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Antibiotic Resistance Profile and Safety Assessment of *Lactobacillus acidophilus* and *Lactobacillus plantarum* Isolated from Milk

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In the era where developing antibiotic resistance is an emerging issue, probiotics are amongst the most promising therapeutic as well as prophylactic measures. One of the most commonly used probiotics in cheese, yoghurt, silage and preserved food such as pickles is lactobacilli. However, some studies have shown that some lactic acid bacteria (LAB) strains are resistant to antibiotics which pose a threat to human health. Hence, to evaluate the safety of *L. acidophilus* and *L. plantarum*, they were subjected to antibiotic susceptibility tests using the disc diffusion method against a total of 26 antibiotics. 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. The present study showed that antibiotic resistance is prevalent in different species of probiotic strains, which may pose a food safety concern. Hence, antibiotic sensitivity should be considered an important part of safety assessment for the evaluation of probiotics. Therefore, the current study concluded that antibiotic resistance is prevalent among *L. acidophilus* and *L. plantarum*, which is major concern of food safety. Furthermore, studies to evaluate the presence of antibiotic resistance genes in commercially available probiotics should be conducted. Antimicrobial susceptibility tests should be considered as an essential measure for the assessment of the safety of probiotics.

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 and are generally considered safe [1]. They are widely used in food production and biopreservation. Of all the genera of LAB, *Lactobacillus* is the most economically important microbiota, which is harbored mainly in the gut of man and animals [2]. They are beneficial for human health because of their antimicrobial and immunomodulatory activity [3]. 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 starter culture [4,5]. There have been numerous studies on the commercial production and use of *Lactobacillus*-based probiotics because of their 'generally recognized as safe' (GRAS) status [6].

Antibiotics are an important therapeutic tool in tackling many infectious diseases of bacterial origin [7]. 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 [8], which has become a global cause of health concern [9]. 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 [10]. 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) [11].

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 [12]. Several reviews [13] on LAB have recommended safety criteria for probiotics such as the absence of antibiotic resistance genes (ARGs) which are responsible for resistance to clinically important antibiotics [14]. Although LABs are regarded as safe, they can act as a reservoir for ARGs, which can persist in our food chain [15,16] and can be responsible for the transfer of ARGs via horizontal gene transfer [17]. Some studies have reported the transfer of ARGs between various lactobacilli [18]. *In vitro* and *in vivo* studies have also been conducted to evaluate the transfer of ARGs from lactic acid bacteria to other pathogenic bacteria [19,20]. 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. MATERIALS AND METHODS

2.1 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.

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*

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

2.2 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 against the procured probiotics. The details of the name of the drug, concentration, antibiotic group, and mode of action are described in Table 1.

2.2.1 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 [21]. 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 was 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 [11] (Table 2) as described by Sharma et al. [22].

2.3 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 [11]. Comparative evaluation of the diameter of the zone of inhibition was shown in graphical form (Fig. 1). The growths of both tested LAB strains i.e. *L. acidophilus* and *L. plantarum* were homogenous over MRS. The results for the reference LAB strains was documented in terms of resistant (R), susceptible (S) and intermediate (I) (Table 3).

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*.

Lactobacilli are generally considered susceptible to the cell wall synthesis inhibitors (β-Lactams) [15,23] and more resistant towards cephalosporins [24] which corroborated with our study. However, *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. [25] and Nawaz et al. [18]. In addition, *L. plantarum* showed intermediate susceptibility towards ampicillin/cloxacillin while *L. acidophilus* was susceptible to it. Similarly, resistance towards cephalosporins in our study for both strains was reported by Karapetkov et al. [24]. Lactobacilli were reported to have intrinsic resistance to aminoglycosides [26,22,27,28] which was similar to our study except for gentamicin and amikacin. In a study conducted by Pell et al. [29], they also observed the susceptibility of *L. plantarum* towards gentamicin.

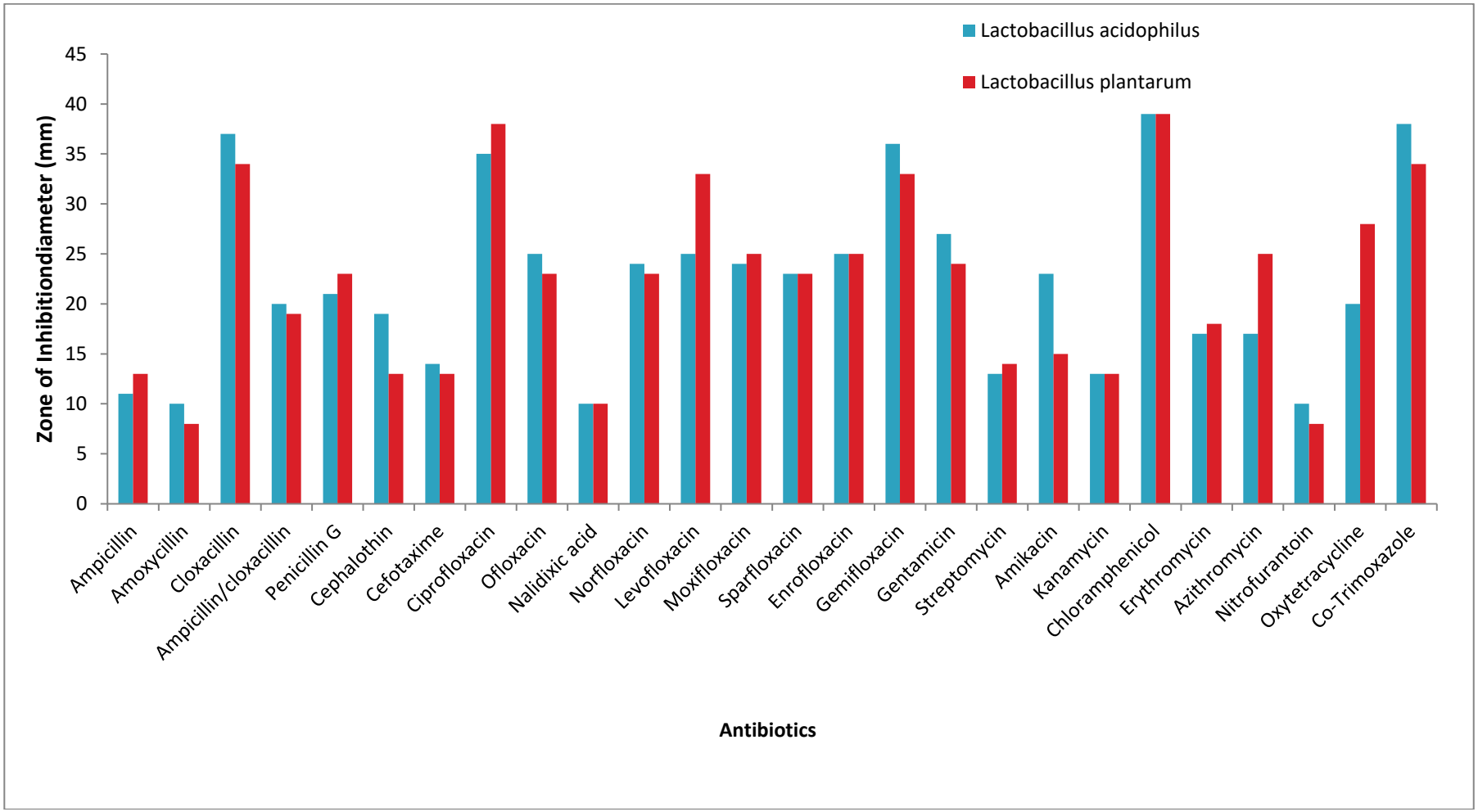

Fig. 1. Comparative evaluation of Diameter of zone of inhibition of *L. acidophilus* and *L. plantarum*

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)
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)

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 should be done to evaluate the transferability of genes responsible for resistance to most commonly used antibiotics.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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