Original Research Article

Antibiotic resistance profile and safety assessment of *Lactobacillus acidophilus* and *Lactobacillus plantarum* isolated from milk

ABSTRACT

Aims: The present study was undertaken to evaluate the safety of probiotics viz., *Lactobacillus acidophilus* and *Lactobacillus plantarum* through antibiotic susceptibility tests.

Study design: The study was conducted on two reference *Lactobacillus* strains viz., *Lactobacillus acidophilus* (NCDC 13) and *Lactobacillus plantarum* (NCDC 20) procured from the National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal, India.

Place and Duration of Study: Department of Veterinary Public Health and Epidemiology, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, between March - April 2021.

Methodology: The strains of *L. acidophilus* and *L. plantarum* were subjected to antibiotic susceptibility tests using the disc diffusion method against 26 antibiotics.

Results: The isolates were found to exhibit multiple resistance against some of the most commonly used antibiotics. The isolates showed a high level of resistance toward ampicillin, amoxycillin, cefotaxime, nalidixic acid, streptomycin, kanamycin and nitrofurantoin. The isolates showed low levels of resistance toward cephalothin, amikacin, erythromycin and azithromycin. They were susceptible to ciprofloxacin, penicillin G, cloxacilln, ofloxacillin, norfloxacin, levofloxacin, moxifloxacin, sparfloxacin, enrofloxacin, gemifloxacin, chloramphenicol, gentamicin, co-trimoxazole and oxytetracycline.

Conclusion: The current study concluded that antibiotic resistance is prevalent among *L. acidophilus* and *L. plantarum*, which is a major concern for food safety. Hence, antibiotic susceptibility tests should be considered as an essential measure for the assessment of the safety of probiotics. Furthermore, studies to evaluate the presence of antibiotic-resistant genes in commercially available probiotics should be conducted.

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Keywords: Lactobacilli; safety; probiotics; antibiotic resistance.

1. INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive bacteria that can produce lactic acid as an end product of carbohydrate fermentation. They are widely used in food production and preservation (Lahtinen *et al.,* 2011). Of all the genera of LAB, *Lactobacillus* is the most economically important microbiota which is harbored mainly in the gut of man and animals (Tannock, 2005). They are beneficial for human health because of their antimicrobial and immunomodulatory activity. They can also help to restore the healthy gut microflora. Keeping in view of their benefits, they are included in our diet as probiotics, food preservatives or starters (Salminen *et al.,*1998; Rozman *et al.,* 2020). There have been numerous studies on the commercial production and use of *Lactobacillus*-based probiotics because of their 'generally recognized as safe' (GRAS) status (García-Fruitós, 2012).

Antibiotics are an important therapeutic tool in tackling many infectious diseases of bacterial origin (Aminov, 2017). However, misuse and exploitation of antibiotics by human beings in animal husbandry as well as medical fields has caused the emergence of antibiotic-resistant bacteria (EFSA/ECDC, 2018), which has become a global cause of health concern (World Health Organization, 2014). The bacteria may become resistant in two ways i.e. naturally to escape the action of antibiotics or genetically horizontally resistant gene transfer through transposons or plasmids (Belletti *et al.,* 2009). Furthermore, there has been a rise in the frequency of documented cases of antibiotic-resistant LAB strains over the past decade. To assess the risk, it is important to evaluate if commonly used lactobacillus species isolates display phenotypic resistance to antibiotics and if they do, to determine the extent of resistance and identify the responsible genes. Standardized methods have been developed to determine levels of antibiotic resistance and, as they allow comparisons of results between laboratories, they are recommended by several international agencies including the Clinical and Laboratory Standards Institute (CLSI), International Organization for Standardization (ISO), European Food Safety Authority (EFSA) and the International Dairy Federation (IDF).

Since, probiotics are used in doses unlimited, ensuring their safety is of utmost importance, while there are as such no legislation/acts/rules for microorganisms that are deliberately added to our food as probiotics. It is therefore recommended that these products follow similar requirements as feed additives as precautionary measures (EFSA, 2007). Several reviews (Saarela *et al*., 2000) on LAB have recommended safety criteria for probiotics such as the absence of ARGs which are responsible for resistance to clinically important antibiotics (EFSA-FEEDAP, 2018). Although LABs are

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regarded as safe, they can act as a reservoir for ARGs, which can persist in our food chain (Guo *et al.,* 2017; Duranti *et al.,* 2017) and can be responsible for the transfer of ARGs via horizontal gene transfer (Liu *et al*., 2012). Some studies have reported the transfer of ARGs between various lactobacilli (Nawaz *et al.,* 2011). *In vitro* and *in vivo* studies have also been conducted to evaluate the transfer of ARGs from lactic acid bacteria to other pathogenic bacteria (Kazimierczak *et al.,* 2006; Jacobsen *et al.,* 2007). Thus, the present study was conducted to characterize the phenotypic antibiotic resistance profile in isolates of *Lactobacillus acidophilus* and *Lactobacillus plantarum* using the disc diffusion method. It provides an insight into the safety of probiotics available in terms of antibiotic resistance.

2. MATERIAL AND METHODS

Bacterial strains and propagation

Two reference *Lactobacillus* strains viz., *Lactobacillus acidophilus* (NCDC 13) and *Lactobacillus plantarum* (NCDC 20) were procured from the National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal, India. *Lactobacilli* were maintained and propagated in Lactobacillus de Man Rogosa Sharpe (MRS) broth (Himedia Laboratories Pvt. Ltd.). It was prepared according to the instructions of the manufacturers. The final pH of the media was maintained at 6.5 ± 0.2. Strains were incubated at 37 °C for 48 h in anaerobic atmospheric conditions. They were further sub-cultured thrice before the experiments. It was followed by plating on MRS agar under the same above-mentioned conditions. The bacterial cultures were preserved in glycerol stocks at -80° C.

Antibiotic discs

Twenty-six antibiotics that are used most commonly from different classes were purchased from Hi-media Laboratories Pvt. Ltd. Mumbai, India, and tested for procured probiotics. The details of the name of the drug, concentration, antibiotic group, and mode of action are described in Table 1.

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Table 1. Antibiotics used for antibiotic-resistant profile and their mode of action

S. No.	Name of drug	Concentration (mcg)	Group antibiotics	Mode of action
1.	Ampicillin	10	β-Lactams	Cell wall synthesis inhibition
2.	Amoxycillin	30		
3.	Cloxacillin	30		

4.	Ampicillin/ cloxacillin	10		
5.	Penicillin G	10[a]		
6.	Cephalothin	30	First generation Cephalosporins	
7.	Cefotaxime	10	Third generation Cephalosporins	
8.	Ciprofloxacin	5	Quinolones	DNA replication and transcription inhibition
9.	Ofloxacin	5		
10.	Nalidixic acid	30		
11.	Norfloxacin	10	Fluoroquinolones	
12.	Levofloxacin	5		
13.	Moxifloxacin	5		
14.	Sparfloxacin	5		
15.	Enrofloxacin	10		
16.	Gemifloxacin	5		
17.	Gentamicin	10	Aminoglycosides	Protein synthesis inhibition
18.	Streptomycin	300		
19.	Amikacin	30		
20.	Kanamycin	30		
21.	Chloramphenicol	30	Other	
22.	Erythromycin	15	Macrolides	
23.	Azithromycin	15		

24.	Nitrofurantoin	300	Other	
25.	Oxytetracycline	30	Tetracyclines	
26.	Co-Trimoxazole	25	Other	Folic acid synthesis inhibitors or anti-metabolites

[a] denotes concentration in units

Antimicrobial susceptibility testing

The sensitivity or resistance of LAB to most commonly used antibiotics was evaluated by an antimicrobial susceptibility test. The standard disc diffusion assay was performed according to the Kirby–Bauer method (Bauer *et al.,* 1966). Isolates were cultured and grown overnight in MRS broth and 100 µl culture (0.5 McFarland equivalent to 10^8 cfu/ml) was spread on Mueller-Hinton agar plates with the help of an L spreader. Antimicrobial disks will be placed with the help of sterile forceps and incubated at 37°C for 24 h under anaerobic conditions. The zone of inhibition diameter was measured by zone reader (Hi Antibiotic zone scale, Hi-Media) and results were read according to the breakpoints recommended by Clinical and Laboratory Standard Institute standards for disc-diffusion assay (CLSI, 2016) (Table 2) as described by Sharma *et al.* (2017).

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Table 2. Susceptible and resistant strains were evaluated after being compared with known standard given by CLSI (2016)

Disc Diffusion Method	Diameter of zone of inhibition (mm)
Susceptible	>20
Intermediate	15–19
Resistant	≤14

Statistical evaluation

The disc diffusion method was performed in triplicate and the average diameters calculated are presented as resistant (R), sensitive (S) or intermediate (I).

3. RESULTS AND DISCUSSION

Antimicrobial disc susceptibility tests were performed according to the procedures described by CLSI (CLSI, 2016). Comparative evaluation of the diameter of the zone of inhibition has been shown in graphical form (Fig. 1). The growths of both tested LAB strains i.e. *L. acidophilus* and *L.*

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plantarum were homogenous over MRS. The results for the reference LAB strains have been documented in terms of resistant (R), susceptible (S) and intermediate (I) (Table 3).

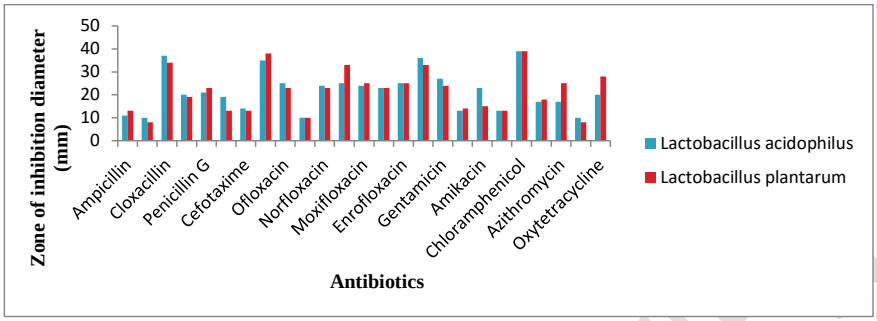

Fig. 1. Comparative evaluation of Diameter of zone of inhibition of *L. acidophilus* and *L. plantarum*

Table 3. Susceptibility of LAB to commonly used antibiotics using the disc diffusion method

S. No.	Antibiotics	Inhibition zone diameter range (mm)	
		Lactobacillus acidophilus	***Lactobacillus plantarum***
1	Ampicillin	R (11)	R (13)
2	Amoxycillin	R (10)	R (8)
3	Cloxacillin	S (37)	S (34)
4	Ampicillin/cloxacillin	S (20)	I (19)
5	Penicillin G	S (21)	S (23)
6	Cephalothin	I (19)	R (13)
7	Cefotaxime	R (14)	R (13)
8	Ciprofloxacin	S (35)	S (38)
9	Ofloxacin	S (25)	S (23)
10	Nalidixic acid	R (10)	R (10)
11	Norfloxacin	S (24)	S (23)
12	Levofloxacin	S (25)	S (33)
13	Moxifloxacin	S (24)	S (25)
14	Sparfloxacin	S (23)	S (23)
15	Enrofloxacin	S (25)	S (25)
16	Gemifloxacin	S (36)	S (33)

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17	Gentamicin	S (27)	S (24)
18	Streptomycin	R (13)	R (14)
19	Amikacin	S (23)	I (15)
20	Kanamycin	R (13)	R (13)
21	Chloramphenicol	S (39)	S (39)
22	Erythromycin	I (17)	I (18)
23	Azithromycin	I (17)	S (25)
24	Nitrofurantoin	R (10)	R (8)
25	Oxytetracycline	S (20)	S (28)
26	Co-Trimoxazole	S (38)	S (34)

In our study, phenotypic resistance to ampicillin, amoxycillin, cefotaxime, nalidixic acid, streptomycin, kanamycin and nitrofurantoin was exhibited by *L. acidophilus*. While *L. plantarum* showed phenotypic resistance to cephalothin in addition to the above-mentioned antibiotics. There were no significant differences observed in the antibiotic resistance profile of *L. acidophilus* and *L. plantarum*. A low level of resistance was exhibited by *L. acidophilus* toward cephalothin, erythromycin and azithromycin. Whereas, *L. plantarum* showed a low level of resistance toward ampicillin/cloxacillin, amikacin and erythromycin. High susceptibility was exhibited by both isolates toward cell wall synthesis inhibitors (β-Lactams- cloxacillin and penicillin G), DNA replication and transcription inhibitors (Quinolones- ciprofloxacin and ofloxacin; Fluoroquinolones) and protein synthesis inhibitors (chloramphenicol, oxytetracycline and co-trimoxazole). There was no significant difference in the antibiotic susceptibility pattern of *L. acidophilus* and *L. plantarum*.

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Lactobacilli are generally considered susceptible to the cell wall synthesis inhibitors (β-Lactams) (Guo *et al.,* 2017: Gueimonde *et al.,* 2013) and more resistant towards cephalosporins (Karapetkov *et al*., 2011) which corroborated with our study. In our study, *L. acidophilus* displayed susceptibility toward β-lactam antibiotics except for ampicillin and amoxycillin for *L. acidophilus* and *L. plantarum* which was contrary to the findings of Klare *et al*. (2007) and Nawaz *et al*. (2011). In addition, *L. plantarum* showed intermediate susceptibility towards ampicillin/cloxacillin while *L. acidophilus* was susceptible to it. Resistance towards cephalosporins in our study for both strains was reported which was similar to the study conducted by Karapetkov *et al*. (2011). Lactobacilli were reported to have intrinsic resistance to aminoglycosides (Abriouel *et al.,* 2015; Sharma *et al*., 2017; Štšepetova *et al*., 2017; Zhou *et al*., 2005) which was similar to our study except for gentamicin

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and amikacin. In a study conducted by Pell *et al.* (2021), they also observed the susceptibility of *L. plantarum* towards gentamicin. Lactobacilli are considered to have intrinsic resistance to aminoglycosides because of the absence of cytochrome-mediated electron transport, which is responsible for antibiotic uptake (Charteris *et al.*, 2001). Resistance to other antibiotics varies greatly among lactobacilli. The genetic determinants often present in mobile elements like transposons and plasmids have the potential to transfer antibiotic resistance genes primarily through conjugation mechanisms (Aquilanti *et al.,* 2007). Another mechanism of resistance against antimicrobials is known to be present in certain lactobacilli which are mediated by multidrug resistance (MDR) transporters (Gueimonde *et al.,* 2009).

4. CONCLUSION

Foods and food supplements having naturally occurring or intentionally added bacteria, such as probiotics can serve as a potential reservoir for antibiotic resistance genes. Hence, the safety of probiotics requires the assessment of resistance /resistance genes carried by them against the clinically significant antimicrobials. Future research exploring their presence and transferability will certainly be a step towards safety in true terms. As the probiotics in our study possessed resistance levels exceeding the limit recommended by CLSI, it is suggested that the proposed limit should be re-examined. Regulatory guidelines/legislation for the assessment of the safety of lactobacilli for their approval as starter cultures or probiotics should be made. It will also facilitate screening of probiotics from a safety point of view. Further studies can be done to evaluate the transferability of genes responsible for resistance to most commonly used antibiotics.

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