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ANTIBIOTIC RESISTANCE PROFILE OF THE NEWLY ISOLATED LACTIC ACID BACTERIA STRAINS FROM TRADITIONAL FERMENTED FOODS

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Abstract
The use of antibiotics is a major problem everywhere around the globe and in step with this, there's currently an increased public and scientific interest. Microbial resistance is a vital issue for the organizations like EFSA, WHO, FDA, and FAO because it is developing rapidly and is an increasingly serious health concern within the world. Microbial resistance comes as a result of continuous exposure of microorganisms to antibiotics. Lactic acid bacteria (LAB) constitute a really important group of microorganisms that can inhabit different conditions and environments, as the different parts of the gastrointestinal tract of humans and animals, and are an important element of the microbiota of fermented foods. The FDA and EFSA authorities have given them the status referred to as GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety). Continuous exposure of LAB to environments with antibiotics may prompt them to become an intrinsic or extrinsic reservoir of genes accountable for antibiotic resistance. Knowledge of antibiotic susceptibility of bacteria with probiotic potential is important. Also, it's essential to define their resistance profile. Ten newly isolated strains from traditional fermented foods were used for determining the antibiotic resistance profile. The resistance to antibiotics varied among the examined strains and a few of the antibiotics resulted in complete resistance. Chromosomal DNA of LAB was analyzed for the antibiotic resistance genes. Only five of eight vancomycin-resistant strains have shown that they contain the resistance gene in chromosomal DNA. None of the genes that determined the resistance to other antibiotics have been detected in chromosomal DNA.

Keywords: antibiotic resistance, lactic acid bacteria.

1. INTRODUCTION

Antibiotics over the years are used as an instrument to kill bacteria and cure infections. The constant exposure of microorganisms to antibiotics has made it possible for bacteria to achieve adaptation and resistance by making changes within the DNA. Various organizations like EFSA, WHO, FDA, and FAO have focused on microbial resistance, which is often already a heavy problem worldwide (EFSA 2022; FAO 2021; FDA 2021; WHO 2020). Antibiotics are affected by preventing microbial reproduction or through various inhibition mechanisms like blocking the DNA copying process, protein synthesis, cell wall synthesis, or cytoplasmic membrane synthesis. The rise of resistant bacterial strains comes as a result of the increasing use of antibiotics in both humans and animals. Bacteria have gained a spectrum of resistance mechanisms to withstand challenging environmental conditions (Prestinaci et al., 2015). Bacteria can respond immediately to changing environmental conditions by acquiring characteristic traits, altering genome functionality, or

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accepting the competent genome from other species. As a general term for all existing in nature genes for resistance was defined as resistome (Cox & Wright, 2013; Zhang & Fang, 2016; Bello-Lopez et al., 2019). Resistance is split into "internal or innate" and "external or acquired". The group of genes that are found on chromosomes and participate in innate resistance is defined as the intrinsic resistome, which is not associated with gene transfer. Intrinsic resistance is resistance that's naturally present in microorganisms. It's a property controlled by chromosomes and relates to the physiology of microorganisms. The group of genes acquired as result of mutations within the genome, which might be transferred to other bacteria, is termed as extrinsic resistome. Within the case of the external resistance mechanism, bacteria acquire the resistance gene from other bacteria which have already got resistance to harsh environmental conditions (Cox & Wright, 2013; Zhang & Fang, 2016; Bello-Lopez et al., 2019). There are three main ways for the transfer of the acquired resistance genes: through conjugation by using the conjugated plasmids or transposons, through transformation, or transduction. Many authors indicated that conjugation as a transfer tool has the main role in the spread of genes for resistance within the environment. (Thomas & Nielsen, 2005; Villa et al., 2019). Antibiotic-resistant bacteria were first described in the 1940s. As new antibiotics have been discovered at a steady pace, the steps of assessing the consequences of this phenomenon are too slow (Capita & Alonso-Calleja, 2013).

LAB are microorganisms that produce mainly lactic acid during metabolic activity. These bacteria are important in many food applications. LAB are widely used as probiotics with therapeutic properties for human health. According to FAO/WHO - "Probiotics are the living microorganisms that are administered/consumed in an adequate amount which produces beneficial effects on the host". They're a part of the microbiota of some foods like dairy products, fermented meats, and fermented vegetables and fruits and also are present within the digestive tract of humans and animals (Ayivi et al., 2020). LAB are considered as QPS (qualified presumption of safety) and are the foremost studied bacteria thanks to their health benefits. These bacteria to be selected as probiotics, many aspects need to be measured which include: safety, stability and feasibility, functional and technological aspects, and proper internal control. Continuous exposure of LAB to environments with antibiotics may prompt them to become an intrinsic or extrinsic reservoir of antibiotic resistance genes (Álvarez-Cisneros & Ponce-Alquicira, 2018).

For this reason, we undertook to screen the profile of our newly isolated LAB for resistance to antibiotics and also the presence of genes of resistance.

2. MATERIALS AND METHODS

LAB strains

The research in the present work was conducted with ten LAB strains (KC5-12, KC5-13, KC5-14, KZC8-21-1, KZC8-23-5, C10-31-3, KO4-4, KO3-7-5, KZM2-11-3, and KZM2-11-1) preselected for the purpose. All of the strains were newly isolated from different traditional fermented foods. The strains were primarily identified as Lactiplantibacillus plantarum – five isolates; Pediococcus pentosaceus – one isolate; Latilactobacillus sakei – one isolate; Loigolactobacillus coryniformis – one isolate; Lactobacillus delbrueckii subsp. bulgaricus – two isolates (unpublished data). All studied strains were incubated for 24 hours in MRS broth (Merck, Germany).

Antibiotic susceptibility test

The antibiotic resistance test was performed using Kirby – Bauer disk diffusion method (Sharma et al., 2017). Bacterial culture after incubation for 24h (1 ml of 10^8 CFU/ml) of each strain was inoculated in a Petri dish and 19 ml of MRS agar (Merck, Germany) were overlayered,

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homogenized well, allowed to solidify the medium, and then antibiotic discs were aseptically placed to the agar plates. Antibiotics discs (HiMedia) used for the test are vancomycin VA (5 µg), ampicillin AMP (10 µg), streptomycin S (10 µg), tetracycline TE (30 µg), chloramphenicol C (30 µg), clindamycin CD (2 µg), gentamicin GEN (10 µg), kanamycin KA (30 µg), trimethoprim TR (5 µg) and antibiotic discs (oxoid) are neomycin N (30 µg), erythromycin E (15 µg), ciprofloxacin CIP (5 µg) and rifampicin RD (5 µg). Plates were incubated to the corresponding temperature of strains and the zones of inhibition were noted after incubation for 24h. Resistance pattern data were interpreted as per the Clinical Laboratory Standard Institute (CLSI, 2020) and the strains were classified in categories R – resistant, ≤ 14 mm zone; I – intermediate, 15-19 mm zone; S, SS – susceptible, ≥ 20 mm zone. The experiments were performed in triplicate.

Antibiotic resistance gene profile

Chromosomal DNA of 10 strains of LAB was analyzed for the antibiotic resistance gene profile by polymerase chain reaction (PCR) using gene-specific primers (Merck). Twenty-eight pairs of gene-specific primers forward and reverse according to Guo H, et al., (2019) were used for 12 different antibiotics. All PCR reactions were performed according to Dec et al. (2017) with modification at annealing temperature following temperature program: pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 50-66°C for 45 sec, and extension at 72°C for 75 sec with a final extension at 72°C for 8 min. Amplified PCR products were visualized by 1% agarose gel electrophoresis to confirm the gene presence.

3. RESULTS AND DISCUSSIONS

3.1. Antibiotic susceptibility of newly isolated LAB

Because of their health benefits, it is important to determine the antibiotic susceptibility of newly isolated bacteria with probiotic potential. The profile of antibiotic resistance is one of the important criteria for the selection of probiotics. This is very useful in cases where it is necessary to use a combination therapy of antibiotics with probiotics, especially for restore of normal microbiota to gastrointestinal and urogenital tracts. In cases, such as diarrhea, probiotics are used in combination with antibiotics and they must be themselves resistant to antibiotics (Ouwehand et al., 2016). Also, it is very important to determine the resistance profile of strains used in fermented foods. The use of LAB in fermented foods as a preservative or as a probiotic may help reduce the rate of development of antibiotic-resistant strains with the increasingly widespread use of antibiotics (Varankovich et al., 2015).

For these reasons, one of the conditions for characterizing potential probiotic strains is screening for their antibiotic susceptibility. For this purpose, the sensitivity of the studied strains to 13 antibiotics with a different mechanism of action from the main groups used in medical practice was determined: inhibitors of cell wall synthesis - vancomycin, erythromycin, and ampicillin; protein synthesis inhibitors - streptomycin, tetracycline, chloramphenicol, clindamycin, kanamycin, neomycin, and gentamicin; inhibitors of DNA synthesis - trimethoprim, ciprofloxacin, and rifampicin (Sharma et al., 2017).

The results obtained from the studies performed by the diffusion method are present in Table 1. Given the safety aspects, antibiotic resistance varies in the strains studied, with a specific profile observed in some strains. Resistance to a wider range of antibiotics was found mainly in the studied strains KO 4-4, KZC 8-23-5, and C 10-31-3. Strain KZM 2-11-1 was susceptible to vancomycin and KZM 2-11-3 was intermediate to vancomycin. All strains have shown complete resistance to kanamycin, neomycin, and ciprofloxacin. All of the studied strains were susceptible or

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intermediately resistant to ampicillin and only one strain C 10-31-3 was resistant to rifampicin. Other researchers have published similar results. All tested strains of LAB and Bifidobacterium spp. isolated from dairy and pharmaceutical products were susceptible to ampicillin, clindamycin, erythromycin, penicillin G, rifampicin, and the susceptibility for chloramphenicol, gentamicin, neomycin, streptomycin, tetracycline, and vancomycin was variable and depending on the species (D'Aimmo et al., 2007). LABs from fermented food products were susceptible to ampicillin and intrinsically resistant to kanamycin, and vancomycin except L. bulgaricus, L. acidophilus, and S. thermophilus, which were susceptible to vancomycin. Some strains had acquired resistance to penicillin, erythromycin, clindamycin, and tetracycline (Nawaz, M. et al., 2011). Intrinsic resistance to vancomycin was confirmed for L. paracasei, L. salivarius, and L. plantarum (Blandino et al., 2008).

Table 1. Antibiotic susceptibility pattern

Antibiotics	**KZM 2-11-1**	**KZM 2-11-3**	**KO 3-7-5**	**KO 4-4**	**KC 5-12**	**KC 5-13**	**KC 5-14**	**KZC 8-21-1**	**KZC 8-23-5**	**C 10-31-3**
Vancomicin (VA)	S	I	R	R	R	R	R	R	R	R
Ampicillin (AMP)	SS	SS	SS	I	SS	I	S	SS	I	I
Streptomycin (S)	I	I	R	R	R	R	R	R	R	R
Tetracycline (TE)	SS	SS	S	R	S	R	R	I	R	I
Chloramphenicol (C)	SS	SS	S	R	SS	S	I	I	I	S
Clindamycin (CD)	R	R	SS	R	SS	S	I	I	R	I
Gentamicin (GEN)	R	I	R	R	R	R	R	R	R	R
Kanamycin (K)	R	R	R	R	R	R	R	R	R	R
Neomycin (N)	R	R	R	R	R	R	R	R	R	R
Trimethoprim (TR)	R	R	SS	S	S	R	I	S	S	R
Erythromycin (E)	SS	SS	I	I	I	I	I	I	R	I
Ciprofloxacin (CIP)	R	R	R	R	R	R	R	R	R	R
Rifampicin (RD)	SS	SS	S	I	I	I	S	S	I	R

According to CLSI, 2020: R – resistant, ≤ 14 mm zone; I – intermediate, 15-19 mm zone; S, SS – susceptible, ≥ 20 mm zone

3.2 Detection of antibiotic resistance genes in new LAB isolates

The presence of transmissible antibiotic resistance markers in food-associated and probiotic bacteria has become one among the important safety criteria. Probiotics shouldn't carry transferable determinants for resistance to antibiotics. Many authors indicate that bacteria from normal microbiota can become sources for the transfer of genes for resistance to pathogens (Imperial and Ibana, 2016). Considering that LAB may contain transferable antibiotic resistance genes, the strains intended to be used in food or food additives should be monitored for their safety (Stefańska et al., 2021).

Genes for acquired resistance to different antibiotics like tetracycline, erythromycin, and vancomycin have been detected in different LAB strains, isolated from different fermented milk and meat products. Transposable genetic determinants such as plasmids and conjugative transposons have a large distribution in the environment and are common in LAB, making these bacteria potential for the transfer of these genes for resistance (Devirgiliis et al., 2011, Devirgiliis et al., 2013). In food production, bacteria that are identified as containing acquired resistance quite

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often are obligate homofermentative bacteria like *L. acidophilus, L. helveticus, L. delbrueckii*, obligate heterofermentative like *L. reuteri, L. fermentum*, and facultative heterofermentative like *L. plantarum, L. rhamnosus, L. paracasei, Lactococcus lactis, Streptococcus thermophilus, Pediococcus spp., Leuconostoc spp., and Enterococcus spp.* (Gueimonde et al., 2013; Álvarez-Cisneros & Ponce-Alquicira, 2018).

Characterization of antibiotic resistance profile and evaluation of the transferability of corresponding resistance genes to other bacteria have been analyzed by many researchers. Some authors indicated that antibiotic resistance can't be transferred from LAB to the pathogens like *Staphylococcus aureus subsp. aureus, Staphylococcus haemolyticus, Staphylococcus epidermidis, Listeria monocytogenes, Acinetobacter baumannii, Citrobacter freundii,* and *Escherichia coli* (Anisimova and Yarullina, 2018; Guo et al., 2019). Similar studies show that the possibility of the resistant genes found in the LAB being transferred to other bacteria is relatively low, making these bacteria safer for various uses, including food production, from the angle of the spread of antibiotic resistance.

During the study of the newly isolated LAB strains was determined the profile of antibiotic-resistant genes, and the results are presented in Table 2. Only five of eight in total, phenotypically vancomycin-resistant strains have shown the presence of the gene vanX in chromosomal DNA. None of the other genes for antibiotic resistance are detected in chromosomal DNA. For this reason, it's necessary to do some more detailed study of the entire genome, to point out if these genes are present in some plasmids also to analyze the chance of their horizontal gene transfer to other bacteria. Such data will complement the safety profile when implementing the study strains into new products.

Table 2. Antibiotic resistance gene profile of newly isolated lactic acid bacteria strains

Antibiotics	**Primers** (Guo H, et al., 2019)	**KZM 2-11-1**	**KZM 2-11-3**	**KO 3-7-5**	**KO 4-4**	**KC 5-12**	**KC 5-13**	**KC 5-14**	**KZC 8-21-1**	**KZC 8-23-5**	**C 10-31-3**
VA	vanE	-	-	-	-	-	-	-	-	-	-
	vanX	-	-	-	+	+	-	+	+	+	-
AMP	blaZ	-	-	-	-	-	-	-	-	-	-
	bla	-	-	-	-	-	-	-	-	-	-
	mecA	-	-	-	-	-	-	-	-	-	-
S	aadA	-	-	-	-	-	-	-	-	-	-
	aadE	-	-	-	-	-	-	-	-	-	-
	ant(6)	-	-	-	-	-	-	-	-	-	-
TE	tet(M)	-	-	-	-	-	-	-	-	-	-
	tet(K)	-	-	-	-	-	-	-	-	-	-
	tet(W)	-	-	-	-	-	-	-	-	-	-
C	catA	-	-	-	-	-	-	-	-	-	-
	catA	-	-	-	-	-	-	-	-	-	-
CD	Inu(A)	-	-	-	-	-	-	-	-	-	-
	Inu(B)	-	-	-	-	-	-	-	-	-	-
GEN	aac(6')-aph(2")	-	-	-	-	-	-	-	-	-	-

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	aac(6')Ie-aph(2")Ia	-	-	-	-	-	-	-	-	-	-
K	aph(3")-III	-	-	-	-	-	-	-	-	-	-
	ant(2")-I	-	-	-	-	-	-	-	-	-	-
N	aph(3")-I	-	-	-	-	-	-	-	-	-	-
	aph(3")-III	-	-	-	-	-	-	-	-	-	-
TR	dfrA	-	-	-	-	-	-	-	-	-	-
	dfrD	-	-	-	-	-	-	-	-	-	-
E	erm(B)	-	-	-	-	-	-	-	-	-	-
	erm(B-1)	-	-	-	-	-	-	-	-	-	-
	erm(C)	-	-	-	-	-	-	-	-	-	-
CIP	gyrA	-	-	-	-	-	-	-	-	-	-
	parC	-	-	-	-	-	-	-	-	-	-

4. CONCLUSIONS

Some fermented foods such as dairy products have an extremely high bacterial density, mainly composed of LAB. We analyzed 10 strains of LAB isolated from different traditional fermented foods. The study of antibiotic resistance in these bacteria is an important step in preventing the spread of resistant genes. Antibiotic resistance varies between strains. Most strains are susceptible to ampicillin and rifampicin. All strains are resistant to kanamycin, neomycin, and ciprofloxacin. Isolated strains were analyzed for the presence of resistant genes in chromosomal DNA of LAB and concluded in only five of eight vancomycin-resistant strains were intrinsic. Although the presence of genes in plasmids and transposons has been established, some studies have shown that the potential for transfer to pathogenic bacteria is low. This leads to the need for some more detailed study of the presence of these genes in plasmids as well as to analyze the possibility of their horizontal gene transfer to pathogens.

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