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Indian Fermented Foods: A source of bacteria with probiotic attributes

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ABSTRACT An unbalance can occur in gut microflora because of indiscriminate antibiotic use, alterations in diet and under the influence of stress, thereby damaging human health. This balance can be restored by using appropriate probiotics. Probiotics are live microbial cells which confer health benefits to host when given in adequate amounts. Fermented foods are known to harbor microbes with probiotic properties. To explore the diversity of potential probiotic bacteria in Indian fermented foods. Isolation of bacteria was done on MRS agar, followed by evaluation of properties such as acid tolerance, bile salt tolerance, hemolysis on blood agar and effect on survival of nematodes. Preliminary identification of the isolates was done using morphological and biochemical characteristics. In the present investigation, 32 bacterial strains were isolated from Indian fermented foods such as butter, dhokla dough, curd, butter milk, cheese, bread dough, dosa batter, jalebi batter and bhatura dough. Out of these isolates, 12 could survive in the environment analogous to the human gut, such as low pH (2.5) and bile salts (2%) resistance. Among them six bacterial isolates did not exhibit hemolysis on blood agar plate thereby indicating their safe usage. Further these isolates were supplemented in the growth

medium of *Caenorhabditis elegans* to evaluate their effect on the survival of the nematode. Two bacterial isolates among six tested enhanced the survival of *C. elegans* significantly. The preliminary identification revealed hat the isolated bacteria may belong to the class of lactic acid bacteria. Our findings indicate the presence of lactic acid bacteria in these fermented foods with potential probiotic properties such as tolerance to low pH, bile salt, absence of hemolysis, improving the lifespan and reproduction of *C. elegans.* Thus these isolated cultures could be promising probiotic candidates.

Keywords: Probiotic, *C. elegans*, fermented food, lactic acid bacteria, health, gut microflora.

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INTRODUCTION

Human health, to a large extent, depends on the gut microbiota. These gut microorganisms have several functions such as stimulation of the immune system, protection from pathogenic microbes, aiding in digestion and maintaining the integrity of the gut mucosa. Dysbiosis or imbalance in the gut microbiota can have severe consequences. The balance can be restored by consuming probiotics.[1, 2] The term probiotic, meaning, 'for life', is derived from the Greek word 'probios'. These are living microorganisms, which, if given in appropriate quantities, are beneficial for the host. The most common probiotic microorganisms are *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*.[3] Fermented foods are known to harbor these probiotic organisms, and their consumption can improve health.[4]

Fermented food is an important component of our diet. These foods have increased shelf life, enhanced flavor, improved digestibility, and better nutritional and pharmacological properties.[5] This benefit is attributed due to the presence of microflora present in fermented foods.[6] Lactic acid bacteria (LAB), having a GRAS (generally-recognized-as-safe) status,

has a key role to play in the preservation and production of wholesome fermented foods.[7, 8] Consumption of foods containing LAB offer several health benefits, such as immuno-modulation, better digestion by providing several enzymes, lowering blood cholesterol, improved utilization of lactose, reducing obesity, and allergic reactions.[9, 10] Studies have clearly indicated that probiotics have helped patients with irritable bowel syndrome, diarrhea, intestinal infections, *Clostridium difficile* infection, antibiotic-associated diarrhea, necrotizing enterocolitis. [11,12] A wide variety of probiotic food products are available commercially and the global market share of probiotics is expected to reach USD 74.69 billion by the end of 2025.[13, 14]

A probiotic microorganism must be able to survive, grow and exert beneficial influence in the gastrointestinal tract. Hence, it must be acid-tolerant, bile salt-tolerant, non-pathogenic, and be able to produce anti-microbial metabolites. All these characteristics enable them to be established in the intestinal tract.[15, 16]

Caenorhabditis elegans, a non-parasitic nematode is used as a suitable model organism to investigate the probiotic properties of beneficial microorganisms. The probiotics can provide to the worm a longer lifespan, an increased resistance to pathogens and to oxidative or heat stresses.[17] In a report lactic acid bacteria exerted a protective function on *C. elegans* by acting on its intestinal permeability.[18, 19]

The current study was undertaken with the objective to isolate bacterial strains from Indian fermented food products and to study their probiotic potential.

MATERIAL AND METHODS

Sample collection

Twenty fermented food sample like batter of dosa, jalebi, dhokla, bread as well as dairy products like curd, cheese, butter milk were collected from local vendors and household kitchens. They were stored aseptically in sterilized containers and kept at 4^{o}C until further

use.

Isolation procedure

A 10-fold serial dilutions of the sample were prepared in sterile saline solution (0.85 % NaCl, pH 7) and spread plated on freshly prepared Man Rogosa Sharpe (MRS) agar (Hi Media), incubated at 37°C for 24-48 h. After incubation, single morphologically formed colonies were isolated, observed microscopically, sub cultured and preserved as glycerol stocks at -20ºC for further study.

Evaluating the acid tolerance of isolates

The effect of low pH on the viability of the strains was examined by inoculating 1% freshly prepared seed culture in 20 ml MRS broth of pH 2.5 (adjusted by 2 M HCl) and incubated at 37°C, 200 rpm for 90 min. Growth was measured by spreading 100 µl of culture broth on MRS plates after 0, 30, 60 and 90 min. The plates were incubated at 37°C for 48 h and colony forming unit (log CFU/ ml) was calculated.[20]

Evaluating the bile salt tolerance of isolates

The effect of bile on the survival of strains were examined by inoculating 1% freshly prepared seed culture in 20 ml MRS broth containing 2% (w/v) bile salts and incubated at 37°C and 200 rpm for 4h. Growth was measured by spreading 100 µl of culture broth on MRS agar after 0, 30, 60 and 90 min. The plates were incubated at 37°C for 48 h. and colony forming unit (log CFU/ml) was calculated.[20]

Evaluating the hemolytic activity of isolates

Hemolytic activity of strains was examined by inoculating the culture on blood agar plates containing 5% (v/v) sheep blood followed by 24h incubation at 37ºC. The plates were examined for alpha, beta and gamma hemolysis.[21]

Evaluating the effect of probiotic strain on of *Caenorhabditis elegans*

C. elegans strains AB1 (wild type), CB61 (strong dumpy, early larvae non-Dpy) and WLZ3

(nematode model for Parkinson disease) were used in this study. The strains were routinely maintained on nematode growth medium (NGM) plates seeded with *E. coli* (OP-50) at 20°C. *E. coli* is used as a control bacterium in the study as it is known to have little effect in *C. elegans*. The plates were observed for growth under 10X of bright field microscope [22]. For the evaluation of beneficial effects of the isolated bacterial strains, they were spread with OP50 on NGM plates. *C. elegans* (4 to 5) were transferred on these pre-incubated plates followed by incubation at 20°C. Each day morphology and behavior of *C. elegans* was compared with control using light microscopy.

Lifespan assays started when the progeny became fertile. Animals were transferred to NGM plates supplemented with the isolated bacterial strains (DS1, DS8, DS28 and DS40) and OP50 as control . They were incubated at 20°C and were observed for the survival of the nematode. They were scored as dead when they no longer responded to gentle prodding with a platinum wire. Worms that crawled off the plates were not included in the analysis.

Phenotypic/ physiological identification of isolates

Preliminary identification of the potential probiotic isolates was done based on growth in MRS media, Gram's reaction, endospore production and shape of the cell along with biochemical characterization.[23]

RESULTS AND DISCUSSION

Isolation of Lactobacilli strain

In this study, we isolated thirty two bacterial strains from the various fermented food samples. *Lactobacillus* species are known to be present in the Indian fermented foods [24, 25]. reported 46 bacterial and 9 yeasts isolate from 26 fermented food samples isolated twenty yeast strains from the fermented foods (idli and jalebi batter). [26]

Testing of acid tolerance

Survival of a probiotic organism to gastric acidic conditions (pH of 1.5-3.5) is an important

criterion for its selection of a probiotic.[27] This characteristic also contributes to their usage as dietary adjuncts in acidic food preparations.[28] In this study 19 bacterial isolates showed growth at pH 2.5 after exposure to varying time intervals of 30, 60 and 90 min. The reference probiotic strain of *Lactobacillus casei* (DSLC) along with the isolates DS8, DS40, showed high acid tolerance at pH 2.5 after an exposure up to 90 min. The isolates DS1 and DS28 were found to be viable after 60 min exposure but the viability decreased significantly after 90 min. (Fig. 1). Srudies have shown that probiotic bacteria such as *Lactobacillus plantarum* was found to tolerant to acidic pH 2.5 for 2 hrs.[29]

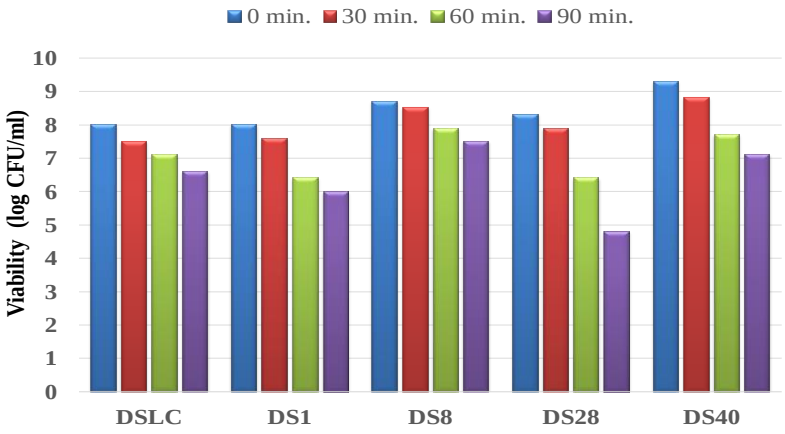

Fig.1. Acid tolerance survival (log CFU/ml) of isolates (DS1, DS8, DS28, and DS40) including *Lactobacillus casei* (DSLC) after 0, 30, 60 and 90 min exposure at pH 2.5 determined by viable plate count method.

Testing of bile tolerance

For an organism to be used as a probiotic, it must be able to tolerate the bile salt concentrations in the small intestine. This is one of the important criteria for selection of a probiotic isolate.[30, 31] In this study out of the 19 acid tolerant bacteria tested for their tolerance to 2% bile salt, 12 were found to be viable after 90 min exposure time. Isolates

DS1, DS8, DS28 showed fair growth after 90 min. DS40 were showed fair tolerance to bile salts even after 90 min. In comparison to DSLC (*Lactobacillus casei*) isolates DS1 and DS8 showed good tolerance after 60 min (Fig. 2). No significant decline occurred in the viability of the isolates after 90 minutes exposure to bile salts. Similar findings were reported by Hoque *et al.*[32] The LAB strains isolated from coconut palm nectar, were found to have a survival rate above 50% at low pH and 0.3% bile salt concentration after 4-h exposure.[6] *Lactobacillus helveticus* and *Lactobacillus plantarum* isolated from traditional Pakistani yoghurt showed resistance to acidic pH (6-6.5), bile salt (0.01-1%), and 1-7% NaCl salt and showed good growth at acidic pH and antibacterial activity against ten different foodborne pathogens.[33]

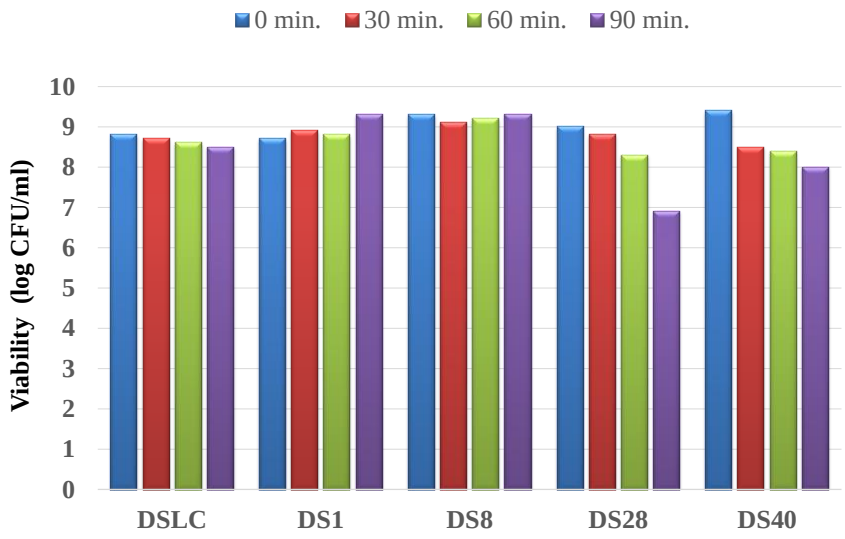

Fig.2. Bile salt tolerance survival (log CFU/ml) of isolates (DS1, DS4, DS8, DS11, DS28, DS34, DS36 and DS40) including *Lactobacillus casei* (DSLC) after 0, 30, 60 and 90 min exposure at 2% bile salt determined by viable plate count method on MRS medium.

Hemolytic activity

Out of 12 acid and bile tolerant strains, eight isolates DS1, DS8, DS28, DS40 showed gamma

hemolytic activity and incapable of lysing of RBC on the agar media containing 5% blood. Isolates showing alpha or beta hemolysis were not considered for further study. Only isolates with gamma hemolysis were further used in this study (Fig. 3). If a bacteria is to be considered as a probiotic, it should not cause lysis of red blood cells in the body. Evaluation of the safety of the isolates obtained from coconut palm nectar, revealed no hemolytic or DNAse activities, which confirmed their non-pathogenic status.[6] Lactobacilli are usually non-hemolytic in nature. Our results are in agreement with the reports of lactic acid bacteria and *Bifidobacteria* species being non-hemolytic in nature.[34]

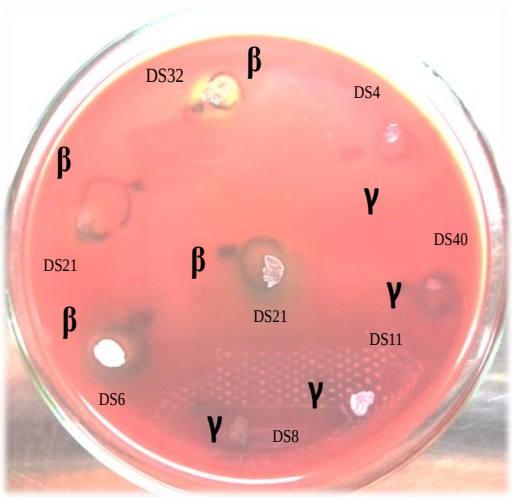

Fig.3. Haemolysis test of isolates DS4 DS8, DS11, DS40, show gamma haemolysis (around the area of colony unchanged) and DS21, DS6, DS32, DS21 show beta haemolysis (area appear lightened yellow around the colony) on blood agar

Evaluating the effect of probiotic strain on of *Caenorhabditis elegans*

A non-parasitic nematode, *Caenorhabditis elegans*, represents a relevant and powerful model for, microorganisms-microorganisms and microorganisms-host interactions studies. Experiments have shown that the probiotic bacteria can increase the lifespan of the worm and improve resistance to pathogens and to oxidative or heat stresses.[35] By regulating molecular pathways, such as insulin/insulin-like growth factor-1 (IIS) and p38 mitogen-

activated protein kinase (p38 MAPK), probiotic bacteria are able to apply anti-ageing effects and thereby controlling life span in nematodes.[17] The toxicity of graphene oxide (GO) on the functions of both primary and secondary targeted organs in wild-type nematodes was prevented by LAB. The translocation of GO was blocked into secondary targeted organs by LAB by maintaining normal intestinal permeability in wild-type nematodes. LAB exerted a protective function on *C. elegans* by influencing its intestinal permeability.[18]

This is in line with our resuts obtained in this study. The bacterial isolates which were acid and bile salt tolerant and non-hemolytic, were selected for supplementing the NGM plates containing *E. coli* OP50 along with *C. elegans*. Light microscopy studies showed that the nematode exhibited normal morphology and behavior in the presence of probiotic bacteria (Fig. 4).

The survival rate of *C. elegans* monitored in the presence of the probiotic isolates showed that isolates DS8 and DS40 showed a positive effect on the survival rate of AB1 and CB61 after 25days (Fig. 5A, 5B). Isolate DS40 significantly enhanced the survival of the nematode WLZ3 after 25 days (Fig. 5C). In this current investigation, inclusion of our bacterial isolates in the diet of the nematode increased the life span of *C. elegans* as compared to when they were feed only with *E. coli* (OP50). This study suggests the role of probiotic supplementation in increasing the life span of the host.

Research has indicated that *Lactobacillus gasseri* SBT2055 (LG2055) has the beneficial effects on longevity and anti-aging of *C. elegans* by increasing the amounts of mitochondria, strengthening resistance to oxidative stress and by stimulating the innate immune response signaling including p38MAPK signaling pathway and others.[36]

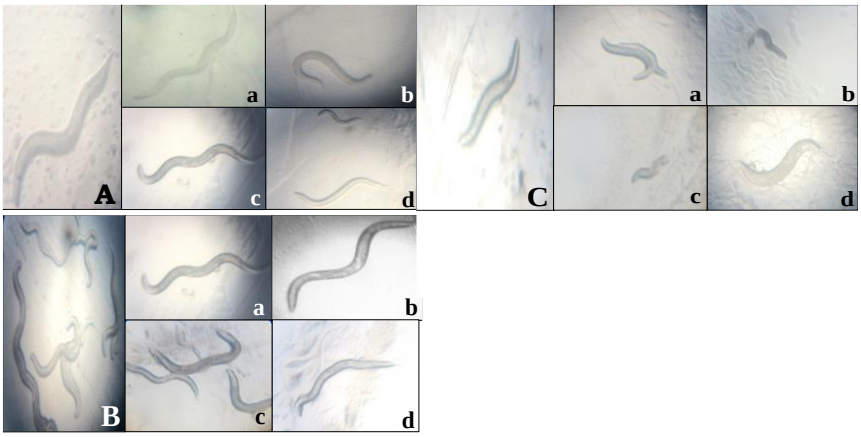

Fig.4. Evaluating the effect of isolated *Lactobacillus* strains [(a) DS1, (b) DS8, (c) DS28 and (d) DS40] on the morphology and behaviour of *C. elegans* under light microscope 10X magnification. (A) Wild strain, (B) CB61 (strong dumpy, early larvae non-Dpy) and (C) WL25 (nematode model for Parkinson disease

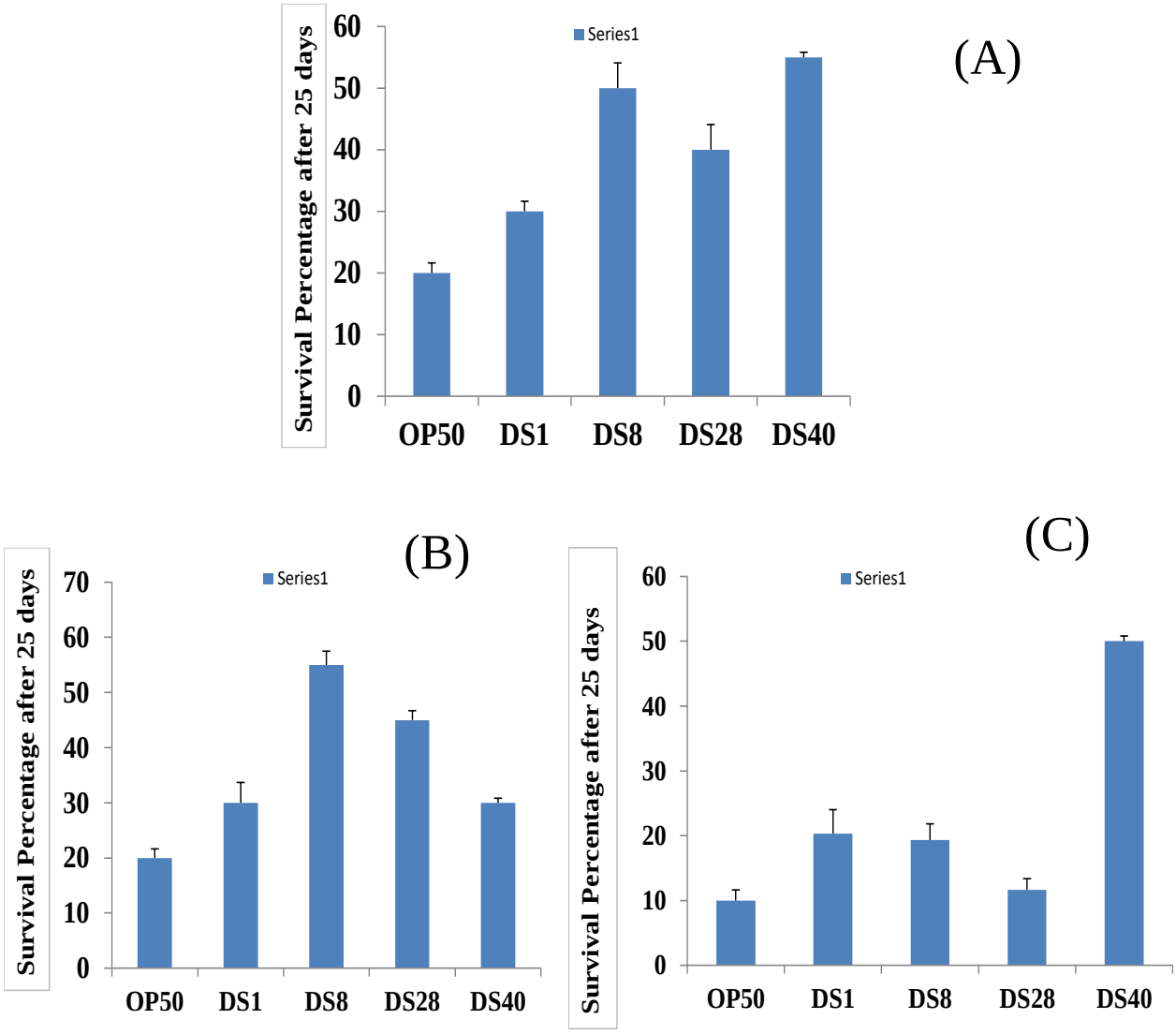

Fig.5. Effect of *Lactobacillus* feeding (Strain DS1, DS8, DS28, DS40) on the lifespan of *Caenerorhabitis elegans.* In control nematodes were fed only *Escherichia coli* OP50. (A) AB1 wild strain of *C.elegans*, (B) CB61 (strong dumpy, early larvae non-Dpy) and (C) WLZ3 (nematode model for Parkinson disease).

Phenotypic and physiological identification of isolates

White to creamy white glistening, convex colonies were observed on MRS agar plates. Majority of the isolates were Gram positive and non-endospore forming. Fig.6 shows their colony morphology along with the Gram's reaction. They were catalase and oxidase negative.[37] These bacteria could be *Lactobacillus*, however, further confirmation is required

by molecular tests. A list of morphological, biochemical and probiotic characteristics of the isolates is listed in Table 1. Out of the 75 isolates obtained from fermented coconut palm nectar, 40 isolates were Gram-positive, non-spore-forming, catalase-negative and ferment all the tested sugars. They were considered for testing as presumptive LAB isolates.[6]

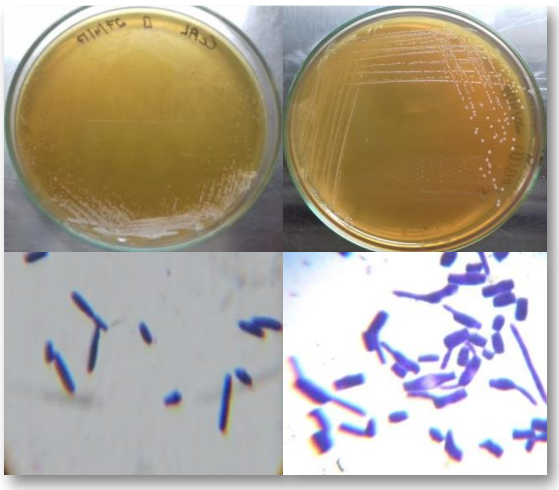

Fig.6. Morphological feature of bacterial isolates on MRS medium (A) Isolate DS8 on MRS medium.(B) Isolate DS40 on MRS medium, (C) Morphology of isolate DS8 under immersion oil after Gram staining and (D) Morphology of isolate DS40 under immersion oil after Gram staining

Table 1: Comparative data on the basis of phenotypic/physiological characteristics of potentials probiotic strain

Characteristics	DS1	DS4	DS8	DS11	DS28	DS34	DS36	DS40	DSLC
Gram Staining	+	+	+	+	+	+	+	+	+
Morphology	LR	LO	LR	LO	LR	LR	LR	LR	LR
Catalase	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-			-	-	-	-
% Acid tolerance viability at 90 min	60.0	73.7	75.0	74.3	48.6	40.7	29.5	71.5	66.9
% Bile tolerance viability at 90 min	93.0	75.0	93.2	63.7	69.9	79.6	50.0	80.0	85.5
Heamolysis	γ	γ	γ	γ	γ	γ	γ	γ	γ
Survival rate	A,	A,B,C	A,B,C	A,B,C	A,B,C	A,B,C	A,B,C	A,B,C	-

percentage of *C. elegans* after 25 days	B,C 5,10,0	1,5,2	10,12,10	2,10,4	5,3,0	10,5,5	0,10,2	15,15,5	

CONCLUSION

There is a rich diversity of microflora in fermented foods. In this study we have isolated bacteri exhibiting probiotic properties such as tolerance to low pH, growth in presence of bile salt, absence of hemolysis, improving longevity of *C. elegans.* These studies are in line with the work done using commercially available probiotic *Lactobacillus casei*. These potential probiotic candidates can be used for further investigation for the development of probiotics foods and feeds.

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