confirmation of the strain species was performed by multiplex-PCR assay. Antibiotic resistance was assessed by disk diffusion (DD) and Minimum Inhibitory Concentration (MIC) methods. Of 54 L. plantarum, 44 strains were genotypically confirmed as L. plantarum and 3 as Lactobacillus pentosus. The highest resistance rates were to ampicillin and streptomycin. The highest susceptibility rates were shown to tetracycline, chloramphenicol and penicillin G. None of the strains showed multidrug resistance. Resistance rates by DD and MIC were not different (P>0.05) for ampicillin, chloramphenicol, erythromycin and penicillin G. Future research should assess the genetic mechanisms underlying the phenotypic resistance in Lactobacillus strains to screen the potential probiotic strains for the development of functional meat products.*
Key words: *meat product, multiplex PCR, probiotic, susceptibility.*

Comparação de métodos para avaliação de resistência microbiana em *Lactobacillus* isolados de embutidos fermentados

RESUMO: *O presente estudo teve como objetivo avaliar a resistência a antibióticos, de 54 cepas nativas de Lactobacillus plantarum isoladas de salames artesanais. A confirmação genotípica da espécie foi realizada por ensaio de PCR multiplex. A resistência aos antibióticos foi avaliada pelos métodos de disco difusão e concentração inibitória mínima. Das 54 cepas, 44 foram confirmadas genotipicamente como L. plantarum e 3 como Lactobacillus pentosus. As maiores frequências de resistência foram para ampicilina e estreptomicina, e as maiores frequências de sensibilidade para tetraciclina, cloranfenicol e penicilina G. Nenhuma das cepas apresentou multirresistência. As frequências de resistência para ampicilina, cloranfenicol, eritromicina e penicilina G foram semelhantes pelos métodos testados (P>0,05). Pesquisas futuras devem ser realizadas para avaliar os mecanismos genéticos envolvidos na resistência fenotípica das cepas de Lactobacillus, no intuito de selecionar as potenciais cepas probióticas para aplicação em produtos cárneos funcionais.*
Palavras-chave: *multiplex PCR, probióticos, produto cárneo, susceptibilidade.*

INTRODUCTION

The most common species of lactic acid bacteria (LAB) used as starter cultures in fermented sausages are *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus casei*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* (KLINGBERG et al., 2005). These microorganisms play an important role in the production of fermented sausages, by increasing the microbiological safety and improving the sensory characteristics. More recently, probiotic starter cultures are also been used to increase the functional value of fermented sausages.

Because meat products conditions are generally not favorable for the growth of most of the LAB already characterized as probiotic; the isolation, identification and assessment of probiotic potential in indigenous LAB from fermented meat products might be an interesting strategy for the development of probiotic cultures for the meat industry (KLINGBERG et al., 2005).

Currently, the GRAS (Generally Recognized as Safe) status of the probiotic bacteria also includes the antibiotic resistance, evaluated by the Qualified

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Prediction Security Program (QPSP), suggested by the European Food Safety Authority (EFSA, 2013). This is an important criterion for the selection of probiotics due to the possibility of the genetic transmission of the antimicrobial resistance among different bacteria.

Intrinsic resistance is the innate ability of a bacterial species or genus to resist to an antimicrobial agent through its inherent structural or functional characteristics. This resistance is known as vertical gene transfer, in which resistance genes are transferred directly to all the bacteria´s progeny during DNA replication. Conversely, acquired resistance occurs through acquisition of exogenous DNA or mutation of wild genes (EFSA, 2008). Thus, genes can be horizontally transferred to other bacteria.

Therefore, acquired resistance has become a concern in the selection of probiotic bacteria because beneficial bacteria can transfer resistant genes to potentially pathogenic bacteria in the gut of the host. A report on global surveillance of antimicrobial resistance by the World Health Organization (WHO, 2014) evidences a link between antimicrobials drugs use in food-producing animals and emergence of resistance among common pathogens. This could lead to an ineffectiveness of antibiotic therapy used for both, treating or preventing infections.

The present study aimed to assess the antibiotic resistance, by two different methodologies, in indigenous *L. plantarum* strains isolated from artisanal fermented sausages, prospecting their future use as probiotics starters in meat products.

MATERIALS AND METHODS

Molecular confirmation of L. plantarum species

Fifty four (54) indigenous *Lactobacillus* strains isolated from fermented sausages (DALLA SANTA et al., 2012) and phenotypically identified by API 50 CHL in a previously research (data not shown) (BioMérieux, Marcy l'Etoile, France) as *L. plantarum* were assessed. Strains were maintained in Man, Rogosa and Sharpe broth (MRS) (Micromed, Duque de Caxias, RJ) with 20% (v/v) of glycerol and stored at -20°C until use.

The molecular confirmation of strain´s species was performed by multiplex-PCR as described by TORRIANI et al. (2001) for the discrimination of the *L. plantarum* group strains. Strain DNA was extracted as described by POSPIECH & NEUMANN (1995), from 2mL samples of overnight cultures grown in MRS broth at 37°C. The recA gene was amplified by multiplex-PCR using the forward primers: planF (5' -CCG TTT ATG CGG AAC ACC TA-3'), pentF (5' -GGT TTT CAG CGC TGA TAT C-3') and paraF (5' -GTC ACA GGC ATT ACG AAA AC-3'), and a single reverse primer pREV (5' -TCG CCA AAC TTA ATC GGA AC-3'). Three species-specific amplification products of different length were obtained: 318bp for *L. plantarum*, 218bp for *L. pentosus* and 107bp for *Lactobacillus paraplantarum*.

PCRs were performed on a DNA Engine Systems (PTC 200, Bio Rad, Foster City, USA) with initial denaturation at 94°C for 3min, followed by 30 cycles at 94°C (30s), 1 cycle at 56°C (10s), 1 cycle at 72°C (30s) and a final cycle at 72°C for 5min (TORRIANI et al., 2001). The amplification products were subjected to gel electrophoresis in agarose gel (1.5%) followed by ethidium bromide staining.

Antibiotic Resistant Phenotypes

Minimal inhibitory concentration (MIC)

MIC was determined using Etest® strips (bioMérieux; Marcy-l'Étoile, France) of ampicillin; streptomycin, chloramphenicol; erythromycin; penicillin G and tetracycline (0.016 to 256µg mL^{-1}). Resistance to penicillin G was determined according to the breakpoint proposed for *Lactobacillus* sp. by the CLSI (2010). Resistance to the other antibiotics was determined according to the breakpoints proposed for *Lactobacillus* by the EFSA (2008).

Disk diffusion (DD) assay

The assay was performed with standard disks of ampicillin (10µg); ciprofloxacin (5µg); chloramphenicol (30µg); erythromycin (15µg); gentamicin (10µg); streptomycin (10µg); norfloxacin (10µg); penicillin G (10U); tetracycline (30µg); vancomycin (30µg) (Laborclin; Pinhais, Brazil) and trimethoprim (5µg) (Sensifar; São Paulo, Brazil) on MRS (Micromed, Duque de Caxias, RJ) agar plates, incubated at 37°C for 24h.

The inhibition zone diameters obtained for penicillin G (≤19mm), vancomycin (≤14mm), tetracycline (≤14mm), chloramphenicol (≤13mm) and erythromycin (≤13mm) were determined according to the breakpoints proposed for *Lactobacillus* sp. by CHARTERIS et al. (2001). For ampicillin (≤16mm) and for trimethoprim (≤10mm), breakpoints recommended for *Enterococcus* sp. and for *Staphylococcus* sp., respectively, were used (CLSI 2011). Inhibition zone diameters lower than 9mm were considered as resistance (CLSI 2011).

Statistical analysis

Results were compared by Chi-square test ($\chi 2$) with a significance level of 5%.

RESULTS AND DISCUSSION

Of 54 *L. plantarum* strains, 47 strains were distinguished based on recA multiplex-PCR assay. Forty-four (44) strains were confirmed as belonging to the species *L. plantarum* and 3 strains as *L. pentosus*. Although they were previously phenotypically identified as *L. plantarum*, seven (7) strains showed negative PCR results for the primers used and might not belong to the species *L. plantarum*, *L. pentosus* or *L. paraplantarum* (Figure 1). The use of primers synthesized from the recA protein gene sequences has been proposed by several authors for the differentiation of genetically similar species of *Lactobacillus*, such as *L. plantarum*, *L. paraplantarum* and *L. pentosus* (TORRIANI et al., 2001; PENNACCHIA et al., 2006).

Resistance to 4 of 6 antibiotics tested by the gradient technique in scale for the determination of MIC was detected. The highest frequencies of resistance by the MIC method were observed for ampicillin and streptomycin (58.8% and 54.2%, respectively). There was also resistance to erythromycin and chloramphenicol (7.69% and 1.92%, respectively). All strains were susceptible to tetracycline and penicillin G.

By the DD method, resistance to 9 of 11 antibiotics was reported. All strains (100%) were resistant to ciprofloxacin, gentamicin, norfloxacin, streptomycin and vancomycin, 92.6% were resistant to trimethoprim, 59.3% to ampicillin, 42.6% to tetracycline and 5.56% to erythromycin. Conversely, 100% of the strains were susceptible to chloramphenicol and to penicillin G.

Antibiotic resistance or susceptibility results obtained by MIC and DD were similar ($P>0.05$) for 4 of 6 antibiotics tested. The highest resistance frequencies to the antibiotics tested in both methods were observed to streptomycin and ampicillin. The methods showed different results ($P<0.05$) for streptomycin and tetracycline, for which higher frequency of resistance by DD was reported. For ampicillin, chloramphenicol, erythromycin and penicillin G no statistical difference ($P>0.05$) between the methodologies was reported.

Studies on the comparison of antibiotic resistance by DD and MIC in *Lactobacillus* sp. are scarce. MAYRHOFER et al. (2008) reported high correlation coefficient ($r=0.84$ to 0.98) between DD and MIC for susceptibility of *L. acidophilus* to erythromycin, streptomycin and tetracycline, and moderate correlation coefficient ($r=0.63$ to 0.83) to ampicillin, gentamicin and vancomycin. Conversely, in the present study, significant difference ($P<0.05$) between DD and MIC for the resistance to streptomycin and tetracycline was reported (Table 1).

THORNSBERRY (1985) recommends that the agreement between DD and microdilution methods should be ≥90%. In the present study, the percentages of agreement for the susceptibility to ampicillin, erythromycin, chloramphenicol and penicillin G were >90%.

Lactobacilli have intrinsic resistance to bacitracin, kanamycin, gentamicin, metronidazole, streptomycin, sulfamethoxazole and vancomycin. The vancomycin-resistant phenotype of some *Lactobacillus* is perhaps the best characterized intrinsic resistance in LAB (FRAQUEZA, 2015). Several *Lactobacillus* species are intrinsically resistant to vancomycin because they have D-Ala-D-lactate in the peptidoglycan instead of the dipeptide D-Ala-D-Ala, which prevents vancomycin binding. This resistance is chromosomally encoded and not inducible or transferable (GUEIMONDE et al., 2013).

Resistance to aminoglycosides (neomycin, kanamycin, streptomycin) is also considered intrinsic in *Lactobacillus* due to the absence of cytochrome-mediated electron transport, which mediates drug uptake. *Lactobacilli* are also naturally resistant to quinolones (ciprofloxacin, norfloxacin, nalidixic acid) by an unknown resistance mechanism and are usually resistant to trimethoprim because they have limited biosynthetic capabilities and lack the folic acid synthesis pathway (CHARTERIS et al., 2001).

Although, the high frequency of resistance to vancomycin, ciprofloxacin, norfloxacin, gentamicin, streptomycin and trimethoprim shown by the *Lactobacillus* strains, it does not represent a major safety concern itself since intrinsic resistance is estimated to present a minimal potential for horizontal spread (FRAQUEZA, 2015). Conversely, *Lactobacilli* are usually susceptible to antibiotics that inhibit protein synthesis, such as chloramphenicol, erythromycin, lincomycin, clindamycin and tetracyclines (FEDERICI et al., 2014) and to antibiotic that inhibit cell wall synthesis such as penicillins (piperacillin and ampicillin) and b-lactamase inhibitors.

With regards to the resistance of *Lactobacillus* strains to ampicillin, chloramphenicol, erythromycin, penicillin and tetracycline by DD, 48.1% of strains were resistant to only one of the antibiotics, 29.6% to 2 antibiotics and 22.3% were not resistant to any of those antibiotics. By MIC method, 60.8% of strains showed resistance to 1 antibiotic, 3.9% to 2, whereas 35.3% showed no resistance to those antibiotics. Thus, none of the *Lactobacillus*

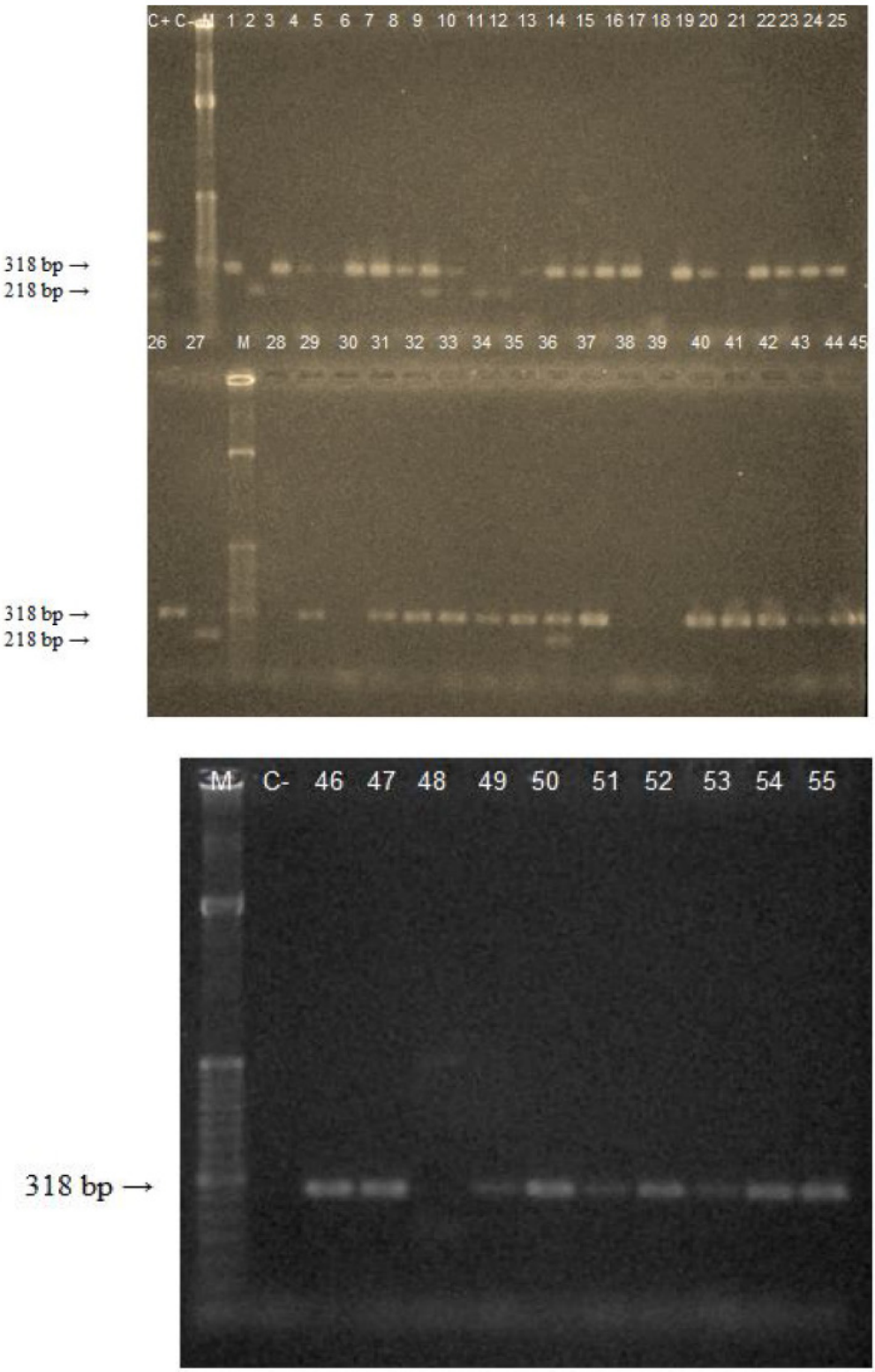

Figure 1 - Agarose gel electrophoresis of DNA fragments generated by multiplex PCR for the *L. plantarum* group species (*L. plantarum* = strains 1, 3-10, 13-17, 19, 20, 22-27, 29, 31-37, 40-45; *L. pentosus* = strains 2, 11, 12; strains 18, 21, 28, 30, 38, 39, 48 did not amplified; C+ - *E. coli* ATCC 25922; C- - negative control; M – molecular size marker (50bp).

Table 1 - Comparison of frequency (%) and agreement (%) of antibiotic resistance and susceptibility in *Lactobacillus* sp. isolated from fermented sausages tested by disk diffusion and MIC methods.

Antibiotic	Resistance			Susceptibility		
	DD%	MIC%	Agr.%	DD%	MIC%	Agr%
AMP	59.3	58.8	99.15	40.7	41.2	98.78
STR*	100	54.2	54.2	0	45.8	***
ERY	5.56	7.69	72.3	94.44	92.31	97.74
CHL	0	1.92	***	100	98.08	98.08
TET*	42.6	0	***	57.40	100	57.40
PEN	0	0	100	100	100	100

AMP (Ampicillin), STR (Streptomycin), ERY (Erythromycin), CHL (Chloramphenicol), TET (Tetracycline), PEN (Penicillin G) in concentration of 0.016 to 256 µg mL^{-1}. DD = disk diffusion; Agr. = agreement (%) between methodologies. *P<0.05 *** = insufficient numbers of results for test application.

strains tested could be considered multidrug-resistant (resistant to 3 or more classes of antibiotics). This is consistent with the results reported by TURCHI et al. (2013), who did not find multidrug resistance in *L. plantarum* isolated from Italian food products intended to be used as probiotics. This result might emphasize the classification of QPS status granted to *L. plantarum* by EFSA (2013).

Considering the MIC doses, 41% of strains were inhibited at concentration ≤2µg mL^{-1} of ampicillin, 92% were inhibited at ≤1µg mL^{-1} of erythromycin, 98% were inhibited at ≤8µg mL^{-1} of penicillin G or chloramphenicol and 100% of strains were inhibited at concentrations <64µg mL^{-1} of streptomycin and ≤16µg mL^{-1} of tetracycline (Table 2).

The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013) report MIC values for 120 *L. plantarum* strains; for which 38.33% of these strains were inhibited at concentration of 0.25µg mL^{-1} of ampicillin, 73.33% at 1µg mL^{-1} of erythromycin, 65.83% at 128µg mL^{-1} of streptomycin and 87.5% at 16µg mL^{-1} of tetracycline. In the present study, most of the strains were inhibited at lower concentrations of tetracycline, erythromycin and streptomycin when compared to the EUCAST study.

Multiplicity of methods available for the antibiotic resistance evaluation brings a lack of agreement regarding the resistance-susceptibility breakpoints for most antibiotics in *Lactobacillus*. AMMOR et al. (2008) report higher breakpoints than those used in the present study for the resistance of *L. plantarum* to ampicillin (4µg mL^{-1}) and to erythromycin (4µg mL^{-1}). However, in another study, these authors reported that 96.5% of *L. plantarum* strains were inhibited at concentrations of erythromycin lower than the breakpoint reported (≤1µg mL^{-1}). They also reported that 96.5% of *L. plantarum* strains were inhibited at concentrations

Table 2 - Distribution of MICs (Minimum Inhibitory Concentrations) for *Lactobacillus* sp. strains isolated from fermented sausages.

Antib.	MIC (µg mL^{-1})/ Isolates (%)																	
	0.094	0.125	0.19	0.25	0.38	0.50	0.75	1	1.5	2	3	4	6	8	12	16	24	32
AMP	4								2	35	51	8						
CHL										17	52	21	8		2			
ERY		4	2	11	35	28	8	4		4				2	2			
PEN		2	9	9	36	26	2	7	7									2
STR												8			4	33	38	17
TET									4	8	29	15	19	13	10	2		

Antibiotic/ Resistance breakpoint (µg mL^{-1}): AMP (Ampicillin/ 2), CHL (Chloramphenicol/ >8), ERY (Erythromycin/ 1), PEN (Penicillin G/ >8), STR (Streptomycin/ 64), TET (Tetracycline/ 32).

≤8μg mL^{-1} of chloramphenicol, ≤32μg mL^{-1} of tetracycline and ≤64μg mL^{-1} of streptomycin.

Results obtained for resistance to streptomycin and tetracycline showed higher accuracy of the DD method than the MIC in detecting resistant *Lactobacillus* strains. In fact, 45.8% of strains identified as resistant to streptomycin by DD were not identified as resistant by MIC, whereas 42.6% of strains identified as resistant to tetracycline by DD were identified as susceptible to this antibiotic by MIC. This confirmed the necessity of more specific inhibition zone range values for interpretation of data from strains belonging to the species *L. plantarum*, since neither CLSI nor EFSA established breakpoints for the assessment of antibiotic resistance by DD in this species.

In this study, a breakpoint data set was generated for 54 indigenous *Lactobacillus* strains to 06 antimicrobials used in food animals. Distribution of MIC values enables the proposal of consistent breakpoints data for *L. plantarum* since there is no consensus on the breakpoints for this genus and species by CLSI and EFSA.

CONCLUSION

Of 54 indigenous *Lactobacillus* strains isolated from fermented sausages, 44 were genotypically confirmed as belonging to the *L. plantarum* species and 3 as *L. pentosus*. None of the strains showed multidrug resistance (non-intrinsic resistance). DD showed higher accuracy than MIC in detecting streptomycin and tetracycline resistant strains. Future research should focus on the genetic mechanisms underlying the phenotypic resistance by analyzing antibiotic resistance genes in the potential probiotic *Lactobacillus* strains in order to estimate the possibility of transferring genes to other bacteria present in the gastrointestinal tract of consumers.

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