ANTIBIOTIC SUSCEPTIBILITY OF LACTOBACILLUS PLANTARUM STRAINS, ISOLATED FROM KATAK

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Summary


Several Lactobacillus species are accepted as microorganisms with Qualified Presumption of Safety (QPS) in the EFSA’s list. One of them, Lactobacillus plantarum is a widely distributed species with a proven probiotic potential and technological relevance. In addition, every strain must complete several requirements, before implementation. Antibiotic susceptibility is one of EFSA’s important criteria regarding the safety of probiotics. The reason is to avoid any possibility of antibiotic resistance genes transfer to opportunistic pathogens in the gut. In the present study 14 Lactobacillus plantarum strains were assessed for susceptibility to 21 antibiotics from different groups. A high number of resistant strains was determined toward 12 antibiotics (penicillins – penicillin, piperacillin; IIIth generation cephalosporins – cefotaxime, ceftriaxone, ceftazidime; glycopeptides – vancomycin; tetracyclines – tetracycline; aminoglycosides – gentamicin; macrolides – clarithromycin; quinolones – nalidixic acid, ciprofloxacin, levofloxacin). Concerning the other tested antibiotics, strain-specific antibiotic-sensitivity patterns were observed. Antibiotic resistance was also discussed as an advantage in the selection of probiotic strains, however only when it is not transferable. Estimated susceptibility patterns of some of tested candidate probiotic strains are also important, considering the use of the latter as agents accompanying antibiotic therapy

Key words: antibiotic susceptibility, Lactobacillus spp., probiotics

INTRODUCTION

Recently, antibiotic resistance has received more attention worldwide due to the increased possibility for emergence of some resistant bacteria. The complex inter-connections and interaction between humans, drugs and the environment sometimes result in appearance of bacterial antibiotic resistance (Barbosa & Levy, 2000; O’Brien, 2002). Thus, several selection pressures from the environment can result in variation in different geographic regions (McCormick et al., 2003). The mechanism of antibiotic resistance most often occurs through horizontal gene
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transfer (McCormick et al., 2003). In this aspect, pathogenic bacteria have gained great attention, due to direct threat (Neut et al., 2017), however scarce information exists to friendly bacteria, such as Lactobacillus (Horowitz et al., 1994). The reason is that lactic acid bacteria (LAB) have obtained a generally regarded as safe status – GRAS (Generally Recognised as Safe) or QPS (Qualified Presumption of Safety), according to FDA and EFSA, respectively. They have been extensively used in food preservation throughout history and are naturally abundant in fermented food. LAB are a great part of the microbiota of mouth, gastrointestinal tract, urogenital tract etc.

Several Lactobacillus strains have been considered beneficial since they confer health benefits on humans and animals and are considered probiotics (Casas & Dobrogosz, 2000). The term probiotic is derived from the Greek words "pro – for" and "bio – life". Probiotics are usually living microorganisms that, when taken at the required dose, provide benefits to the health of the host (Hill et al., 2014). They naturally benefit the health of the host. Moreover, Lactobacillus species are well-known starters and some of them emerge naturally as fermented microflora. Lactobacillus plantarum is a widespread species and a major participant in fermentations in the plant, dairy, meat products and often used as probiotics (Danova & Georgieva, 2013).

Recently, a possible co-application of probiotic and antibiotic is widely discussed. The advantage of such a form of combined therapy is recognised and widely used for preventing antibiotic-associated diarrhoea and induced dysbiosis.

Due to the increased application of lactobacilli as probiotics, EFSA has developed a number of requirements, concerning their safety and functionality. Every commercial probiotic should be able to obtain QPS status. Even though QPS is a status attributed to species, genome content varies widely between species, including those from Lactobacillus genus (Broadbent et al., 2012; Raftis et al., 2014). The bacteria with infectious history and strains that may possess virulence or antibiotic resistance genes should not be used to prevent gene transmission to other species (EFSA, 2012a).

The human and animal GIT, due to the immense amount of bacteria and the close contact between them, is a possible place for a gene transfer. The main hazard is antibiotic resistance determinants transmission from commensal bacteria and the emergence of resistance to common microbial infections, impairing successful antibiotic treatment (Snydman, 2008). Therefore, the lack of acquired or transferable resistance factors need to be justified for candidate probiotics and starter cultures, so they can obtain QPS status (EFSA, 2012a). In the context of co-administration of probiotics with antibiotics, probiotic’s resistance toward the antibiotic used may also be discussed as desired. Before the general application for commercial use, the resistance’s nature should be clarified.

The antibiotic resistance genetic determinants are often plasmid-associated genes. Their passage occurs through horizontal gene transfer. Plasmid carried genes and conjugative transposons could be passed from one LAB to other. They have been commonly found in many strains (Teuber et al., 1999). This is considered acquired resistance since it is found in strains that are typically susceptible. The transmissible genes have been determined in strains belonging to the species Lactobacillus fermentum, Lacto-
bacillus reuteri and L. plantarum (Tannock et al., 1994; Fons et al., 1997). In the opposite, antibiotics resistance of numerous LAB strains have been considered as intrinsic (natural) and non-transmissible (Adams & Marteau, 1995; Salminen et al., 1998). The difference between intrinsic/plasmid antibiotic resistance should be determined. The strains, with a plasmid-derived resistance, should not be used as probiotic products in animal and human products, while intrinsically resistant strains could be useful during antibiotic treatment in patients with unbalanced microbiota (Salminen et al., 1998). Since lactobacilli are widely used as starters and probiotics and co-administered with antibiotics in therapy, they are obtained in great quantity and thus are able to interact with the host microbiota. Therefore, they should be carefully checked for lack of transferable genes and should not add up to the total genes for antibiotic resistance (EFSA, 2012b) in food and gut microbiome. According to the safety criteria of LAB, intrinsic and acquired resistance differences should be carefully distinguished. This is an important requirement for the safety assessment of each newly characterised candidate probiotic and/or starter LAB.

With this aim, the antibiotic susceptibility of 14 L. plantarum strains, newly isolated from homemade samples of the Bulgarian dairy product katak to 21 antibiotics was characterised. The antibiotics belong to the groups of inhibitors of cell wall synthesis; inhibitors of protein synthesis and nucleic acid synthesis and were tested on the group of pre-selected lactobacilli.

**MATERIALS AND METHODS**

**Lactobacillus strains and culture conditions**

Fourteen strains, newly identified as Lactobacillus plantarum strains (unpublished data), were included in the present study. They were part of the laboratory collection of lactic acid bacteria (LAB), isolated from different habitats. All 14 strains were isolated from a homemade sample of a traditional dairy product katak from Lukovit, Bulgaria.

The 14 Lactobacillus cultures were stored at −20 °C in MRS broth supplemented with glycerol (20% v/v) and were pre-cultivated twice at 37 °C in De Man Rogosa Sharpe (MRS) broth (Hi-Media Pvt. Ltd., India), prior to assays. MRS agar, pH 6.5 (Hi-Media Pvt. Ltd., India) was used to cultivate them for antibiotic susceptibility tests. All media were sterilised by autoclaving at 121 °C for 20 min.

**In vitro antibiotic susceptibility tests**

Twenty-one antibiotics, divided into 3 groups according to their mode of action were included in the study:

1. Inhibitors of cell wall synthesis: amoxicillin – amoxicillin (10 μg/disk, HiMedia); penicillins – ampicillin (10 μg/disk), penicillin (15 μg/disk), piperacillin (100 μg/disk); carbapenems – meropenem (10 μg/disk); II generation cephalosporins – cefuroxime (30 μg/disk); III generation cephalosporins – ceftriaxone (30 μg/disk), ceftazidime (30 μg/disk), cefotaxime (30 μg/disk); glycopeptides – vancomycin (5 μg/disk) – all from BB-NCIPD Ltd.
2. Protein synthesis inhibitors: tetracyclines – tetracycline (30 μg/disk, Oxoid, UK), doxycycline (30 μg/disk, HiMe
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(3) Inhibitors of nucleic acid synthesis: quinolones – nalidixic acid (30 µg/disk); ciprofloxacin (5 µg/disk); levofloxacin (5 µg/disk); rifampicins – rifampin (5 µg/disk) – all from BB-NCIPD Ltd.

The Bauer Kurby disk method (Bauer et al., 1966) was used to determine the antibiotic susceptibility to antibiotics from different groups. Petri dishes (9 cm) were seeded with 100 µL of active 24-hour cultures (0.5 MacFarland standard) and overlaid with 15 mL melted MRS agar (2% v/v), cooled to about 37–42 °C, mixed gently. Upon agar solidification, the antibiotic paper disks were dispensed and placed on the agar surface. The Petri dishes were cultivated for 24 h at 37 °C in anaerobic conditions (BBL GasPack anaerocult system). The diameters of inhibition zones (around the disks) in mm were measured and the results (average of 3 readings) were expressed as S (sensitive), I (intermediate) and R (resistant) as per Clinical Laboratory Standards Institute Performance Standards for Antimicrobial Disk Susceptibility (CLSI, 2006).

RESULTS

Strain-specific antibiotic susceptibility patterns were obtained for each of the tested 14 strains (Table 1). Overall, antibiotic susceptibility pattern varied between tested lactobacilli and antibiotics (Fig. 1–3).

DISCUSSION

Before application each candidate probiotic strain has to be individually assessed, first in vitro. With this aim, we selected 14 Lactobacillus strains, isolated from...
**Table 1.** Antibiotic susceptibility patterns of 14 newly characterised *L. plantarum* strains from a homemade fermented dairy product katak

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Fig. 1. Antibiotic susceptibility of 14 L. plantarum strains to antibiotics – inhibitors of cell wall synthesis: beta-lactams: penicillins (ampicillin, A; penicillin, P; piperacillin, Pip); aminopenicillins (amoxicillin, Amx); carbapenems (meropenem, Mer); II generation cephalosporins (cefuroxime, Cxm); III generation cephalosporins (ceftriaxone, Cft; ceftazidime, Caz; ceftotaxim, Ctx); glycopeptides (vancomycin, Va).

Fig. 2. Antibiotic susceptibility of 14 L. plantarum strains to antibiotics – inhibitors of protein synthesis: tetracyclines (doxycycline, D; tetracycline, T); aminoglycosides (gentamicin, Gm; streptomycin, S); amphenicols (chloramphenicol, C); macrolides (clarithromycin, Clr; erythromycin, E).

Traditional Bulgarian “katak”. They were identified as L. plantarum (unpublished data). This fermented milk product is famous with a long shelf life, up to one year without preservatives (Danova & Georgieva, 2013). However, limited data exist on its autochthonous lactic acid microbiota, which is probably responsible for such stability and safety of the product. All lactobacilli originated from one sample of homemade katak and observed variety in the spectrum of antibiotic susceptibility was unexpected.
Antibiotics in current human/animal use have some limitations, concerning their spectrum of antibacterial activity. Therefore, 21 different antibiotics were included in the *in vitro* tests. Only 4 out of 21 antibiotics did not inhibit the growth of all 14 lactobacilli, while for the other 17, a strain-specific variety was observed (Table 1). A higher number of resistant strains was found towards the group of antibiotics inhibitors of cell wall synthesis. Even though generally *Lactobacillus* have been found to be susceptible to penicillins (ampicillin and penicillin) (Ammor et al. 2007), we revealed high prevalence of resistance towards penicillin and piperacillin. Our results confirm the report of Zarazaga et al. (1999). Different studies have shown penicillin’s resistance in *L. plantarum* strains from different habitats: fermented vegetables (Pulido et al., 2005; Lapsiri et al., 2011); home-made Spanish (Herrero et al., 1996) and other cheeses (Flórez et al., 2005; Belleti et al., 2009). Data for *Lactobacillus* spp. from yogurt (Savadogo et al., 2010) and different fermented milk products (Yüksekdağ & Beyatlı, 2008) are also reported. In addition, widespread penicillin resistance has been observed in probiotic and starter lactic acid bacteria (Charteris et al., 1998; Danielsen & Wind, 2003).

Resistance to β-lactams is a disturbing and increasingly spread phenomenon. A supposed mechanism according to Condon (1983) is cell wall impermeability. Other mechanisms implied are nonspecific, such as multidrug transporters (Putman et al., 2001) and defective cell wall autolytic systems (Kim et al., 1982). Currently, there are no evidence and reports on *Lactobacillus* suggesting the transferability of resistance genes for β-lactam antibiotics (Devika et al., 2019).

The tested strains were also found to be resistant toward III generation cephalosporins – cefotaxime; ceftriaxone, ceftazidime (Fig. 1). Resistance towards cephalosporins is found across numerous *Lactobacillus* spp. (Abriouel et al., 2015) including isolates from different types of cheeses (Danielsen & Wind, 2003). Charteris et al. (1997) reported a high level of resistance toward IIrd generation cephalosporins. However, our strains with the

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**Fig. 3.** Antibiotic susceptibility of 14 *L. plantarum* strains to antibiotics – inhibitors of nucleic synthesis: quinolones (nalidixic acid, Nx; ciprofloxacin, Cip; levofloxacin, Lev); rifampicins (rifampin, R).
exception of a single strain were susceptible to cefuroxime (Fig. 1).

All tested strains from katak tolerated well the glycopeptide antibiotic vancomycin (Fig. 1 and Table 1). Such a feature, which is widespread among Lactobacillus spp., is considered chromosomally encoded (Holliman & Bone, 1988; Nicas et al., 1989) and thus is non-transmissible. Such intrinsic resistance could be preferred because antibiotics often cause alteration in the microbiome, dysbiosis and induce antibiotic-associated diarrhoea or other health issues. Therefore, it is desired that the probiotic remains viable and not affected by the antimicrobials applied (Neut et al., 2017).

Lactobacilli are most often susceptible to different antibiotics such as cell wall inhibitors penicillins (Danielsen & Wind, 2003), as well as to low concentrations of most inhibitors of protein synthesis (e.g. chloramphenicol, macrolides and tetracyclines) (Ammor et al., 2007). On the other side, they tend to be resistant to protein cell wall inhibitors – aminoglycosides (kanamycin, gentamicin and streptomycin) (Ammor et al., 2007), intrinsically resistant to cell wall inhibitors – glycopeptides (vancomycin and teicoplanin) (Charteris et al., 1998; Danielsen & Wind, 2003) and some of them to cephalosporins (cefuroxime, ceftriaxone and cefoxitin) (Danielsen & Wind, 2003; Belleti et al., 2009). In addition, they also show intrinsic resistance to inhibitors of nucleic acid synthesis – quinolones (e.g. ciprofloxacin and nalidixic acid) (Hummel et al., 2007).

The tested strains revealed high resistance >90% toward gentamicin (Fig. 2) and 30% to tetracycline. Commonly acquired resistance genes in probiotics and lactobacilli isolated from fermented food are the genes Tet (M), Tet (S) for tetracycline resistance (Thumu & Halami, 2012). Chloramphenicol resistance is dependent on cat genes (Ahn et al., 1992, Hummel et al., 2007). Tetracycline and chloramphenicol resistance is a frequent phenotype that is due to acquired resistance genes. Neut et al. (2017) have detected the same pattern for Lactobacillus spp. from fermented food. L. plantarum strains from katak demonstrated high resistance toward tetracycline, but not to chloramphenicol (Fig. 2). Supposedly, due to the mobile gene homology of mobile genetic elements, lactobacilli from fermented food could have acquired resistance genes from staphylococci and enterococci (Abriouel et al., 2015).

Clarithromycin resistance has been observed in commercial probiotic L. plantarum strains (Sharma et al., 2015). However, other probiotic strains from commercial dairy products were susceptible (Bilal et al., 2010), and intermediate and high susceptibility was determined for 13 out of the 14 tested L. plantarum (Fig. 2). Clarithromycin is applied in Helicobacter pylori treatment and co-administration with probiotic is often recommended.

With regard to inhibitors of nucleic acid synthesis, resistance to quinolones e.g. ciprofloxacin is common (Hummel et al., 2007). This is confirmed by our results showing resistance of lactobacilli from katak to nalidixic acid, ciprofloxacin, levofloxacin (Fig. 3). Resistance towards nalidixic acid was observed for 12 L. plantarum strains (Cebeci et al., 2003) and is also in line with the findings of Horowitz et al. (1994) and Charteris et al. (1998) for Lactobacillus spp. The resistance towards quinolones is due to intrinsic factors such as the structure of cell wall, efflux mechanism and permeability. A lack of sensitivity to rifampicin is a result of mutations (Ezekiel & Hutchins, 2003).
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1968) and is therefore not likely to be transferable.
Marketed probiotic strains have been found resistant toward levofloxacin (Neut et al., 2017) and our results revealed the same characteristic (Fig. 3).

CONCLUSION

The present study is a part of the safety assessment of newly isolated lactobacilli from a not well-studied fermented milk product – katak. The EFSA requires every strain intended for human/animal consumption to be tested for resistance to ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, and chloramphenicol. In this aspect, tested L. plantarum strains L2, L3, L9 and L14 are pre-selected as more sensitive, only except for vancomycin and gentamicin resistance. In addition, the exclusion of strains that could carry antibiotic resistance genes is required as ingested in large numbers, they could possibly transfer antibiotic resistance determinants to the intestinal microbiota. The data from our study revealed that transmissible resistance genes could be possibly present only in limited number of strains. The L. plantarum L1, L10 and L11 showed undesirable for QPS resistance to erythromycin and L1 – to chloramphenicol; they will be subject to additional tests. The minimal inhibitory concentration will be determined and only the strains, under the EFSA’s MIC border values could be further explored.

The tested 14 L. plantarum also revealed atypical penicillin resistance. Overall, the majority of pre-selected strains from katak completed in vitro safety criteria of EFSA and did not present a reservoir of antibiotic resistance genes. As a part of autochthonous lactic acid microbiota of the traditional fermented product, they probably may contribute to the safety quality of katak.

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REFERENCES

Bauer, A. W., W. M. M. Kirby, J. C. Sheris & M. Tuck, 1966. Antibiotic susceptibility testing by a standardized single disk meth-
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European Food Safety Authority (EFSA), 2012a. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal, 10*, 2740–2750.


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