Indian J Dairy Sci 77(6): 526-533
https://doi.org/10.33785/IJDS.2024.v77i06.006

RESEARCH ARTICLE

Microbiological, Biochemical, and Antibiotic susceptibility analysis of the lactic acid bacterial culture *Lactobacillus acidophilus* (MTCC No: 10307)

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Received: 13 June 2023 / Accepted: 23 November 2023 / Published online: 23 December 2024

Abstract: The link between health and nutrition has spurred a growing fascination with functional foods fortified with physiologically active constituents, delivering added advantages beyond fundamental nourishment. Probiotics, falling within the category of functional foods, are living microorganisms that impart health benefits when consumed in appropriate quantities. This research aimed to evaluate the susceptibility of *Lactobacillus acidophilus*, a widely used probiotic culture, to antibiotics. The probiotic culture's purity was examined via multiple microbiological examinations. The antibiotic susceptibility test was executed employing the standard disc diffusion assay based on the modified Kirby-Bauer technique. A total of thirty-six antibiotics underwent testing, and the outcomes were interpreted in line with recognized guidelines. Statistical analysis was carried out utilizing the One-way ANOVA approach. The results indicated the presence of innate resistance within certain antibiotic categories; however, this intrinsic resistance is typically non-transmissible and poses no significant risk. The findings of this investigation are set to enhance the comprehension of the safety and potential antibiotic resistance of lactic acid cultures utilized in various food products.

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Keywords: Lactobacillus, Probiotic, Safety, Antibiotic-susceptibility, Food

Introduction

There is an extensive focus on exploring the physiological benefits of various food items, which could potentially enhance overall well-being and mitigate the risk of chronic ailments. Recently, the connection between health and diet has sparked a surge of interest in nutritious foods. These functional foods are fortified with active components that include bioactive compounds containing phytochemicals, prebiotics, probiotics, vitamins, minerals, dietary fibers, fish oils, plant sterols, and oligosaccharides (Jan et al. 2023). According to the Food Safety and Standards Authority of India (Anonymous, 2017), functional foods, along with nutraceuticals, special dietary products, and health supplements, are defined as items that might contain plants, botanical extracts, as well as vitamins and minerals, either in their natural food form or in the form of powders, tablets, etc. (Tripathi and Giri, 2014). The discovery of the impact of probiotics is often attributed to Eli Metchnikoff, who correlated the robust physical health and longevity of Bulgarian peasants with their consumption of microorganisms found in yogurt, specifically *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. More contemporary definitions emphasize the preventive or therapeutic actions of probiotics. The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) define probiotics as 'live microorganisms that, when administered in sufficient quantities, provide a health benefit to the host'. In 2017, the Food Safety and Standards Authority of India (FSSAI) issued guidelines (Anonymous, 2017) on functional foods, defining probiotic foods as 'foods containing live microorganisms beneficial to human health, which, when consumed in adequate numbers either as a single strain or a combination of cultures, confer one or more specified or demonstrated health benefits in human beings.'

A variety of bacterial strains, including those from the genera *Lactobacillus, Streptococcus, Enterococcus*, and *Bifidobacterium*, are harnessed to produce probiotic food products. Particularly, the Lactic Acid Bacteria (LAB) group is

predominantly employed for this purpose. Typically, these bacteria ferment glucose, generating lactic acid, acetic acid, and hydrogen peroxide, along with diacetyl, low molecular weight antimicrobial substances, and bacteriocins. Acid production aids in reducing the pH of the gut, hindering the growth of pathogenic and putrefactive bacteria that generally thrive in an alkaline environment. Probiotics work to maintain a balanced intestinal flora, fortify the body against unwanted intruders, and safeguard against a leaky gut. Currently, probiotics are consumed globally through food, dietary supplements, or as active constituents of registered medications, and are available in diverse forms. However, it is imperative to ensure their safety, particularly concerning the potential spread of antibiotic resistance (ABR). Despite being a widely consumed bacterial group, the issue of antibiotic resistance in LAB has not received substantial attention compared to the growing concern over antibiotic resistance. Horizontal gene transfer between bacteria in nature and the subsequent dissemination of these resistant strains across populations is highly plausible (Arber, 2014). The past decade has witnessed a surge in reports documenting antibiotic resistance in LAB strains. While LAB are generally considered safe, there remains apprehension regarding the potential transference of resistance determinants to human and animal pathogenic and opportunistic bacteria. Some researchers acknowledge the presence of antibiotic resistance in LAB and endorse the possibility of their co-administration with antibiotic therapy, ensuring the restoration of a healthy gut flora, which is otherwise at risk (Kamath et al. 2023). Instances of resistance-coding genes and their transfer through plasmids and conjugative transposons have been documented in *Lactobacillus* species. Genes that confer resistance to various antimicrobials have been found on transferable genetic elements in several LABs (Kaszab et al. 2023). Consequently, there is a risk of transferring antibiotic resistance from probiotic strains to other bacteria, whether commensal or pathogenic, which could be detrimental. Reports have indicated resistance to current antibiotics in LAB from various commercially available dairy and food products. Therefore, it would be intriguing to assess the spectrum of resistance in cultivable microflora in human milk. Given that humans are routinely exposed to antibiotics, this exposure may influence the susceptibility/resistance profile of the human milk microflora, particularly the LAB group. The close contact of the native microbiota with the human intestine creates an ideal environment for the horizontal transfer of antimicrobial resistance genes facilitated by mobile genetic elements (Partridge et al. 2018). Consequently, routine antibiotic-resistance screening for starter and probiotic cultures is increasingly becoming a standard practice.

Materials and Methods

This study was conducted in the laboratories of the Department of Processing and Food Engineering, Punjab Agricultural University, Ludhiana, and the Department of Dairy Microbiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

Culture Procurement

The *Lactobacillus acidophilus* probiotic culture, identified as MTCC No: 10307, was obtained from the Institute of Microbial Technology (IMTECH) in Chandigarh. This probiotic culture has been designated a 'Generally Regarded as Safe' (GRAS) status.

Maintenance of culture

The freeze-dried form of the probiotic culture, *Lactobacillus acidophilus*, was sourced from the Institute of Microbial Technology (IMTECH) in Chandigarh. The culture was revived on de Man Rogosa and Sharpe (MRS) Media and cultivated for 24 hours at 37°C under aerobic conditions

Purity of probiotic culture

The probiotic culture's purity underwent assessment through a series of tests including microscopic examination, colony morphology, gram staining, and various biochemical evaluations.

Catalase test:

A small amount of culture from a single, isolated colony was obtained with a sterile loop and placed in the center of a clean microscopic slide. The slide was then exposed to a 3-5% hydrogen peroxide solution. The presence of bubbles or effervescence indicated a positive reaction.

Indole test:

The probiotic culture, *Lactobacillus acidophilus*, was introduced into 5ml of tryptone broth and left to incubate for 24 hours. Following this, 0.2-0.3 ml of Kovacs reagent was added to the 24-hour culture, allowing the yellow reagent to settle on the surface. A deep red color on the surface layer indicated a positive reaction.

Hydrogen sulphide test:

A tube of Triple Sugar Iron (TSI) Agar was streaked and then incubated for 24-48 hours. The presence of blackening due to H_2S production was observed.

Methyl red reaction:

The methyl red (MR) indicator was dissolved in 300ml of alcohol and diluted with distilled water to 500ml. The test culture was incubated in MR-VP broth for 48 hours at 37°C. Following this, 5 or 6 drops of the reagent were added to the culture and observed for color changes. A red color was interpreted as MR positive, while a yellow color indicated MR negative.

Urease test:

The urease agar media was inoculated with the probiotic culture, *Lactobacillus acidophilus*, and incubated for 24 hours at 37°C. The presence of urease caused the indicator's color to change from yellow to pink due to the formation of ammonia.

Carbohydrate fermentation test:

Different carbohydrates (Sorbitol, Lactose, Glucose, Dextrose, Mannitol, and Sucrose) were incorporated into phenol red medium. The medium was then transferred to test tubes containing Durham's tubes and autoclaved at 15 psi pressure (121°C) for 15 minutes. After sterilization, 0.1 ml of the bacterial culture was added to each sugar tube separately and incubated at 37°C. Results were noted after 24 hours. Acid production was indicated by a color change from red to yellow in positive cases, while gas formation was observed through the presence of gas bubbles in the Durham's tubes.

Antibiotic Susceptibility Test of the given culture

To examine the susceptibility of the probiotic culture, *Lactobacillus acidophilus*, to commonly used antibiotics, an antibiogram test was conducted. A total of thirty-six (36) antibiotics were acquired from Hi-media Laboratories Pvt. Ltd. in Mumbai, India for the experiment. Details regarding the drug concentrations, antibiotic groups, and modes of action can be found in Table 1.

Table 1: The concentration of various drugs, antibiotic group, and mode of action

S. no.	Antibiotic Susceptibility Test				
	Name of drug		Concentration (µg)	Antibiotic group	Mode of action
1	OX-1	Oxacillin	1	β-Lactams	Inhibitors of the cell wall synthesis
2	MET-5	Methicillin	5		
3	FAR-3	Faropenem	3		
4	CMZ-30	Cefmatazole	30		
5	CEP-30	Cephalothin	30		
6	AMP-10	Ampicillin	10		
7	P-10	Penicillin-G	10 units		
8	MRP-10	Meropenem	10		
9	AMC-30	Amoxyclav	30		
10	IPM-10	Imipenem	10		
11	CX-30	Cefoxitin	30		
12	CF-30	Ce-Factor	30	Cephalosporins	
13	CTX-30	Cefotaxime	30		
14	TEI-30	Teiceplanin	30	Glycopeptides	
15	VA-30	Vanomycin	30		
16	CXM-30	Cefuroxime	30	Second generation	
17	CAZ-30	Ceftazidime	30	Third generation	
18	GEN-10	Gentamycin	10	Aminoglycosides	
19	AK-30	Amikacin	30		
20	K-30	Kanamycin	30		
21	TOB-10	Tobramycin	10		
22	S-10	Streptomycin	10		
23	FC-10	Fusidic Acid	10	Fusidane	Inhibitors of protein synthesis
24	TGC-15	Tigecycline	15	Glycylcycline	
25	CD-2	Clindamycin	2	Lincosamide	
26	AZM-15	Azithromycin	15	Macrolides	
27	E-15	Erythromycin	15		
28	TE-30	Tetracyclin	30	Tetracyclines	
29	NIT-300	Nitrofurantoin	300	Other	
30	C-30	Chloramphenicol	30		
31	GAT-5	Gatifloxanin	5	Quinolones	Inhibiting DNA replication and transcription
32	OF-5	Ofloxacin	5		
33	CIP-5	Ciprofloxacin	5		
34	NA-30	Nalidixic Acid	30		
35	COT-25	Co-trimoxazale	25	Other	Folic acid synthesis inhibitors
36	TR-5	Trimethoprin	5		

The standard disc diffusion assay, following the modified Kirby–Bauer method (Bauer et al. 1966), was utilized to assess the antibiotic susceptibility pattern. The testing of antibiotic susceptibility was conducted using MRS agar medium. A broth culture (100 µl, 0.5 McFarland, equivalent to 10^8 CFU/ml) of the *Lactobacillus acidophilus* probiotic culture under examination was combined with 8 ml of soft agar, which was then layered over a pre-solidified agar plate. Subsequently, antibiotic discs were meticulously placed equidistant to each other with sterile forceps.

The plates were pre-incubated at room temperature (25°C) for 1 hour to ensure proper diffusion. Following this, they were incubated overnight at 37°C. The interpretation of results was carried out according to the standards set by the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines. In this regard, isolates exhibiting a zone of inhibition equal to or less than 14 mm were classified as resistant (R), those with a diameter greater than 20 mm were categorized as susceptible (S), and those with a zone of inhibition ranging between 15 and 19 mm were considered intermediate (I).

Statistical Analysis

Tests were conducted three times to ensure precision. The collected data underwent statistical analysis using the CPCS-1 software to apply One-way Analysis of Variance (ANOVA).

Results and Discussion

Microbiological examination

Lactobacillus acidophilus probiotic culture was cultivated on MRS media. The colonies exhibited a moderate size and appeared creamish-white in color as shown in Figure 1. They were raised with a smooth texture and flat elevation, characterized by an entire margin as shown in Table 2.

Lactobacillus acidophilus is Gram-positive, Rod-shaped (Figure-2), non-motile, and exhibited negative results for catalase, citrate utilization, H_2S formation, indole production, oxidase test, urease activity, and Voges-Proskauer reaction. These observations align with the findings documented by Hawaz (2014).

Carbohydrate fermentation test

Seven sugars (sorbitol, lactose, maltose, mannitol, dextrose, glucose, and sucrose) were used to test the ability of *Lactobacillus acidophilus* to ferment the given sugars as explained in Table 3.

According to the results of the carbohydrate fermentation test, *Lactobacillus acidophilus* effectively fermented various sugars (such as lactose, maltose, glucose, sucrose, and dextrose), leading to acid production, which consequently altered the color from red to yellow. However, the specific probiotic culture, *Lactobacillus acidophilus*, was incapable of fermenting sorbitol and mannitol. Furthermore, no bubbling was observed in the Durham tubes, indicating the absence of gas production. These findings affirmed the homo-fermentative characteristic of *Lactobacillus acidophilus* (MTCC 10307).

Antibiotic susceptibility test of the probiotic culture, *Lactobacillus acidophilus*

The results of the antibiotic susceptibility test were organized in Table 04, indicating the response of *Lactobacillus acidophilus* (MTCC No: 10307) to various antibiotics, classified as resistant (R), intermediate (I), or susceptible (S). *Lactobacillus acidophilus* exhibited sensitivity to tetracycline, imipenem, chloramphenicol, clindamycin, amoxyclav, fusidic acid, tigecycline, and erythromycin (macrolide). Conversely, it displayed resistance to methicillin (β-lactams), oxacillin, cefuroxime, cefoxitin, cefatazimide, gentamicin, co-trimoxale, nalidixic acid, kanamycin, feropenem, and cefmatazole. A mixed response was observed for certain antibiotics: resistance–intermediate pattern for ceftazidime, streptomycin, tobramycin, nitrofurantoin, and cefotaxime; sensitive–intermediate pattern for ofloxacin, azithromycin, gatifloxacin, teicoplanin, vancomycin, penicillin, meropenem, and ciprofloxacin; and resistance–intermediate–sensitive pattern for

Table 2: Morphological and Biochemical characterization of the given culture, *Lactobacillus acidophilus* (MTCC 10307)

Morphological Characters		Biochemical Tests	
Gram Staining	Gram +ve	Catalase Test	-
Type Of Colony	Large	Indole Test	-
Color	Non-Shiny, Creamish white in Color	Methyl Red Test	+
Margin	Entire	Uraese Test	-
Elevation	Flat	Voge'sProskauer Test	-
Opacity	Opaque	Citrate Utilisation	-
Growth On MRS	High Growth	Hydrogen Sulphide Production	-
Endospore Formation	Non-Endospore Forming	+ indicates positive result	
Motility	Non-Motile	- Indicates negative result	

Fig. 1 Colony morphology of *L. acidophilus*

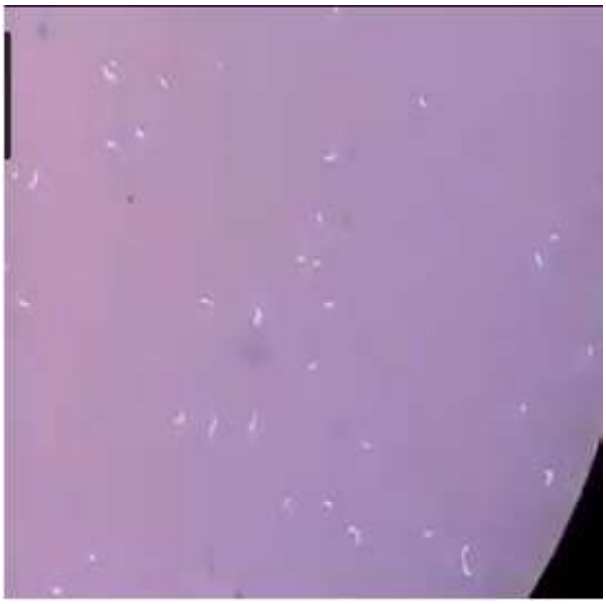

Fig. 2 Negative staining of *L. acidophilus* (40X magnification)

Table 3: Carbohydrate fermentation tests of the given culture, *Lactobacillus acidophilus* (MTCC 10307)

Sugar	Fermentation of Carbohydrates Acid Formation	Gas Formation (Indicated by Bubbling in Durham Tube)
Sorbitol	-	-
Lactose	+	-
Maltose	+	-
Dextrose	+	-
Mannitol	-	-
Sucrose	+	-
Glucose	+	-

+ indicates positive result - Indicates negative result

ampicillin, trimethoprim, and cephalexin. *Lactobacillus acidophilus* demonstrated resistance to 16 out of 36 antibiotics, intermediate susceptibility to 11 out of 36 antibiotics, and susceptibility to 9 out of 36 antibiotics as shown in Figure 3.

A general resistance was noted in *Lactobacillus acidophilus* against the antibiotic discs containing cephalosporins. Additionally, *Lactobacillus acidophilus* showed intermediate susceptibility towards glycopeptides (teicoplanin, vancomycin) and quinolones (ciprofloxacin, ofloxacin). Notably, within the aminoglycoside group, streptomycin and tobramycin exhibited a moderate inhibitory effect on the growth of *Lactobacillus acidophilus*. On the other hand, *Lactobacillus acidophilus* demonstrated sensitivity to chloramphenicol, erythromycin, and tigecycline. A general susceptibility of the tested LAB to β-lactams was also observed. Overall, these findings are consistent with previous studies on *Lactobacillus acidophilus* species. Studies by Sharma et al. (2016) previously reported susceptibility of different LAB species to penicillin and ampicillin. They also documented high sensitivity of *L. acidophilus* to meropenem. Resistance to β-lactams may be attributed to the presence of genes encoding for β-lactamases, which are known to transfer conjugally within different groups. Various studies have reported a high frequency of conjugation in different *Lactobacillus* species. Intermediate susceptibility to glycopeptides (teicoplanin, vancomycin) and quinolones (ciprofloxacin, ofloxacin) demonstrated by *Lactobacillus* in this study can be explained by the existence of intrinsic resistance mechanisms to both antibiotic families (Hawaz, 2014). Intrinsic resistance refers to the insensitivity of bacterial strains to approved drug doses, regulated by permeability barriers and active efflux. Such intrinsic resistance is typically non-transferable and poses no risk in LABs. Resistance to cephalosporins, a structural sub-type of β-lactam antibiotics, can be attributed to the presence of variants of broad-spectrum β-lactamases and the presence of efflux pumps associated with cell wall impermeability (Impey et al. 2020). Resistance to aminoglycosides can further be attributed to the absence of cytochrome-mediated electron transport, which enables antibiotic uptake. The susceptibility of *Lactobacillus* to both erythromycin and chloramphenicol has also been

documented by Jiang et al. (2016). Similarly, Jiang et al. (2016) showed an intermediate pattern of human milk *Lactobacilli* against nitrofurantoin and susceptibility to tetracycline. Anisimova et al. (2022) observed that *Lactobacillus* strains were susceptible to chloramphenicol, erythromycin, tetracycline, and clindamycin.

Dhillon et al. (2021) and Natt and Katyal (2022) separately developed probiotic mango and guava juice enriched with *Lactobacillus acidophilus* MTCC 10307, respectively, each with varying shelf lives. Reddy et al. experimented with muskmelon juice enriched with four lactic acid bacterial cultures, including *Lactobacillus acidophilus* MTCC 10307, while Siddiqui et al. (2023) worked on bacterial nano-conjugates derived from *Lactobacillus acidophilus* MTCC 10307, demonstrating potent anti-oxidant, anti-bacterial, and cytotoxicity activities. Furthermore, Pattnaik et al. (2022) used *Lactobacillus acidophilus* MTCC 10307 to produce flavoring phenolic compounds from sugarcane baggage, as identified by high-performance thin-layer chromatography, while Bhukya and Bhukya (2021) studied *Pediococcus pentosaceus* for its probiotic efficiency, using *Lactobacillus acidophilus* MTCC 10307 as a reference strain.

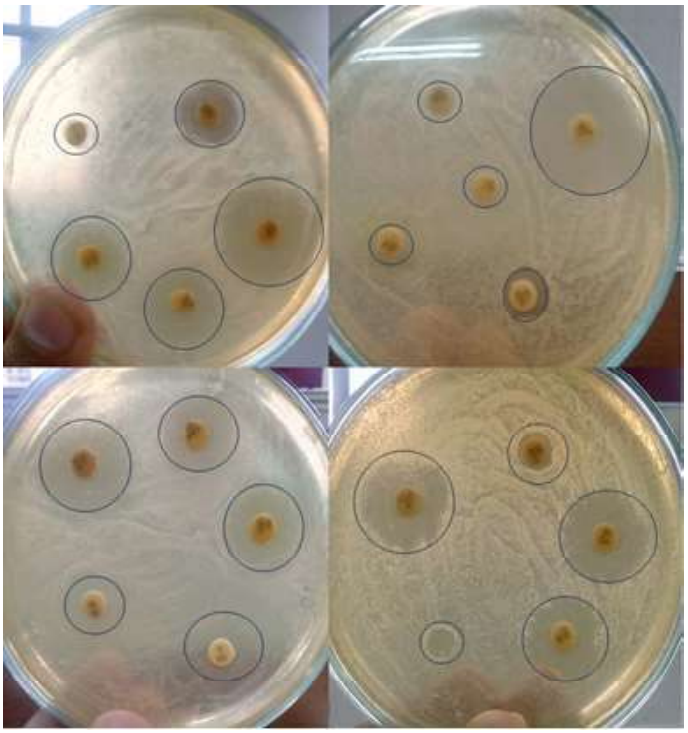

Fig. 3 Antibiotic discs showing zone of inhibition

Lactobacillus possesses a natural resistance to nucleic acid synthesis inhibitors, like trimethoprim and sulphonamides (co-trimoxazole). The combination of trimethoprim and co-trimoxazole has been widely used for various clinical conditions in humans since the late 1960s, mainly due to its cost-effectiveness, low toxicity, and high bactericidal activity, making it a preferable option, particularly in the developing world (Goldberg and Bishara 2012). The study emphasized the importance of inherent and non-transferrable resistance. Sharma et al. (2017) pointed out the

Table 4: Antibiotic susceptibility test of the probiotic culture, *Lactobacillus acidophilus* (MTCC No: 10307) in terms of resistant (R), intermediate (I) and susceptible (S)

S. no.	Name of drug		ZOI (Diameter in mm)	Interference
1	CAZ-30	Ceftazidime	11.3±1.15	R
2	OX-1	Oxacillin	10.3±0.58	R
3	CXM-30	Cefuroxime	10.7±0.58	R
4	CX-30	Cefoxitin	10±0	R
5	TR-5	Trimethoprin	11.7±2.89	R
6	GEN-10	Gentamycin	10.3±0.58	R
7	NA-30	Nalidixic Acid	10.3±0.58	R
8	MET-5	Methicillin	10.3±0.58	R
9	AK-30	Amikacin	10.7±1.15	R
10	K-30	Kanamycin	11±1	R
11	FAR-3	Faropenem	10.3±0.58	R
12	CMZ-30	Cefmatazole	12.3±2.08	R
13	COT-25	Co-trimoxazale	10.3±0.58	R
14	CF-30	Ce-Factor	10.7±1.15	R
15	TOB-10	Tobramycin	12±1	R
16	S-10	Streptomycin	12.7±0.58	R
17	NIT-300	Nitrofurantoin	16±3	I

18	CTX-30	Cefotaxime	16.3±3.21	I
19	MRP-10	Meropenem	16.7±2.08	I
20	CEP-30	Cephalothin	15.3±0.58	I
21	AMP-10	Ampicillin	15.3±0.58	I
22	AZM-15	Azithromycin	18.7±3.06	I
23	GAT-5	Gatifloxanin	16.7±1.15	I
24	OF-5	Ofloxacin	14.3±2.08	I
25	TEI-30	Teiceplanin	17.7±0.58	I
26	VA-30	Vanomycin	18±3.61	I
27	P-10	Penicillin-G	17±1.73	I
28	CIP-5	Ciprofloxacin	19.3±0.58	S
29	TGC-15	Tigecycline	19.3±2.08	S
30	FC-10	Fusidic Acid	23±1	S
31	AMC-30	Amoxyclav	21.3±2.08	S
32	C-30	Chloramphenicol	24.3±0.58	S
33	CD-2	Clindamycin	25±2.10	S
34	TE-30	Tetracyclin	25.3±1.15	S
35	E-15	Erythromycin	24.7±2.31	S
36	IPM-10	Imipenem	30.3±2.52	S

potential of the natural antimicrobial properties of LABs to work synergistically with antibiotic therapy in eliminating pathogenic strains.

Goldberg and Bishara (2012) examined the antimicrobial susceptibility of various *Lactobacillus acidophilus* strains from probiotics, nutritional foods, animals, and human sources, reporting susceptibility to certain antibiotics like penicillin, ampicillin, vanomycin, erythromycin, and clindamycin, while showing resistance to others including trimethoprim, gentamycin, fusidic acid, and chloramphenicol. Additionally, Anisimova et al. (2022) highlighted similar findings regarding the resistance and susceptibility of *Lactobacillus acidophilus* strains to specific antibiotics.

Conclusion

The freeze-dried form of the probiotic culture, *Lactobacillus acidophilus*, was acquired from IMTECH, Chandigarh. The maintenance of the culture was done on de Man Rogosa and Sharpe (MRS) media. Detailed analysis of the morphological and biochemical properties of the *Lactobacillus acidophilus* culture was performed.

On the MRS agar plate, the colonies exhibited moderate size, were creamish-white in color, raised, with an entire margin, slimy texture, and flat elevation. Biochemically, the culture displayed a positive methyl red test and exhibited acid formation from glucose. However, negative results were obtained for the catalase test, indole production test, urease test, Voges-Proskauer test, and citrate utilization test. In the carbohydrate fermentation tests, no bubbling was observed in Durham's tubes, indicating the homo-fermentative nature of *Lactobacillus acidophilus*. The culture was found capable of fermenting lactose, maltose, dextrose, and glucose, while no fermentation of sorbitol and mannitol was observed.

Antibiogram test was conducted to assess the susceptibility of the culture to commonly available antibiotics. Among all the antibiotic discs tested, imipenem, a β-lactam group antibiotic, demonstrated the most significant effectiveness. The results indicated the presence of intrinsic resistance within certain antibiotic families; however, this inherent resistance was found to be non-transferrable and posed no significant risk.

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