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Original Article

Determining Acid-Bile Optimization and Correlation between Optical Density and the Colony Forming Units of *Lactobacilli* Species

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ABSTRACT

Different research practices are being done with time to check the fruitful aspects of probiotics administration in food. In this regard, a specific number of probiotics is mixed in the food and presented to the experimental animals in laboratory to the fruitful impacts of probiotics on host. **Objective:** To design the standard curve between optical density and the number of colony-forming units. **Methods:** *Lactobacilli* species were grown in broth and their colony forming units (CFU) were calculated at different dilutions. In addition to this, relationship between optical density and number of colony forming units and acid-bile tolerance of these Lactobacilli species were also found out. **Results:** It was observed that *L. paracasii, L. delbrueckii, L. rhamnosus, and L. brevis* showed optimum rates with the Mean±SEM values of 2.56±0.04 on pH 7, 2.51±0.04 on pH 7.5, 2.46±0.04 on pH 7.5 and 2.54±0.02 on pH 7 respectively ($p < 0.0001$). Similarly, all these isolates (*L. paracasii, L. delbrueckii, L. rhamnosus,* and *L. brevis*) showed maximum growth rates on bile concentration of 0.1, and their Mean±SEM values of optical densities were 1.83±0.10, 2.36±0.25, 2.50±0.31, 1.58±0.10 respectively ($p < 0.0001$). Means were compared by ANOVA employing SPSS 20.0. **Conclusions:** The study provided insights in conducting different research practices on probiotics in future.

INTRODUCTION

Probiotics, also known as lactic acid bacteria, are added to food as nutritional supplements to offer a range of health advantages [1]. Many therapeutic properties, including antimicrobial, anticholesterol, improved lactose utilization, and anticarcinogenic properties, may be attributed to these bacteria, especially *Lactobacilli* and *Bifidobacteria* [2-10]. There isn't much proof that probiotics regularly enhance animal models' health. According to Renner theory, insufficient experimental design and the unpredictability of strain features employed in clinical research are the reasons behind the absence of definitive proof for biological activity [11]. This is untrue, but it's evident that more study is required to conclusively prove that the strains that have been identified have positive effects on animal health [12]. Numerous kinds of lactic acid bacteria are used in the food business, and they are found in large quantities in nature. Many environments with an abundance of carbohydrate-bearing substrates, such as plant materials, fermented foods, and the mucous membranes of humans and animals (primarily the oral,

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intestinal, and vaginal regions), are home to lactic acid bacteria. Lactic acid bacteria are exclusively fermentative, aero-tolerant to anaerobic, acidic, or acidophilic conditions, and have intricate nutritional needs when grown in cheese [13, 14]. Gastric acid tolerance and bile compounds based on both survival and growth investigations are currently the two most commonly employed in vitro tests, according to the criteria for the evaluation of probiotic microorganisms given by the FAO/WHO Joint Working Group [15]. The capacity of test strains to proliferate in culture media containing various quantities of bile components can be assessed during the assessment of bile tolerance using growth tests. One of the following techniques is used to generate these ratings: stresses on pH characteristics, and optical density, capacity to affect modifications to the broth culture media by evaluating its capacity for growth on solid media [16-22]. Colony-forming units are important because, as we will discuss, they are employed to count the amount of viable cells in a bacterial stock. This is crucial when utilizing bacterial stocks in investigations that need knowledge of the expected amount of bacterial growth. On the other hand, mild pH variations affect how amino acid functional groups ionize and break hydrogen bonds, which in turn causes alterations in the molecule's folding that lead to denaturation and the destruction of activity. The pH that is most suited for an organism's growth is known as the optimum growth pH. Another useful characteristic of probiotics is bile resistance. According to a recent study from North Carolina State University, probiotic *Lactobacillus* bacteria regulate bile acids and increase their survival in the stomach by using enzymes in certain situations [3].

The main objectives of the current study were to check optimum culture conditions (for pH and Bile) of some locally isolated *Lactobacilli* species and another objective was to design the standard curve between Optical Density and the number of Colony-Forming Units (CFU).

METHODS

Sample Collection and Isolation of *Lactobacilli*

Yogurt sample was taken in sterilized container from the market of new Mozang Lahore, Punjab. Pure cultures were kept in MRS agar slant and also in the form of glycerol stock [24]. The pure cultures were commercially identified for 16S rRNA genes and identified accordingly using National Center for Biotechnology (NCB) sequencing data.

Culture Revival

In the culture repository of the Microbial Biotechnology Laboratory, Institute of Zoology University of the Punjab Lahore, Pakistan, four randomly selected identified strains of *Lactobacillus paracasii* AP012541.1, *Lactobacillus delbrueckii* CP018614.1, *Lactobacillus rhamnosus* NR-113332-1, and *Lactobacillus brevis* MF179529 were preserved in cryovials (glycerol and bacterial culture grown in broth in 4:1). These vials were taken out of the freezer of the culture repository, allowed to thaw, and then 100 microliter cultures were taken out of each tube and used to inoculate the freshly sterilized MRS broth (De Man–Rogosa–Sharpe) to obtain new growth.

Ability of LAB to Tolerate pH and Bile

Thirteen equal portions of fresh MRS broth (De Man–Rogosa–Sharpe) were prepared, and these subdivisions were given precise pH ranges using 1N HCl [2-8]. Similarly, fresh MRS broth (De Man–Rogosa–Sharpe) broth was prepared and divided into five equal portions to evaluate the impact of bile. This soup was then supplemented with bile salt in precise amounts, ranging from (0.1 0.2 0.3 0.4 0.5g/100ml of MRS broth). Following the division of the broth, the glass vials with a capacity of 20 ml were autoclaved for 15 minutes at 121ºC and 15 psi pressure. After chilling, the bacterial species from which the fresh growths were obtained were added to the vials, and they were then incubated for 48 hours at 37ºC. To measure the bacterial growth, optical densities for both the pH and the bile concentration were measured at 600 nm using a VIS spectrophotometer (V-M5, Italy).

Standard Curve and CFU Determination

Sterilized distilled water was used to serially dilute the broth culture of *Lactobacilli* species. 15 sealed test tubes with 9 mL of distilled water were autoclaved for 20 minutes at 15 psi and 121oC for this purpose. Once the broth had cooled, 1 mL of the enormous growth was moved to test tube number one and serially diluted to test tube number fifteen. After that, 100 µL of each dilution was spread out on an MRS agar plate, and each dilution tube's optical density was promptly measured with 1 mL removed. Using a V-M5 VIS spectrophotometer, it was discovered that 1×10^8 (CFU mL^{-1}) of *Lactobacilli* species displayed an optical density of 0.5 at (600 nm). Every *Lactobacilli* species underwent the above-described method, which was carried out in a laminar airflow cabinet [25].

RESULTS

It was observed that *Lactobacillus paracasii, Lactobacillus delbrueckii, Lactobacillus rhamnosus,* and *Lactobacillus brevis* exhibited the highest level of bacterial growth at pH levels of 7, 7.5, 6, and 8, respectively as shown in Table 1 and figure 1.

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Table 1: Growth of Probiotic Isolates in MRS Broth at varying pH levels after 48 hours Anaerobic Incubation at 37°C

pH	*L. paracasii*	*L. delbrueckii*	*L. rhamnosus*	*L. brevis*	F value	p-value
2	0.05±0.03	0.12±0.04	0.06±0.06	0.28±0.28	<0.0001	<0.0001
2.5	0.09±0.00	0.15±0.03	0.17±0.00	0.04±0.04		
3	0.3±0.16	0.20±0.08	0.21±0.01	0.10±0.10		
3.5	0.32±0.12	0.43±0.27	0.59±0.41	0.12±0.10		
4	1.72±0.11	1.80±0.09	1.44±0.41	0.96±0.84		
4.5	2.07±0.03	2.16±0.07	2.05±0.15	1.19±0.91		
5	2.23±0.18	2.33±0.01	2.29±0.09	2.43±0.06		
5.5	2.26±0.19	2.38±0.07	2.44±0.05	1.99±0.52		
6	2.25±0.21	2.44±0.01	2.46±0.04	2.03±0.43		
6.5	2.43±0.18	2.38±0.01	2.34±0.09	2.35±0.01		
7	2.56±0.04	2.45±0.03	2.44±0.01	2.54±0.02		
7.5	2.55±0.02	2.51±0.04	2.46±0.01	2.49±0.04		
8	2.49±0.02	2.52±0.00	2.41±0.03	2.55±0.01		

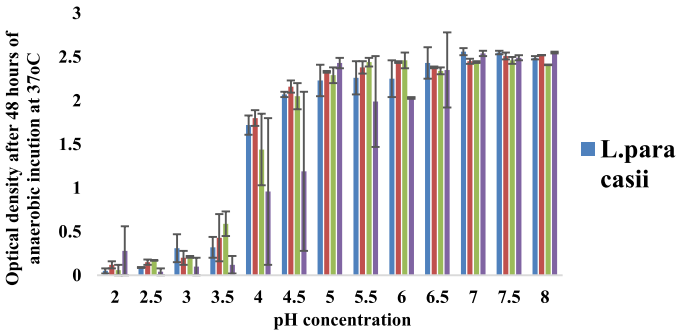

Figure 1: Growth of Probiotic Isolates in MRS Broth at varying pH levels after 48 hours of Anaerobic Incubation at 37°C pH test. A=2, b=2.5, c=3, d=3.5, e=4, f=4.5, g=5, H=5.5, i=6, j=6.5, k=7,l=7.5, m=8

In table 2 and figure 2 Values are Mean±SEM of triplicate. The data were analyzed statistically using one-way ANOVA, means that do not share same letters in the same column are significantly different. The effects were declared highly significant at p<0.05. Moreover, upon assessing the tolerance of bile in these isolates, it was discovered that all four strains exhibited the highest level of bacterial growth at a bile concentration of 0.1%.

Table 2: Assessing the Bile Tolerance of Probiotic Strains Following 48 Hours of Anaerobic Incubation at 37°C

Bile Conc. (g/100ml of MRS Broth)	*L. paracasii*	*L. delbrueckii*	*L. rhamnosus*	*L. brevis*	F-value	p-value
0.1	1.83±0.10	2.36±0.25	2.50±0.31	1.58±0.10	18.40	<0.0001
0.2	0.55±0.27	0.92±0.12	1.06±0.11	0.93±0.01		
0.3	0.52±0.26	0.63±0.15	0.68±0.15	0.69±0.05		
0.4	0.39±0.14	0.42±0.03	0.56±0.12	0.52±0.08		
0.5	0.43±0.02	0.34±0.13	0.49±0.05	0.49±0.10		

Note: Values are Mean±SEM of triplicate. The data were analyzed statistically using one-way ANOVA, means that do not share same letters in the same column are significantly different. The effects were declared highly significant at p<0.05.

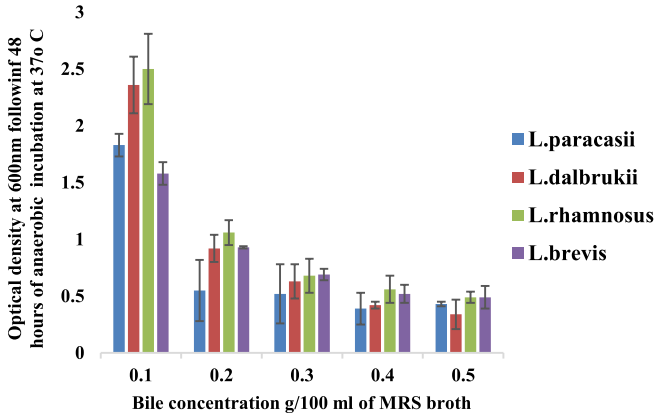

Figure 2: Assessing the Bile tolerance of probiotic strains following 48 Hours of anaerobic incubation at 37°C

In table 3, the bacterial strain showed the highest growth rate at low bile concentrations. Standard curve for CFU vs OD600nm after 48 hours of incubation in the anaerobic jar, the distinct colony-forming units(CFU)were calculated.

Table 3: Calculation of colony forming units (CFU) for *Lactobacillus paracasii*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, and *Lactobacillus brevis* at different dilutions.

Dilutions	*L. paracasii*		*L. delbrueckii*		*L. rhamnosus*		*L. brevis*	
	CFU	OD	CFU	OD	CFU	OD	CFU	OD
D1	3.16×10^{9}	0.806	3.32×10^{9}	0.915	1.76×10^{9}	2.001	2.59×10^{9}	1.089
D2	3.16×10^{8}	0.143	3.32×10^{8}	0.219	1.76×10^{8}	1.029	2.59×10^{8}	0.270
D3	3.16×10^{7}	0.096	3.32×10^{7}	0.194	1.76×10^{7}	0.591	2.59×10^{7}	0.105
D4	3.16×10^{6}	0.073	3.32×10^{6}	0.111	1.76×10^{6}	0.545	2.59×10^{6}	0.092
D5	3.16×10^{5}	0.070	3.32×10^{5}	0.107	1.76×10^{5}	0.421	2.59×10^{5}	0.082
D6	3.16×10^{4}	0.028	3.32×10^{4}	0.102	1.76×10^{4}	0.259	2.59×10^{4}	0.073
D7	3.16×10^{3}	0.022	3.32×10^{3}	0.096	1.760	0.221	2.59×10^{3}	0.051

Based on the number of CFU (N) on each agar plate, we averaged the counts on the duplicated plates for each dilution, and correlated them with the respective OD600 readings to create a standard curve (which is helpful to calculate the unknown concentrations of CFU in dilutions of *Lactobacillus paracasii*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus* and *Lactobacillus brevis* as shown in figure 3.

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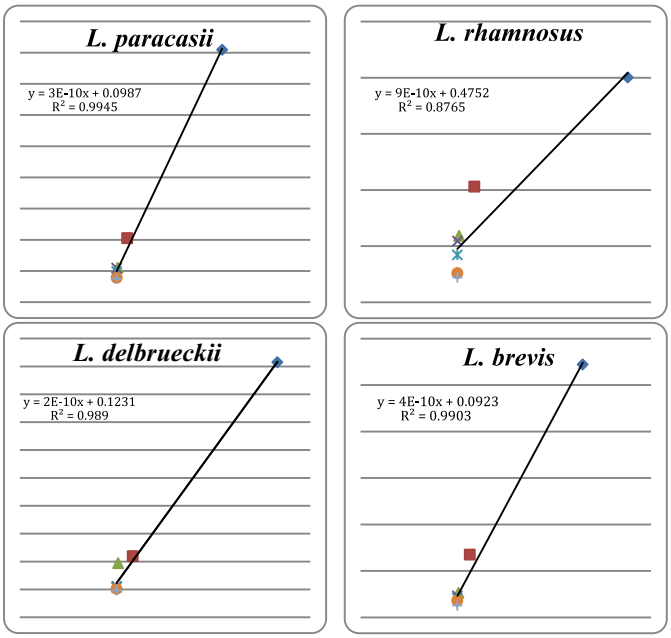

Figure 3: Standard Curves to Calculate the Unknown Concentrations of CFU in Dilutions of *Lactobacillus paracasii, Lactobacillus delbrueckii, Lactobacillus rhamnosus* and *Lactobacillus brevis* by using Optical Density Values

DISCUSSION

The current study is important in such a way that we say the tolerable ranges for model dimensions and other analysis feature parameters are examples of constraints that govern an optimization study while it searches for a solution to an objective (minimization or maximization of an analysis feature parameter). This research study was designed to optimize the culture conditions of newly isolated *Lactobacilli* using a wide range of pH and bile concentrations. On the other hand, Colony Forming Units (CFUs) per milliliter can be determined from OD 600 data by relating the two parameters, which will save time and resources in subsequent studies. In this investigation, graphs were plotted to find out the correlative relationship between optical density and number of colony forming units as shown in figure 3. So, there were two main objective of the current investigation. One was to optimize the culture conditions while growing the isolates in ranges of bile and pH. It was done because probiotics have ability to tolerate low pH and bile concentration. Secondly, the experiment was also designed to calculate specific doses of *Lactobacilli* species which can be presented to the experimental host. On the experimentation purposes, we normally make master stock (aliquots) of probiotics that is preserved in a refrigerator in normal saline and in eppendorf tubes at low temperature, from where the eppendorf tubes with required quantity of bacteria is withdrawn n daily basis, and can be administered to the experimental animal on daily basis till the termination of an experiment. The pH tolerance assessment test was performed because, according to Chan *et al* in 2011, biological macromolecules including fatty acids, proteins, and DNA are disrupted by acids like Hydrochloric Acid (HCl), which is also present in the human stomach [26]. Low pH conditions can hinder metabolism and lessen lactobacilli's capacity to grow and survive. Another research has verified that, following a 3-hour incubation period, exposure to gastric acid with a pH of less than two results in a significant reduction of bacterial viability [27, 28]. Because this mimics the presence of bacteria in the stomach, and set the acid resistance threshold at pH = 2 and pH = 3 for a 3-hour incubation [21-26]. In the current investigation, it was observed that *L. paracasii, L. delbrueckii, L. rhamnosus,* and *L. brevis* showed optimum rates with the Mean±SEM values of 2.56±0.04 on pH 7, 2.51±0.04 on pH 7.5, 2.46±0.04 on pH 7.5 and 2.54±0.02 on pH 7 respectively ($p < 0.0001$). Similarly, all these isolates (*L. paracasii, L. delbrueckii, L. rhamnosus,* and *L. brevis*) showed maximum growth rates on bile concentration of 0.1, and their Mean±SEM values of optical densities were 1.83±0.10, 2.36±0.25, 2.50±0.31, 1.58±0.10 respectively ($p < 0.0001$).

CONCLUSIONS

The current methodology was performed to optimize the growth conditions for locally isolated probiotics. The results of this study confirm the preposition acid-bile tolerance do exist in population of locally isolated species from dairy product. In this way, introduction and development of native strains with an identified origin and specific probiotic features can be very valuable. As in vitro studies can only partially mimic the actual in situ conditions in the gut ecosystem, survival of strain under conditions more similar to the human GI tract could provide more clear information regarding the characterization of native probiotic strain. Moreover, the study will be helpful in conducting different research practices on probiotics in future.

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Authors Contribution

Conceptualization: SJ
Methodology: AH
Formal analysis: MR
Writing-review and editing: AH, MAR, SA, AH

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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