Research Article

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COMPARATIVE EVALUATION OF PROBIOTIC PROPERTIES OF LACTOBACILLUS AND ITS MUTANTS WITH AN ISOLATE FROM A COMMERCIAL PROBIOTIC PREPARATION VSL#3®

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ABSTRACT

The main objective of the research was to isolate *Lactobacilli* from curd and to study their worthiness to be used as probiotic. The screened *Lactobacillus* isolate A_2 obtained from curd sample was subjected to physical and chemical mutagenesis and the two mutants were named as isolate M_1 and M_2 respectively. Another isolate S was obtained from a standard probiotic preparation VSL#3®. All four isolates were subjected to antibiotic susceptibility testing and characterized for probiotic properties according to ICMR guidelines. The study of antibiotic susceptibility testing on all four isolates showed difference in their antibiotic resistance patterns, mainly in isolates A_2 and its two mutants M_1 and M_2, thus confirming successful mutagenesis in the isolate A_2. The isolates were highly resistant under *in vitro* gastric conditions (pH 3) and at 0.3 to 0.5% bile salt concentrations. All isolates showed antagonistic activity against gut pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus* as well as bile salt deconjugation activity. Overall, all the three *Lactobacillus* isolates A_2, M_1 and M_2 were having a probiotic potential similar to that of the standard isolate S.

Keywords: *Lactobacillus*, Mutation, Antibiotic susceptibility, Probiotic properties

INTRODUCTION

Probiotics are living microorganisms when administered in adequate amount; confer a health benefit on the host. Probiotics are used either as preventives (prophylactics) or as curatives (biotherapeutics) for particular diseases[1]. Considering the great importance of probiotics to the society in the context of both clinical as well as industrial applications, isolation and characterization of more and more bacterial strains for their ability to be used as probiotics in therapeutics and also in foods is a challenging task. Probiotics are commercially available and may be administered in different forms, comprising foods, mainly in a fermented state, and pharmaceutical products, mainly as capsules or in microencapsulated form. Probiotic foods comprise 60 to 70% of the total functional food market. A continued increase is observed among the dairy-type probiotic foods, but even in the range of non-dairy probiotic food products such as fermented meats, vegetable and fruit juices. Taking into account the wide range of potential (fermentable) substrates and the different conditions under which LAB strains may be challenged for "functional performance," it can be expected that developments toward new food-based probiotics will proceed further in the future[2].

Gram positive bacteria belonging to two genera, *Lactobacillus* and *Bifidobacterium* are extensively used in probiotics. Other microorganisms like *Streptococcus*, *Escherichia*, *Enterococcus* and *Saccharomyces* have often been used but concerns have been expressed regarding their safe use. The available evidence indicates that the beneficial effects of probiotics are not only species-specific but also strain-specific[3]. Thus, comprehensive study of each isolated organism along with its identification up to the strain level is necessary before its introduction into the market. The selection of new strains presents a major challenge, both to science and industry. The primary objective is to select microbial strains with one or more proven functional properties[2]. A commercial probiotic preparation VSL#3® was used as a standard for the comparative studies. VSL#3® is a medical food for the dietary management of irritable bowel syndrome (IBS) or ulcerative colitis (UC). It contains a mixed culture of live freeze dried lactic acid bacteria including *Streptococcus thermophilus*, three species of the Genus *Bifidiobacterium* (*Bifidiobacterium breve*, *B. longum* and *B. infantis*) and four species of the Genus *Lactobacillus* (*Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei* and *L. delbrueckii* subsp. *bulgaricus*). The *Lactobacilli* were used for the research study as they are considered under Generally Recognized as Safe (GRAS) category[4]. In this study, curd was used as a source for isolation of the *Lactobacilli* because it is commonly used in diet and easily available. Also, concern regarding their source among people could not be the problem.

MATERIALS AND METHODS

Sample collection and pre-treatment

A curd sample was taken from a home-made curd of buffalo milk. The sample was kept in refrigerator at 4°C for 7 days for the sake of its pre-treatment. Tablets of a commercial probiotic preparation VSL#3® were purchased from the local medical in Ahmednagar and stored in a refrigerator until use

Isolation of bacteria

Isolation of bacteria from curd sample and VSL#3® capsule (56.25 cfu/ml) was done by serial dilution and spread plate technique. The dilutions were spread on to sterile de Man-Rogosa-Sharpe (MRS) agar (Hi Media) plates, all plates were wrapped with paraffin paper and incubated at 37°C for 45

hours. After incubation, pure cultures of 11 well isolated colonies from the plates of the curd sample and a single well isolated colony from the plate of VSL#3® spread sample were prepared by streak plate method followed by incubation at 37°C for 18 hours. After incubation, pure cultures of all isolates were subjected to catalase test by placing a drop of 30% H_2O_2 onto previously spotted cultures on a glass slide. Two isolates showing a negative catalase test and Gram positive nature were chosen randomly along with a standard isolate and named them as isolates A_2, A_6 and S respectively. Pure cultures of all three selected isolates were maintained on sterile MRS agar slants at 4^0C in a refrigerator for future use.

Detection of long term low temperature survival

Pure cultures of all three isolates were streaked on to sterile MRS agar slants and incubated at 37°C for 48 hours. After incubation, half of the slants were overlaid with sterile glycerol. All slants were kept in a refrigerator at 4°C for 65 days. After 65 days of storage, only isolates A_2 and S were chosen for further research work.

Identification of the Genus

Genus identification of the two isolates was done according to Bergey's manual of determinative bacteriology (9^{th} edition) using various morphological, physiological and biochemical tests. These included colony characterization, Gram staining, spore staining, motility testing, oxygen requirements, nitrate reduction test, gelatinse test and sugar fermentation profile.

Classical mutagenesis in *Lactobacillus* isolate A_2

The *Lactobacillus* isolate A_2 was subjected to the classical mutagenesis by means of both physical and chemical mutagenesis approaches.

Physical mutagenesis

Cell suspension of the isolate A_2 was exposed to the ultraviolet (UV) radiation for 10 minutes. Plate was covered immediately using dark paper after exposure period. This suspension was spread on to sterile MRS agar plate and the plate was incubated at 37°C for 48 hours. The UV induced mutant of the isolate A_2 was named as isolate M_1 and stored on sterile MRS agar slant for further use.

Chemical mutagenesis

Formaldehyde (5 µl) was added to 10 ml suspension of the isolate A_2 and mixed thoroughly for 5 minutes. 0.2 ml of this suspension was spread on to sterile MRS agar plate and the plate was incubated at 37°C for 48 hours. After incubation, loopful culture from the mat growth was streaked on to sterile MRS agar plate and the plate was incubated at 30°C for 48 hours. The formaldehyde induced mutant of the isolate A_2 was named as isolate M_2 and stored on sterile MRS agar slant for further use

Detection of long term low temperature survival

Pure cultures of the two mutant isolates M_1 and M_2 were streaked on to sterile MRS agar slants and incubated at 37°C for 48 hours. After incubation, half of the slants were overlaid with sterile glycerol. All slants were kept in a refrigerator at 4°C for 65 day.

Antibiotic susceptibility testing

The antibiotic susceptibility of all the four *Lactobacillus* isolates S, A_2, M_1 and M_2 was determined towards eight antibiotics (Hi Media), namely, ampicillin (10µg/ml), chloramphenicol (30 µg/ml), erythromycin (15 µg/ml), gentamycin (10 µg/ml), tetracycline (30 µg/ml), penicillin G (10 µg/ml), amoxicillin (10 µg/ml) and streptomycin (10 µg/ml) by agar well diffusion method. The antibiotic solutions of various concentrations were prepared from 1 mg/ml of stock solutions of each[5]. Culture (4 hours old grown) of each of the four isolates was spread using sterile cotton buds on to sterile MRS agar plates, wells were created and 30 µl of each of the antibiotic solution was poured in to the respective well. After addition of antibiotic solutions in to the wells, the plates were kept in a refrigerator for 20 minutes to allow diffusion of antibiotics and then incubated at 37°C for 48 hours. After incubation, plates were observed for presence or absence of zone of inhibition and results were expressed as either sensitive or resistant[6].

Characterization of *Lactobacillus* isolates for probiotic properties

Resistance to gastric acidity

Lactobacilli cultures were grown for overnight in MRS broth at 37°C. An aliquot of 0.5 ml of the overnight culture was inoculated into 50 ml MRS broth whose pH had been adjusted to 3 and 7. Bacterial growth was monitored by determination of optical density at 620 nm after 6 and 24 h incubation period at 37°C. The percent difference between the variation of optical density (OD) at pH 7.0 (ΔODpH7) and the variation of optical density (OD) at pH 3 (ΔODpH3) would give an index of isolates surviving that can be expressed as follows[5]:

$$\text{Surviving (\%)} = \Delta ODpH7 - \Delta ODpH3 / \Delta ODpH7 \times 100$$

Resistance to bile salts

Bile salt resistance ability of all four *Lactobacillus* isolates was determined by simply streaking or spotting the cultures of each of them separately onto sterile MRS agar supplemented with 0.3%, 0.4% and 0.5% (w/v) bile salts and incubating the plates at 37°C for 48 hours. After incubation, plates were observed for the presence or absence of growth and results were interpreted as either resistant or sensitive to bile salts.

Ability to reduce pathogen adhesion to surfaces

Ability of all four *Lactobacillus* isolates to reduce pathogen adhesion to surfaces was determined using bacterial antagonism assay[7].Two pathogenic organisms *Escherichia coli* and *Staphylococcus aureus* were selected as test organisms and grown in 5 ml sterile nutrient broth for 3 hours. These cultures were then spread on to sterile nutrient agar plates using sterile cotton buds, wells were created and 30 µl of overnight grown cultures of each of the four isolates were poured in to the respective labeled wells. The plates were kept in a refrigerator for 20 minutes to allow diffusion of cultures and then incubated at 37°C for 48 hours. After incubation, plates were observed for presence or absence of zone of inhibition.

Antimicrobial activity

All four isolates were grown in sterile MRS broth (10 ml) tubes overnight and cultures were centrifuged at 9000 rpm at 4°C for 30 minutes. The pH of cell free supernatants was measured and neutralized to pH 7.3 using 1 N NaOH. The neutralized cell free supernatants were filter sterilized through 0.22 µm syringe filters and stored in a refrigerator until used. Three pathogenic organisms *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* were selected as test organisms and grown in 5 ml sterile nutrient broth for 3 hours. These cultures were then spread on to sterile nutrient agar plates using sterile cotton buds, wells were created and 30 µl of neutralized and filter sterilized cell free supernatants of each of the four isolates were poured in to the respective labeled wells. The plates were kept in a refrigerator for 20 minutes to allow diffusion of cultures and then incubated at 37°C for 48 hours. After incubation, plates were observed for presence or absence of zone of inhibition. The assay was performed in triplicates[6].

Bile salt hydrolase activity

Overnight grown cultures of all of the four isolates were spotted with the help of sterile cotton buds on to sterile MRS agar supplemented with 0.5% (w/v) ox-bile (Hi Media) and 0.37 g/l $CaCl_2$. Plates were incubated anaerobically at 37°C for 72 hours. After incubation, plates were observed for the appearance of precipitation zones[4].

Table 1: Colony characteristics of the isolate S and A_2

Sr. no.	Colony character	Isolate S	Isolate A_2
1.	Size	Punctiform	Punctiform
2.	Shape	Round	Round
3.	Colour	White	White
4.	Margin	Entire	Entire
5.	Opacity	Opaque	Opaque
6.	Consistency	Butyrous	Butyrous
7.	Elevation	Convex	Convex
8.	Surface	Smooth, glistening	Smooth, glistening

Table 2: Physiological and biochemical characteristics of the isolate S and A_2

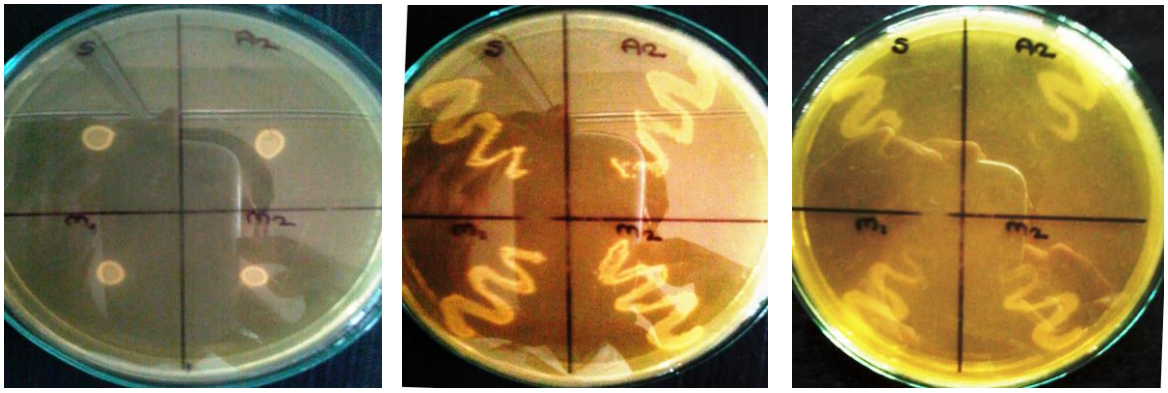
Sr. no.	Physiological/ Biochemical test	Isolate S	Isolate A_2
1.	Catalase production	Catalase negative	Catalase negative
2.	Gram staining	Gram positive, long rod (bacillus)	Gram positive, short rod (bacillus)
3.	Spore staining	Non- spore forming	Non- spore forming
4.	Motility testing	Non-motile	Non-motile
5.	Oxygen requirement	Facultative anaerobic	Facultative anaerobic
6.	Nitrate reduction	Nitrates are not reduced	Nitrates are not reduced
7.	Gelatinase production	Gelatinase negative	Gelatinase negative
8.	Metabolism	Fermentative and saccharoclastic	Fermentative and saccharoclastic
9.	Gas production from glucose	No gas formation	No gas formation
10.	Optimum growth temperature	30 to 37°C	30 to 37°C
11.	Growth period	48 hours	48 hours

Table 3: Antibiotic susceptibility testing of the four isolates S, A_2, M_1 and M_2. ('S': Sensitive, 'R': Resistant.)

Sr. no.	Antibiotic	Concent-ration (µg/ml)	Lactobacillus isolates			
			S	A_2	M_1	M_2
1.	Ampicillin	10	S	S	S	S
2.	Chloramphenicol	30	R	R	R	R
3.	Erythromycin	15	R	R	R	R
4.	Gentamycin	10	R	R	R	R
5.	Tetracycline	30	R	R	R	R
6.	Penicillin G	10	R	S	R	R
7.	Amoxycillin	10	R	S	S	R
8.	Streptomycin	10	R	R	R	R

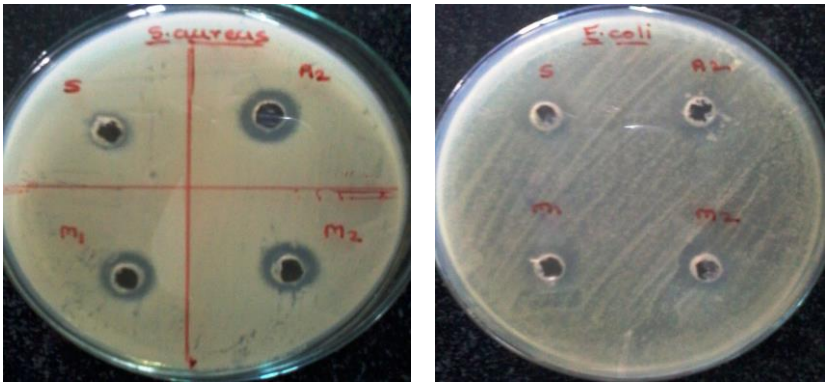
Table 4: Survival percentage of four isolates S, A_2, M_1 and M_2 at pH 3

Sr. no.	Isolate	Percent survival at pH 3 (%)	
		After 6 hours of incubation	After 24 hours of incubation
1.	S	96.09	97.94
2.	A_2	93.55	98.26
3.	M_1	86.45	98.75
4.	M_2	94.59	99.08



0.3% bile salts **0.4% bile salts** **0.5% bile salts**

Figure 1: Resistance to bile salts (0.3, 0.4 and 0.5%) by four isolates S, A_2, M_1 and M_2



Antagonism against *S. aureus* **Antagonism against *E. coli***

Figure 2: Antagonism against pathogens by four isolates S, A_2, M_1 and M_2

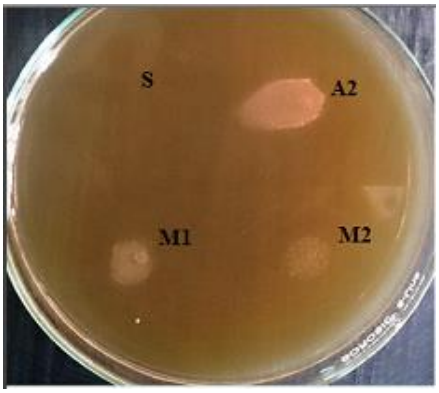


Figure 3: Bile salt hydrolase activity of four isolates S, A_2, M_1 and M_2

RESULTS

Two isolates from curd sample and a single isolate from standard VSL#3® sample showing a negative catalase test (no formation of bubbles) were selected randomly for further research work and named as isolates A_2, A_6 and S respectively.

Out of the three, only isolates A_2 and S were able to survive after long term storage of 65 days at low temperature (4°C) on both MRS agar slants and glycerol stocks. However, the isolate A_6 did not survive at all on MRS agar slants as well as glycerol stocks. Hence, isolate A_6 was rejected and only isolates A_2 and S were selected for further research work.

Colony characteristics of both the isolates as well as all the results of morphological, physiological and biochemical tests showed similar characteristics as the *Lactobacillus* as described in the Bergey's Manual of Determinative Bacteriology (9th edition) (Table 1 and 2). Both isolates were found to produce acid from four carbohydrates glucose, fructose, lactose and sucrose. However, all of them were found unable to produce gas from these carbohydrates. Thus, on the basis of these results, the two isolates 'S' and 'A_2' were confirmed as *Lactobacilli*.

Two new isolates were obtained through classical mutagenesis in isolate A_2 by physical (UV induced) and chemical (formaldehyde induced) methods and named as isolates M_1 and M_2 respectively. In the study of survival of the isolates at low temperature (4°C) for long term of 65 days, it was found that mutant isolates M_1 and M_2 were able to survive at this condition.

Table 3: Out of the eight test antibiotics, isolate S and M_2 were resistant to seven antibiotics, isolate M_1 and A_2 were resistant to six and five antibiotics respectively. All four isolates were sensitive to the ampicillin. The mutant isolate M_2 proved beneficial as it showed resistance to all antibiotics except ampicillin compared to its wild type isolate A_2, which was sensitive to three antibiotics. Also, isolate A_2 was sensitive to penicillin G and amoxicillin and isolate M_1 was sensitive to amoxicillin

Table 4: In the study of the ability of four *Lactobacillus* isolates, all the four organisms were highly resistant to the acid conditions with almost all of them showing a survival percentage greater than 90% (except for isolate M_1 with 86.45% survival after 6 hours of incubation in MRS broth at pH 3) after 6 and 24 hours of incubation in MRS broth at pH 3. All four isolates were able to survive as well as grow on to the MRS agar medium in presence of 0.3, 0.4 and 0.5% (w/v) of bile salts (Figure 1).

The antagonistic activity of four isolates tested against two indicator pathogens *Staphylcoccus aureus* and *Escherichia coli* revealed that all the four isolates were able to inhibit the growth of both indicator organisms. Also, in case of *S. aureus*, three isolates showed larger zone of inhibition than that of the standard isolate S (Figure 2).

In the study of antimicrobial activity of the four isolates against three pathogenic organisms *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* by means of production of bacteriocin (antibiotic) like substances, it was found that none of the isolate showed this ability. The neutralized cell free supernatants of all of the four isolates did not exhibit any inhibitory activity against three indicator pathogens.

Out of the four isolates screened for the bile salt hydrolase activity, three of them namely, A_2, M_1 and M_2 showed a distinct zone of hydrolysis of ox-bile while isolate S did not (Figure 3).

DISCUSSION

Even when probiotic microorganisms are suggested to promote health and well-being, the challenge remains to define particular end points or biomarkers by which such strains can be characterized and particular claims are sustained either by in vivo or validated in vitro tests even when all the mechanisms involved have not yet been fully elucidated[2]. Research work was conducted to characterize the isolated *Lactobacilli* for their ability to be used as probiotic at the preliminary level.

Several strains of probiotic bacteria lack the ability to survive harsh conditions such as low temperature and may not be suitable for use as dietary adjunct[8]. The test species of *Lactobacillus* genus could be stored at refrigerator and freezing temperatures to be used as probiotics. Most of the food preparations such as yoghurt, probiotic drinks, baby foods, buttermilk, cheese, wine etc. as well as probiotic preparations used for therapeutic use such as tablets are stored at low temperatures[9]. All of the isolates under study were found suitable to be used as probiotics in foods as well as in therapeutic preparations.

The inter-genus and inter-species differences in the antibiotic resistance patterns among lactic acid bacteria do exist[10]. The study of antibiotic susceptibility testing on all four isolates showed difference in their antibiotic resistance patterns, mainly in isolates A_2 and its two mutants M_1 and M_2, thus confirming successful mutagenesis (by both physical and chemical method) in the isolate A_2. A prevalent and vexing problem is antibiotic-associated diarrhoea (AAD). Numerous controlled trials have shown that probiotics can be efficacious in preventing AAD[2]. Isolates S and M_2 were found better in the context of antibiotic resistance than two others (A_2 and M_1). Thus, a standard isolate S and mutant isolate M_2 were equally good in terms of their antibiotic resistance ability.

Resistance to gastric acidity is one of the important criteria recommended by ICMR-DBT to detect the probiotic potency of the bacterial isolate(s) under study. Unless a probiotic is going to survive transit through the stomach and duodenum, it is going to be of little or no benefit. The high-acid conditions of the stomach require that the organism should have a high tolerance to acid. Acid resistance is frequently measured by evaluating its ability to survive pH 3 or lower for 3 hours, an average passage time through the stomach. Isolates need sufficient tolerance to bile to enable safe passage through the duodenum to their site of action. This is generally measured by simply plating out isolates on media containing bile salts. This process, however, largely measures direct resistance to bile rather than just tolerance[2].

The preliminary study conducted to study the usefulness of the *Lactobacilli* isolated from curd to be used as probiotics in both therapeutic and food preparations showed that all the three isolates A_2, M_1 and M_2 well deserved candidates for the same as they showed much similarities in their probiotic properties as those of the *Lactobacillus* isolated from a standard probiotic preparation VSL$^{\#}$3® capsule. All of them were found to fulfill *in vitro* tests' criteria for their selection as probiotics according to the guidelines provided by ICMR-DBT (2002).

In the study of antimicrobial activity of the four isolates against three pathogenic organisms *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* by means of production of bacteriocin (antibiotic) like substances, it was found that none of the isolate showed this ability. However, there may also be possibility that the antimicrobial peptides or antibiotic like substances (e. g. bacteriocin) may not have been produced by any isolate after 24 hours of incubation within medium. Thus, the assay can be repeated for further incubation periods to detect the exhibition of antibiotics by the four isolates. Acid production is contributing significantly to the antagonistic activity against pathogenic microbes[2]. Thus all four isolates showing antimicrobial/antagonistic activity against two pathogenic bacteria may be due to the acid production.

All the three *Lactobacillus* isolates A_2, M_1 and M_2 were having a probiotic potential similar to that of the standard isolate S, hence further investigations such as strain identification, determination of possible risk of transferable antibiotic resistance at the gene level, in vivo efficacy and safety studies in animal models as well as in humans etc. can be done as

future aspect of research study to determine their worthiness for introduction into the market.

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