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Development and Evaluation of Vaginal gel Containing Anti-Fungal Agent in Combination with Probiotic Bacteria Without API

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ABSTRACT
Probiotic Lactobacillus strains have garnered significant interest due to their potential health benefits, including improving gut health and immune function. This study investigates the characteristics and growth of four subcultured Lactobacillus strains—Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, and Lactobacillus rhamnosus (LGG)—in various conditions. Growth patterns were monitored in MRS broth, and morphological, microscopic, and colony characteristics were recorded. The study further explores the viability of Lactobacillus strains under acidic pH and bile salt conditions, assessing the impact of these factors on their growth and survival. Results show that while Lactobacillus strains demonstrated considerable resilience in acidic environments (pH 1 and pH 3), their growth was notably affected by higher bile concentrations. L. rhamnosus exhibited the highest tolerance to bile salts, whereas L. plantarum showed the greatest resilience at low pH. The viable cell counts across all strains varied significantly under different conditions, providing valuable insight into the probiotic potential of these strains for applications in functional foods and supplements. The findings suggest that specific Lactobacillus strains possess unique traits, offering avenues for their use in formulations designed to survive harsh gastrointestinal conditions.

Keywords: Lactobacillus strains, Antioxidant activity, Microbial stability, Bile salt tolerance, Gut health

1. INTRODUCTION

The vaginal cavity, extending from the cervix to the external genitalia, serves as an important site for drug delivery, offering both local and systemic therapeutic benefits. Due to its high vascularity and direct connection to the bloodstream via the internal iliac veins, the vagina enables drugs to bypass first-pass metabolism, potentially enhancing bioavailability compared to oral administration. However, the vaginal microbiota plays a critical role in maintaining the vaginal health and can influence drug absorption. Therefore, drug formulations must be designed to avoid disrupting the normal microbiota and to respect the vaginal environment. A successful vaginal drug delivery system (VDDS) should meet several criteria: it must dissolve in the vaginal environment, be non-toxic and non-irritating, have appropriate physical properties (e.g., wetting, emulsifying, viscosity), and ensure prolonged contact with the mucosa for continuous drug delivery. Furthermore, the system should be patient-friendly, odorless, colorless, and not interfere with sexual activity, leakage, or cause irritation. By fulfilling these requirements, a VDDS can enhance therapeutic outcomes while promoting patient compliance.[1-3]

The development of vaginal drug delivery systems (VDDS) requires careful consideration of both aesthetic and functional qualities to optimize therapeutic efficacy and patient compliance. Future formulations are likely to include innovative systems such as liposomes, vaginal rings, cubic gels, and materials based on polystyrene and silicone elastomers. A promising area of research is the use of mucoadhesive polymers, which enhance drug retention and provide controlled, sustained release by adhering to the vaginal mucosa. These polymers improve bioadhesion, distribution, retention time, and release profiles, ensuring prolonged therapeutic effects. By focusing on these attributes, novel VDDS can improve therapeutic outcomes while maintaining user comfort and convenience.[4-6]

The development of a successful novel vaginal drug delivery system (VDDS) requires a comprehensive understanding of the physiological, anatomical, and microbiological features of the vaginal tract. The vaginal

epithelium is covered by a thin fluid film, composed of cervical mucus, gland secretions, transudates, leukocytes, and other fluids. In women of reproductive age, this fluid is produced at a rate of 3-4 grams every four hours, with a significant decrease post-menopause. The volume of vaginal secretions also increases during sexual arousal, influencing drug absorption and action. The viscosity, pH, and composition of this fluid can affect drug solubility and bioavailability, while the vaginal microbiota can further influence drug metabolism and efficacy. Understanding these factors is crucial for designing effective VDDS that optimize drug delivery and therapeutic outcomes.[7-10]

2. PROBIOTIC BACTERIA

Probiotics, derived from the Greek word meaning "for life," are live microorganisms that provide health benefits by enhancing the balance of intestinal flora, promoting "eubiosis" (a healthy microbial balance) and preventing "dysbiosis" (microbial imbalance). These beneficial bacteria, such as Lactobacillus and Bifidobacterium strains, contribute to digestive health, immune support, and the prevention of infections. Prebiotics, non-digestible food ingredients, selectively stimulate beneficial bacteria, while synbiotics combine both probiotics and prebiotics to maximize health benefits. Ideal probiotic characteristics include non-pathogenicity, stability in the gastrointestinal tract, and the ability to adhere to mucosal surfaces. Probiotics exert their effects through mechanisms like producing antibacterial compounds, competing for nutrients and adhesion sites, and stimulating immune responses. They are used to treat gastrointestinal disorders, infections, immune deficiencies, and atopic diseases. Probiotics are commercially available in various forms, including liquids, freeze-dried powders, capsules, and chewable tablets, and are produced through methods like subculturing, ultrafiltration, and freeze-drying.[11-15]

3. MATERIALS AND METHODS

1. Materials

Ampicillin discs, supplied by Dynamicro, Thane; Sodium Taurocholate (Bile Salt), supplied by High Media Labs, Pune; Carbomer 1342 (Batch no. CBP 1342-28), manufactured by Noveon, Mumbai; Carbomer 974 (Batch no. CC7DFBK158), manufactured by Noveon, Mumbai; HPMC K 15 M (Batch no. RH16012N12), supplied by Colorcon Asia Pvt. Ltd., Goa;; MRS Broth (Batch no. 369), supplied by High Media Labs, Pune; PEG 400 (Batch no. NL 2192 6312 V), supplied by Qualigens Fine Chemicals, Mumbai; and Polycarbophil (Batch no. PCP 181-52), supplied by Noveon, Mumbai, and Emcure Pharmaceuticals, Pune. The strain names and their corresponding NCIM/MTCC numbers are as follows: L. plantarum with NCIM No. 2083, and L. casei with NCIM No. 2125, both supplied by the National Collection of Industrial Microorganisms (NCIM), Pune. The cell lines and their descriptions are as follows: Caco-2, a human intestinal epithelial cell line, supplied by the National Centre for Cell Science (NCCS), Pune, and A-431, a human skin carcinoma squamous cell line.

2. Methods:

i. Procurement and characterization of probiotic cultures:

The authentic cultures of Lactobacillus plantarum, Lactobacillus casei and Lactobacillus acidophilus were procured from National Collection of Industrial Microorganisms, NCIM, Pune and Lactobacillus rhamnosus was procured from Microbial Type Culture Collection Bank, MTCC, and Chandigarhas lyophilised powders.

ii. Composition of de Man,Rogosa and Sharpe (MRS) broth:

The broth is prepared with 20.00 g/L dextrose, 10.00 g/L beef extract, 10.00 g/L protease peptone, and 5.00 g/L each of sodium acetate and yeast extract, along with 2.00 g/L ammonium citrate, 2.00 g/L dipotassium phosphate, and 1.00 g/L polysorbate-80. Additionally, 0.10 g/L magnesium sulfate and 0.05 g/L manganese sulfate are included, with the final pH adjusted to 6.5 ± 0.2 at 25°C.

iii. Preparation of MRS broth medium:[16]

MRS broth was prepared by accurately weighing the required quantity of MRS powder, dissolving it in a minimal amount of distilled water, and adjusting the final volume to 100 mL. It was sterilized by autoclaving at 121°C for 15 minutes under moist heat and pressure, then cooled and stored for future use..

iv. Subculturing of pure cultures of Lactobacillus strains:[16]

To subculture lyophilized Lactobacilli cultures, MRS broth and agar are prepared and sterilized, followed by inoculation onto petriplates using the four-quadrant streaking method. The plates are incubated in sealed glass chambers at 25-30°C for 24 hours to allow the bacteria to grow and form visible colonies.

v. Inoculation of Lactobacilli in MRS broth:[17]

A loopful of the four bacterial strains subcultured previously were inoculated aseptically in separate four sets of 10 X 50 ml sterile MRS broth quantities contained in conical flasks. These flasks were tightly closed with cotton plugs and were shaken on laboratory shaker for 48 hrs (25-30^{0}C ^{0} C)

vi. Determination of viable count of lyophilized powders of Lactobacilli: -

Procedure for serial dilution technique:[17]

The procedure involves transferring 5 mg of lyophilized Lactobacilli into test tubes with sterile distilled water, followed by serial dilutions and plating onto MRS agar plates. After incubating the plates at 37°C for 24 hours, the colonies are counted to determine the concentration of Lactobacilli in the original suspension based on the dilution factor.

vii. Determination of acid tolerance of lyophilized powders of Lactobacilli strains:

To determine the viable bacterial cell count (CFU) for individual Lactobacilli cultures, the procedure began with the preparation and sterilization of MRS broth. Two sets of 50 ml of MRS broth were prepared, one set buffered to pH 1 and pH 3 using 1.0 N HCl, while the other set remained unadjusted (control). After sterilization, 5 mg of lyophilized powder from each individual Lactobacillus culture was aseptically transferred into separate conical flasks containing the respective MRS broth. These cultures were then incubated at 37°C ± 10°C for varying time intervals of 0 hours, 2 hours, and 4 hours. Following incubation, the viable bacterial count in each flask was determined using the Standard Plate Count (SPC) method. The test was carried out in duplicate to ensure accuracy and reliability of the results.

viii. Determination of bile tolerance of lyophilized powders of Lactobacilli strains:

To determine the viable bacterial count of Lactobacilli cultures, MRS broth with different bile concentrations (0.3%, 1%, and a control) was prepared and sterilized. After incubating the cultures at 37°C for various time points, the viable bacterial count was determined using the Standard Plate Count method, performed in duplicate for accuracy.

ix. Determination of adhesion of Lactobacilli to cell lines:

Materials:

The bacterial strains used include Lactobacillus plantarum (NCIM No. 2083), Lactobacillus casei (NCIM No. 2125), Lactobacillus acidophilus (NCIM No. 2285), and Lactobacillus rhamnosus (MTCC No. 1408B). The study utilized MRS broth, Eagle's 1959 medium, PBS pH 7.2, formaldehyde, crystal violet stain, and cell lines Caco-2 and A-431, maintained at 37°C in a CO2 incubator, using equipment like 12-well tissue culture plates and a microscope.

Table 1: Composition of Eagle's 1959 medium (MEM) for cell lines:

Sr. No.	Ingredients	Quantity
1	L-glutamine	traces
2	Sodium bicarbonate	adj.of pH
3	Fetal calf serum	5%
4	Antibiotics	-
a	Penicillin	100 UI/ml
b	Streptomycin	100 UI/ml
c	Neomycin	200 mg/ml

Methods:[16]

a. Slicing of cell lines:

The process begins with the cutting of 24-hour-old cell lines on tissue culture plates containing MEM (Modified Eagle's Medium) at a concentration of 1 x 10^5 cells/ml. The cell lines are then washed twice with PBS (phosphate-buffered saline) at pH 7.2 to remove any residual medium or contaminants. After washing, the cell lines are incubated in a CO2 incubator at 37°C for 24 hours to allow for further growth and proper cellular activity before proceeding with any subsequent experiments.

b. Preparation of Lactobacilli cultures:[16]

The procedure began with the inoculation of individual Lactobacilli strains separately into MRS broth, as well as the addition of marketed Lactobacilli tablet contents to separate MRS broth samples. These cultures were then incubated in a CO2 incubator at 37°C for 18 hours to allow for bacterial growth. After this initial incubation, the bacterial cultures were transferred into fresh MRS medium to promote further growth and rejuvenation. The cultures were again incubated at 37°C in the CO2 incubator for an additional 24 hours.

c. Procedure of test for cell adhesion

The procedure began with the transfer of Lactobacilli cultures onto the incubated cell lines. The 24-hour-old Lactobacilli cultures were first diluted using a 1:1 mixture of MRS broth and MEM, and the bacterial concentration was adjusted to 10^8 CFU/ml. This prepared bacterial suspension was then inoculated into the wells containing the cell lines. The inoculated cell lines were incubated in a CO2 incubator at 37°C for 30 minutes to allow the bacteria to adhere to the cells. After incubation, the wells were washed with PBS (4×100 µl) to remove any unbound bacteria. The cell lines in the wells were then fixed using 3.7% v/v formaldehyde for 1 hour. Following fixation, the wells were washed again with PBS (2×100 µl) to remove excess formaldehyde. The bacterial cells adhered to the cell lines were then subjected to Gram staining, and the slides were examined microscopically at 100x magnification using an oil immersion lens.

4. PREPARATION AND EVALUATION OF PLAIN GEL BASES[17-19]

i. Method of preparation:

Required quantities of gelling agents viz Carbomer 1342, 974 and Polycarbophil were soaked in minimum amount of distilled water for 2 to 3 hrs. (Phase I). Ethanol (99.9%), PEG 400 and propylene glycol were mixed in appropriate proportions. (Phase II).

The Phase II was added slowly to the (phase I) while stirring with help of overhead stirrer. The dispersion of gelling agents were neutralized by drop wise addition of triethanolamine (TEA) with continuous stirring. The final weight (gm) was make up using more amount of distilled water.

pH of all formulations was maintained in the range of 4.0 to 4.5.

ii. Preparation of plain gels using Carbomers viz Carbopol 1342, Carbopol 974 and Polycarbophil

In Phase I, Carbopol 1342, Carbopol 974, or Polycarbophil were soaked in distilled water for 2-3 hours. In Phase II, ethanol, PEG 400, and propylene glycol were mixed in appropriate proportions. The Phase II mixture was then slowly added to Phase I while stirring, followed by neutralization of the gel base with triethanolamine (TEA). Finally, the gels were stored for further evaluation (Scheme No. 1).

Table 2. Formulation of plain gel bases

Sr.No.	Formulation ingredients	Quantities (gm)
1	Carbopol 1342/974/Polycarbophil	0.5/1/1.5
2	Ethanol (99.9%)	20.00
3	PEG 400	20.40
4	Propylene glycol	15.52
5	Triethanolamine	q.s. for pH adjustment
6	Distilled water	q.s. to 100 gm

These plain gel bases were evaluated for following parameters;

- Appearance
- pH
- Viscosity
- Spreadability

5. Characterization of gels:[20]

Physical characteristics:

i) Appearance:

All the gel preparations were examined for homogeneity, color, odour, texture, and general appearance.

ii) pH:

The pH meter was calibrated with standard buffer solutions (pH 4, pH 7, and pH 9) before use. Then, the pH of 1-2 gm of each gel was measured using the calibrated pH meter.

iii. Spreadability:

The spreadability of gel formulations was tested using a device with two glass slides, where 0.5 gm of each gel was placed between the slides, and a 100 gm weight was applied for 1-2 minutes. After removing the weight and applying a 5 gm pull, the time taken for the slide to travel 6.5 cm was recorded to assess the gel's spreadability.

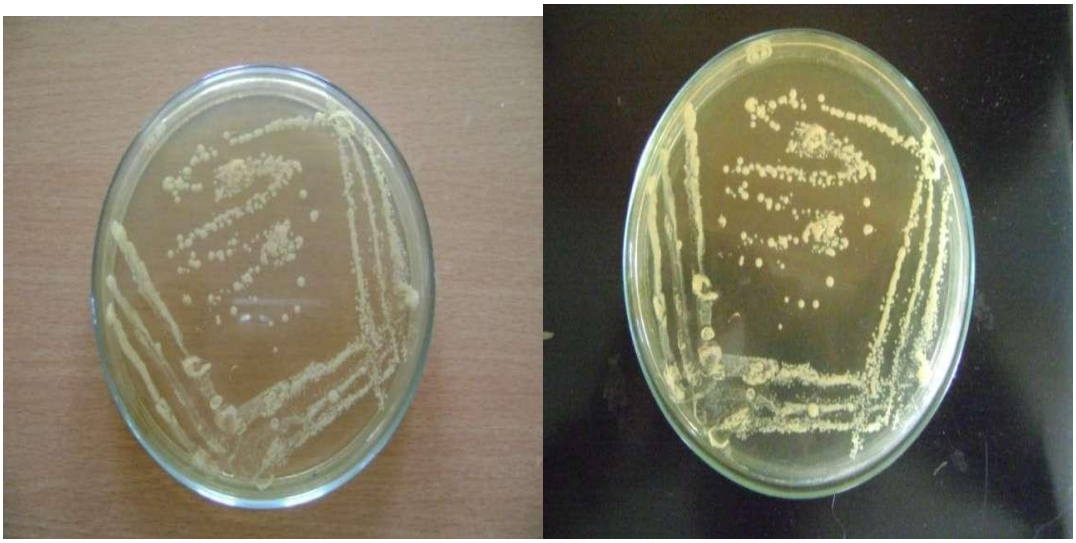
iv. Viscosity (cps):

The viscosity of gel formulations was measured using a Brookfield viscometer with a helipath assembly and Spindle No. S 93, where 50 gm of each gel was tested at varying speeds. Viscosity (centipoises) and torque percentage were recorded at each speed, providing insights into the gel's rheological behavior, shear-thinning or thickening properties, and flow characteristics.

6. RESULT AND DISCUSSION

1. Characteristics of probiotic cultures:

Subcultured Lactobacillus strains:

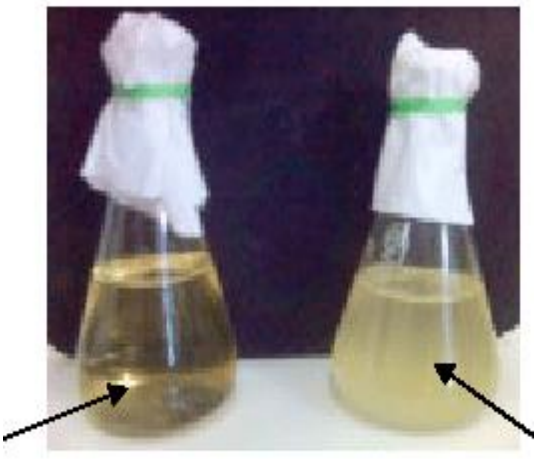


a. **L. plantarum** **b. L. Casei**

c. L. acidophilusd. L. rhamnosus (LGG)

Fig 1: Subcultured Lactobacillus strains.

2. Growth of Lactobacillus strains in MRS broth:



Clear medium turbid medium

Before inoculation after inoculation

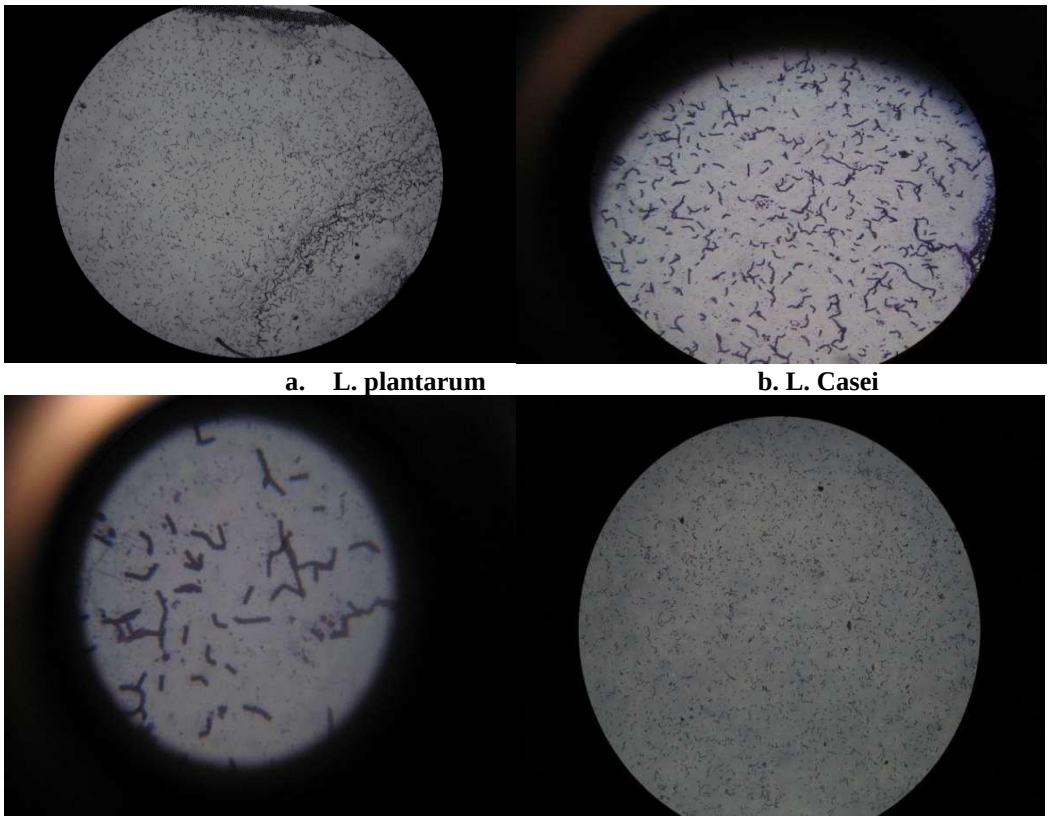
Fig 2: Subcultured Lactobacillus strains

3. Characteristics of Lactobacilli strains

A. Morphological characteristics of Lactobacilli strains

Fig 3: Morphological characteristics of Lactobacilli strains

A. Microscopic characteristics of Lactobacilli strains:



a. L. plantarum **b. L. Casei**

c. L. acidophilus **d. L. rhamnosus (LGG)**

Fig 4: Microscopic characteristics of Lactobacilli strains

Colony characteristics of Lactobacillus strains observed Macroscopically and microscopically

C- i. viable cell count of lyophilized powders (5mg) of Lactobacilli

Table 3 : Viable cell count of Lactobacilli

Characteristics of colony	Lactobacillus plantarum	Lactobacillus casei	Lactobacillus acidophilus	Lactobacillus rhamnosus
Size	2.8-3 mm	3 mm	2.7-3 mm	2.6-3 mm

Shape	Round	Round	Round	Round/Circular
Colour	Light yellow	White to gray	White to gray	White to gray
Margin	Entire	Entire	Entire	Entire
Elevation	Convex	Raised	Flat	Convex
Consistency	Mucoid/ smooth	Mucoid/ smooth	Mucoid/ smooth	Mucoid/ smooth
Opacity	Opaque	Opaque	Opaque	Opaque
Gram Staining	Gram +ve, short rods	Gram +ve, short rods	Gram +ve, short rods	Gram +ve, short rods
Motility	Motile	Motile	Motile	Motile

Table 4. Viable cell count of Lactobacillus plantarum.

Plate No.	**Dilution (a)**	**No. of colonies (b)**	**Corresponding no. of microorganisms (a×b)**	**Average cell count (approx.)**
6	10^6	dense growth	-	10^9-10^{10}
8	10^8	270	27×10^{10}	
9	10^9	31	31×10^9	
10	10^{10}	6	6×10^{10}	

Table 5. Viable cell count ofL. casei.

Plate No.	**Dilution (a)**	**No. of colonies (b)**	**Corresponding no. of microorganisms (a×b)**	**Average cell count (approx.)**
6	10^6	dense growth	-	10^9-10^{10}
8	10^8	240	24×10^9	
9	10^9	42	42×10^9	
10	10^{10}	3	3×10^{10}	

Table 6. Viable cell count of L. acidophillus**.**

Plate No.	**Dilution (a)**	**No. of colonies (b)**	**Corresponding no. of microorganisms (a×b)**	**Average cell count (approx.)**
6	10^6	dense growth	-	10^9-10^{10}
8	10^8	290	29×10^9	
9	10^9	65	65×10^9	
10	10^{10}	9	9×10^9	

Table 7. Viable cell count of L. rhamnosus.

Plate No.	**Dilution (a)**	**No. of colonies (b)**	**Corresponding no. of microorganisms (a×b)**	**Average cell count (approx.)**
6	10^6	dense growth	-	10^9-10^{10}
8	10^8	160	16×10^9	
9	10^9	35	35×10^9	
10	10^{10}	7	7×10^{10}	

ii. Effect of acidic pH on viable cell counts of individual Lactobacilli species:

Table 10: a. L. plantarum

Incubation time (hrs)	**Serial dilution (a)**	**No. of CFU in MRS (b)**			**Total No. of Microorganisms in MRS (axb)**			**Average count of CFU**
		Control	**pH 1**	**pH 3**	**Control**	**pH 1**	**pH 3**	10^8-10^9

0	10^4	960	793	910	960	793	910	
	10^6	390	362	320	390	362	320	
	10^8	80	75	60	80	75	60	
	10^4	936	854	886	936	854	886	
	10^6	354	290	302	354	290	302	
	10^8	86	65	62	86	65	62	10^8-10^9
2	10^4	968	680	902	968	680	902	
	10^6	396	256	312	396	256	312	
	10^8	92	35	22	92	35	22	
	10^4	940	700	836	940	700	836	
	10^6	362	220	310	362	220	310	
	10^8	88	27	45	88	27	45	10^8-10^9
4	10^4	966	540	812	966	540	812	
	10^6	380	125	282	380	125	282	
	10^8	78	8	27	78	8	27	
	10^4	922	510	812	922	510	812	
	10^6	392	100	254	392	100	254	
	10^8	62	11	22	62	11	22	

*** Note:** The plating procedure was carried out in duplicate.

Table 11: b. L. casei

Incubation time (hrs)	**Serial dilution (a)**	**No. of CFU in MRS (b)**			**Total No. of Microorganisms in MRS (axb)**			**Average count of CFU**
		Control	**pH 1**	**pH 3**	**Control**	**pH 1**	**pH 3**	
0	10^4	760	720	758	760	720	758	10^8-10^9
	10^6	212	390	226	212	390	226	
	10^8	40	78	44	40	78	44	
	10^4	630	648	650	630	648	650	
	10^6	226	410	230	226	410	230	
	10^8	46	60	50	46	60	50	10^8-10^9
2	10^4	764	660	752	764	660	752	
	10^6	212	264	222	212	264	222	
	10^8	52	45	42	52	45	42	
	10^4	642	430	654	642	430	654	
	10^6	236	265	228	236	265	228	
	10^8	54	39	46	54	39	46	10^8-10^9
4	10^4	680	510	748	680	510	748	
	10^6	242	145	218	242	145	218	
	10^8	60	16	40	60	16	40	
	10^4	650	280	652	650	280	652	
	10^6	238	132	222	238	132	222	
	10^8	50	09	39	50	09	39	

*** Note:** The plating procedure was carried out in duplicate.

Table 12: c. L. acidophilu

Incubation time (hrs)	Serial dilution (a)	No. of CFU in MRS (b)			Total No. of Microorganisms in MRS (axb)			Average count of CFU
		Control	pH 1	pH 3	Control	pH 1	pH 3	
0	10^4	820	642	836	820	642	836	10^8-10^9
	10^6	264	380	296	264	380	296	
	10^8	64	62	56	64	62	56	
	10^4	844	810	230	844	810	230	
	10^6	252	405	250	252	405	250	
	10^8	54	52	55	54	52	55	10^8-10^9
2	10^4	824	510	812	824	510	812	
	10^6	268	212	281	268	212	281	
	10^8	71	24	44	71	24	44	
	10^4	846	725	212	846	725	212	
	10^6	256	376	234	256	376	234	
	10^8	54	36	41	54	36	41	10^8-10^9
4	10^4	836	410	800	836	410	800	
	10^6	269	98	262	269	98	262	
	10^8	75	-	30	75	-	30	
	10^4	848	590	200	848	590	200	
	10^6	262	275	110	262	275	110	
	10^8	60	09	30	60	09	30	

*** Note:** The plating procedure was carried out in duplicate.

Table 13: d. L. rhamnosus (LGG)

Incubation time (hrs)	Serial dilution (a)	No. of CFU in MRS (b)			Total No. of Microorganisms in MRS (axb)			Average count of CFU
		Control	pH 1	pH 3	Control	pH 1	pH 3	
0	10^4	920	672	942	920	672	942	10^8-10^9
	10^6	390	352	380	390	352	380	
	10^8	78	72	82	78	72	82	
	10^4	896	630	885	896	630	885	
	10^6	410	340	405	410	340	405	
	10^8	60	66	62	60	66	62	10^8-10^9
2	10^4	926	510	940	926	510	940	
	10^6	394	264	376	394	264	376	
	10^8	81	38	75	81	38	75	
	10^4	896	526	882	896	526	882	
	10^6	414	217	400	414	217	400	
	10^8	70	41	58	70	41	58	10^8-10^9
4	10^4	932	430	938	932	430	938	
	10^6	402	125	366	402	125	366	
	10^8	86	04	64	86	04	64	
	10^4	898	412	878	898	412	878	
	10^6	424	97	396	424	97	396	
	10^8	76	13	54	76	13	54	

*** Note:** The plating procedure was carried out in duplicate.

Table 14: Average count of Lactobacilli cells in acidic pH.

Sr. No.	Incubation time (hrs)	No. of CFU in MRS (b)			Total No. of Microorganisms in MRS (axb)			Average count of CFU
		Control (pH>)	pH 1	pH 3	Control (pH>)	pH 1	pH 3	
a	0	80	75	60	80	75	60	pH1: 10^7-10^8 & pH3: 10^8-10^9
		86	65	62	86	65	62	
	4	78	8	27	78	8	27	
		62	11	22	62	11	22	
b	0	40	78	44	40	78	44	
		46	60	50	46	60	50	
	4	60	16	40	60	16	40	
		50	09	39	50	09	39	
c	0	64	62	56	64	62	56	
		54	52	55	54	52	55	
	4	75	-	30	75	-	30	
		60	09	30	60	09	30	
d	0	78	72	82	78	72	82	
		60	66	62	60	66	62	
	4	86	04	64	86	04	64	
		76	13	54	76	13	54	

*** Note:** The plating procedure was carried out in duplicate. Effect of acidic pH has been noted at dilution factor of 10^8

Table 15: Effect of bile salts on viable cell counts of individual Lactobacilli species: a) L. plantarum

Incubation time (hrs)	Serial dilution (a)	No. of CFU in MRS (b)			Total No. of Microorganisms in MRS (axb)			Average count of CFU
		Control without bile salts	0.3% w/v	1% w/v	Control without bile salts	0.3% w/v	1% w/v	
0	10^4	110	130	121	110	130	121	10^8-10^9
	10^6	72	74	84	72	74	84	
	10^8	38	36	40	38	36	40	
	10^4	98	92	86	98	92	86	
	10^6	64	61	54	64	61	54	
	10^8	42	39	37	42	39	37	10^8-10^9
3	10^4	122	116	214	122	116	214	
	10^6	70	61	73	70	61	73	
	10^8	36	33	38	36	33	38	
	10^4	90	82	98	90	82	98	
	10^6	58	51	66	58	51	66	
	10^8	39	31	44	39	31	44	10^8-10^9
6	10^4	221	120	112	221	120	112	
	10^6	80	66	58	80	66	58	
	10^8	41	34	28	41	34	28	
	10^4	101	88	79	101	88	79	
	10^6	71	57	46	71	57	46	
	10^8	45	38	26	45	38	26	
24	10^4	222	116	107	222	116	107	10^8-10^9
	10^6	86	64	52	86	64	52	
	10^8	42	30	21	42	30	21	
	10^4	109	86	73	109	86	73	
	10^6	75	56	42	75	56	42	
	10^8	45	32	18	45	32	18	
48	10^4	228	113	104	228	113	104	10^8-10^9

	10^6	91	59	50	91	59	50	
	10^8	44	28	16	44	28	16	
	10^4	114	82	64	114	82	64	
	10^6	76	53	39	76	53	39	
	10^8	48	30	14	48	30	14	

*** Note:** The plating procedure was carried out in duplicate.

Table 16: b) L. casei

Incubation time (hrs)	**Serial dilution (a)**	**No. of CFU in MRS (b)**			**Total No. of Microorganisms in MRS (axb)**			**Average count of CFU**
		Control without bile salts	**0.3% w/v**	**1% w/v**	**Control without bile salts**	**0.3% w/v**	**1% w/v**	
0	10^4	178	162	154	178	162	154	10^8-10^9
	10^6	94	86	91	94	86	91	
	10^8	22	26	20	22	26	20	
	10^4	122	140	134	122	140	134	
	10^6	84	83	94	84	83	94	
	10^8	28	24	22	28	24	22	10^8-10^9
3	10^4	178	161	151	178	161	151	
	10^6	96	82	89	96	82	89	
	10^8	22	26	20	22	26	20	
	10^4	126	138	134	126	138	134	
	10^6	85	80	91	85	80	91	
	10^8	28	23	21	28	23	21	10^8-10^9
6	10^4	182	158	146	182	158	146	
	10^6	98	79	82	98	79	82	
	10^8	23	24	18	23	24	18	
	10^4	130	133	130	130	133	130	
	10^6	88	76	81	88	76	81	
	10^8	30	21	17	30	21	17	
24	10^4	185	155	140	185	155	140	10^8-10^9
	10^6	101	72	81	101	72	81	
	10^8	26	24	12	26	24	12	
	10^4	132	130	121	132	130	121	
	10^6	91	72	75	91	72	75	
	10^8	32	20	09	32	20	09	
48	10^4	187	149	136	187	149	136	10^8-10^9
	10^6	104	68	77	104	68	77	
	10^8	28	21	07	28	21	07	
	10^4	135	127	117	135	127	117	
	10^6	94	65	71	94	65	71	
	10^8	36	18	06	36	18	06	

*** Note:** The plating procedure was carried out in duplicate.

Table 17: c) L. acidophilus

Incubation time (hrs)	**Serial dilution (a)**	**No. of CFU in MRS (b)**			**Total No. of Microorganisms in MRS (axb)**			**Average count of CFU**
		Control without bile salts	**0.3% w/v**	**1% w/v**	**Control without bile salts**	**0.3% w/v**	**1% w/v**	
0	10^4	124	112	120	124	112	120	10^8-10^9
	10^6	76	66	66	76	66	66	
	10^8	42	39	44	42	39	44	
	10^4	202	163	181	202	163	181	

	10^6	144	94	78	144	94	78	
	10^8	51	41	43	51	41	43	
3	10^4	125	108	116	125	108	116	10^8-10^9
	10^6	77	62	64	77	62	64	
	10^8	42	35	36	42	35	36	
	10^4	204	154	173	204	154	173	
	10^6	144	88	70	144	88	70	10^8-10^9
	10^8	50	36	31	50	36	31	
6	10^4	127	104	112	127	104	112	
	10^6	80	58	59	80	58	59	
	10^8	40	30	26	40	30	26	
	10^4	208	149	168	208	149	168	
	10^4	204	154	173	204	154	173	
	10^6	144	88	70	144	88	70	
	10^8	50	36	31	50	36	31	
24	10^4	131	101	108	131	101	108	
	10^6	84	51	53	84	51	53	10^8-10^9
	10^8	46	19	20	46	19	20	
	10^4	210	141	163	210	141	163	
	10^6	153	74	60	153	74	60	
	10^8	53	14	19	53	14	19	
48	10^4	136	134	100	136	134	100	
	10^6	91	41	41	91	41	41	
	10^8	47	21	17	47	21	17	10^8-10^9
	10^4	214	133	152	214	133	152	
	10^6	155	68	53	155	68	53	
	10^8	56	19	08	56	19	08	

*** Note:** The plating procedure was carried out in duplicate.

Table 18.: d) L. rhamnosus (LGG)

Incubation time (hrs)	**Serial dilution (a)**	**No. of CFU in MRS (b)**			**Total No. of Microorganisms in MRS (axb)**			**Average count of CFU**
		Control without bile salts	**0.3% w/v**	**1% w/v**	**Control without bile salts**	**0.3% w/v**	**1% w/v**	
0	10^4	98	88	91	98	88	91	
	10^6	64	48	54	64	48	54	
	10^8	23	28	27	23	28	27	10^8-10^9
	10^4	102	104	74	102	104	74	
	10^6	69	76	49	69	76	49	
	10^8	25	26	24	25	26	24	
3	10^4	98	86	90	98	86	90	
	10^6	65	48	52	65	48	52	10^8-10^9
	10^8	23	27	27	23	27	27	
	10^4	104	102	71	104	102	71	
	10^6	71	70	42	71	70	42	
	10^8	26	24	23	26	24	23	
6	10^4	101	84	85	101	84	85	
	10^6	68	44	46	68	44	46	10^8-10^9
	10^8	24	25	24	24	25	24	
	10^4	108	99	62	108	99	62	
	10^6	75	68	38	75	68	38	
	10^8	27	22	20	27	22	20	
24	10^4	106	81	81	106	81	81	
	10^6	67	40	42	67	40	42	10^8-10^9
	10^8	26	23	21	26	23	21	
	10^4	111	95	57	111	95	57	

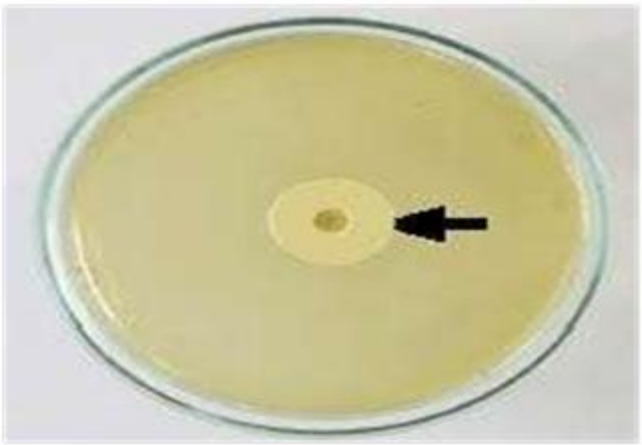
	10^6	78	64	31	78	64	31	
	10^8	27	20	18	27	20	18	
48	10^4	112	74	79	112	74	79	10^8-10^9
	10^6	70	31	38	70	31	38	
	10^8	28	21	17	28	21	17	
	10^4	116	90	52	116	90	52	
	10^6	82	61	28	82	61	28	
	10^8	28	18	14	28	18	14	

*** Note:** The plating procedure was carried out in duplicate.

Table 19: Average count of Lactobacilli cells after exposure to bile salts

Sr. No.	Incubation time (hrs)	No. of CFU in MRS (b)			Total No. of Microorganisms in MRS (axb)			Average count of CFU
		Control without bile salts	0.3% w/v	1% w/v	Control without bile salts	0.3% w/v	1% w/v	
a	0	38	36	40	38	36	40	pH1: 10^7-10^8 & pH3: 10^8-10^9
		42	39	37	42	39	37	
	48	44	28	16	44	28	16	
		48	30	14	48	30	14	
b	0	22	26	20	22	26	20	
		28	24	22	28	24	22	
	48	10^8	28	21	07	28	21	
		36	18	06	36	18	06	
c	0	42	39	44	42	39	44	
		51	41	43	51	41	43	
	48	47	21	17	47	21	17	
		56	19	08	56	19	08	
d	0	23	28	27	23	28	27	
		25	26	24	25	26	24	
	48	28	21	17	28	21	17	
		28	18	14	28	18	14	

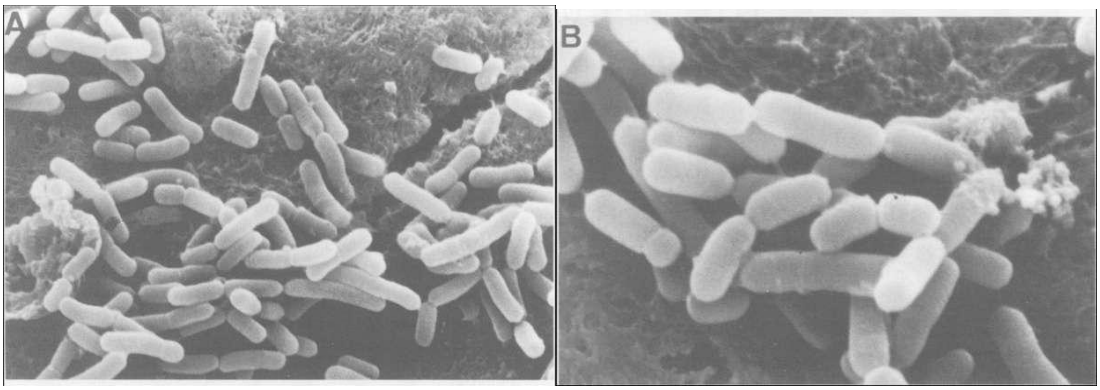
Table 20. Standard zone size interpretation data for Ampicillin



Sensitivity of Lactobacilli	Diameter of zone of inhibition (mm)
Resistant	≤11
Moderate	12 – 13
Sensitive	≥ 14

*** Note:** Values reported on the label of Ampicillin discs manufactured by **Dynmicro, Thane.**

v. Adhesion of Lactobacilli to cell lines:



a. A-431 **b. Caco-2**

Fig. No. 5 Adhesion of Lactobacilli to cell lines a.A-431, b. Caco-2.

viii) Viable Cell Count:

C- i. viable cell count of lyophilized powders of Lactobacilli (Formulation Code La or Lb)

In plate 6, dense growth was observed at a dilution of 10^6, with an approximate cell count of 10^9-10^{10}. For plates 8, 9, and 10, the corresponding colony counts at dilutions 10^8, 10^9, and 10^{10} were 250, 39, and 5, respectively, with corresponding cell counts of 25×10^{10}, 39×10^9, and 5×10^{10}.

Viable cell count of L.casei.

In plate 6, dense growth was observed at a dilution of 10^6, with an approximate cell count of 10^9-10^{10}. For plates 8, 9, and 10, the corresponding colony counts at dilutions 10^8, 10^9, and 10^{10} were 240, 42, and 3, respectively, with corresponding cell counts of 24×10^9, 42×10^9, and 3×10^{10}.

Viable cell count of L. acidophillus.

Plate 6 showed dense growth at a dilution of 10^6, with an approximate cell count of 10^9-10^{10}. Plates 8, 9, and 10, at dilutions of 10^8, 10^9, and 10^{10}, had 290, 65, and 9 colonies, respectively, corresponding to 29×10^9, 65×10^9, and 9×10^9 microorganisms.

Viable cell count of L. rhamnosus.

Plate 6 showed dense growth at a dilution of 10^6, with an approximate cell count of 10^9-10^{10}. Plates 8, 9, and 10, at dilutions of 10^8, 10^9, and 10^{10}, had 160, 35, and 7 colonies, respectively, corresponding to 16×10^9, 35×10^9, and 7×10^{10} microorganisms.

7. Stability studies

i) Appearance:

All the formulations were found to be clear, transparent in appearance.

ii) pH

The pH values of different formulations (La, Lb, Lc, Ld, and G) were measured at various storage conditions (5 ± 10°C, 25°C/75% RH, and 40°C/75% RH) over a 45-day period. Initially, pH values ranged from 4.6 to 4.8, with slight fluctuations observed throughout the storage period, where La and Lb showed minimal changes, while Ld and G exhibited slight decreases in pH at higher temperatures.

iii. Drug contents

The average percentage of drug content of various formulations (La, Lb, Lc, Ld, and G) was measured at different storage conditions (5 ± 10°C, 25°C/75% RH, and 40°C/75% RH) over 45 days. Initial drug content ranged from 98.91% to 101.95%, with slight variations observed during the storage period, showing the most significant decrease in drug content at 40°C, particularly for Lc and Lb formulations.

iv)Viscosity (cps)

The viscosity of different formulations (La, Lb, Lc, Ld, and G) was measured at various storage conditions (5 ± 10°C, 25°C/75% RH, and 40°C/75% RH) over 45 days. Initially, viscosities ranged from 6350 to 6723 cps, with

slight fluctuations observed during the storage period, showing more significant viscosity decreases at 40°C, particularly for Lb and G.

v) Viability count

The viscosity of formulations (La, Lb, Lc, Ld, and G) was measured at different storage conditions (5 ± 10°C, 25°C/75% RH, and 40°C/75% RH) over 45 days. Initially, viscosities ranged from 6350 to 6723 cps, with slight decreases observed over time, especially at 40°C, where Lb and G showed more noticeable viscosity reductions.

8. CONCLUSION

Probiotic Lactobacillus strains (Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, and Lactobacillus rhamnosus) were studied for their growth, viability, and characteristics. All strains grew in MRS broth, showing turbidity and exhibiting Gram-positive, short rod morphology with similar colony characteristics. Viable cell counts ranged from 10^9 to 10^{10} CFU, and survival decreased under acidic conditions, with L. plantarum being more acid-tolerant. Bile salts reduced cell viability, but L. casei and L. rhamnosus showed more resistance. The strains' adhesion to human cell lines varied, and most strains were resistant to ampicillin. These findings support the probiotic strains' potential for gut health and shelf stability.

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