Isolation and Characterization of Commercial Probiotics

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ABSTRACT

Commercial probiotics have bacteria which offer scope for specific treatment as a probiotic against diarrhoea, boosting immune response and relieving stress. Research in probiotics and aquaculture has been done to promote sustainable aquaculture. Studies on microbial ecology in aquaculture and their benefits on human systems are to be assessed. Hence, the present study was aimed at the isolation and characterization of these bacteria obtained from various commercial probiotics. The commercial probiotic samples were collected for isolation using MRS agar. The colonies were selected on the basis of colonial morphology. Isolates were put through cell morphology, physiology and different biochemical tests. Probiotic sample confirmation was done using 16srRNA molecular technique. MEGA7 was used to conduct phylogenetic evolutionary analysis and tabulate a distance matrix. In results, the isolates manifested non-identical growth patterns at dissimilar conc. of NaCl (2.0,4.0 and 6.5) oxygen and at different temperatures (15^0C, 30^0C and 45^0C). On the basis of sugar utilization, physiological testing, biochemical characterization and genetic identification tests, all isolates were established to different species of *Lactobacillus rhamnosus, Pediococcus acidilactici* and *Lactobacillus Plantarum*. A systematic protocol was done to identify, characterize probiotic samples and identify them by genetic analysis. Probiotic use is carefully assessed by regulations.

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INTRODUCTION

Fermented products containing live microorganisms have always been traditionally used to maintain gut health. The utilization of live microbes to improve host health forms the basis of the probiotic concept. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host. Probiotic strain and products should demonstrate safety and efficacy. Probiotics, once ingested, should deal with biological hurdles, including acid and bile in the stomach, intestine to apply health-promoting effects at their place of action. Probiotics give a span of host well being (Sanders, 1998; Tannock, 1999a), comprising pathogen intervention, exclusion & antagonism, immune stimulation, anticarcinogenic activity and antimutagenic activity, alleviation of evidence of blood pressure, reduction in serum cholesterol and lactose intolerance, decreased occurrence

and period of diarrhoea, maintenance of mucosal integrity and prevention of vaginitis.

The probiotics exert their effects by different mechanisms which may require altering stomach pH, antagonistic effect of antimicrobial compounds on pathogens, striving for obtainable growth factors, nutrients receptor and pathogen binding sites, producing lactase and stimulating immunomodulatory cells (Nagpal *et al.*, 2012). Advancement in microbiology and molecular biology have made probiotic species and strain identification easier. While microbiological approaches are key for culture production, phenotypic characterization, selection and enumeration, phylogenetic analysis (PCR amplification, 16SrRNA, identification) is important for taxonomical classification of bacterial cultures (O'sullivan, 2000). Probiotic bacteria adherence to intestinal epithelial cells is a prerequisite to exert beneficial health effects. In vitro adhesion assay on human intestinal epithelial cell lines, like Caco-2 or HT-29, reflect the ability of probiotic to transiently persist in the human gut (Tuomola and Salminen, 1998). The development of a new generation of probiotics has become easier due to novel methodologies for isolation and characterizing new site-specific probiotics (Tannock, 1999b).

MATERIALS AND METHODS

Isolation of bacteria

Commercial probiotic and National centre of industrial microbiology (NCIM) samples such as Enteroplus, Avantbact and NCIM *Lactobacillus sp.* were taken. One gram of samples under aseptic condition was suspended in 9mL saline and vortexed. After mixing 1ml of the sample, enrichment was donein 9mL of sterile De Man, Rogosa and Sharpe agar broth(MRS) for 24h at 37°C.(pH- 6.5$\pm$0.2) MRS agar was inoculated with enriched samples using a sterile loop and incubated at 37°C for 48h and colony growth was observed.

Identification of selected lactobacilli and pediococcus

Identification and further characteristics of lactobacilli and pediococcus grown on MRS agar was done by the procedure described in Bergey's manual of systematic bacteriology with following tests carried out: microscopic examination, catalase test, growth at different temperatures, aerobic and anaerobic conditions, different NaCl concentration, carbohydrates fermentation (Roberfroid, 2000).

Microscopic examination

Purity, morphology and microscopic examination of lactobacillus and pediococcus isolates was done by gram staining where a single colony of isolates was subjected to staining as per protocol and observed under 100X for cell similarity.

Physiological Characterization of Isolates

Once culture purity was confirmed, each isolates were further observed for growth at different oxygen requirement and temperature (Stanton *et al.*, 2001).

Growth of isolates at (10°C, 31°C, 42°C)

The isolates were examined for growth in MRS broth at 10°C for 7 days and 31°C,42°C for 24-48h. 1 %of *Lactobacillus* and *Pediococcus* culture were mixed with 10ml MRS broth, turbidity at different temperatures was observed as positive or negative.

Oxygen requirements for isolates

The isolates were inoculated in MRS broth and kept at different oxygenated conditions (microaerophilic, aerobic, anaerobic). Growth of the isolates were observed for the existence absence of growth.

Effect of bile salt concentration on isolates growth

The isolates were inoculated with different bile salt concentrations (0.1%, 0.3 % and 0.5 %) in MRS broth and incubation done at 37°C for 24-48h. Culture tubes were observed for the existence or absence of growth (Holt *et al.*, 1994).

Effect of NaCl concentration on isolates growth

The isolates were inoculated with different NaCl concentrations (2%, 4% and 6%) in MRS broth and incubated at 37°C for 24-48h. Cultured tubes were observed for the existence or absence of growth.

Biochemical characterization of isolates

Catalase test

Test was carried on isolated cultures for the determination of degradation of hydrogen peroxide by producing enzyme catalase. The culture was placed on the slide using the inoculation needle and a drop of 3% hydrogen peroxide solution was added to the culture and looked for bubble evolution indicating a positive result.

Oxidase test

Cytochrome C oxidase producing bacteria is identified by this test. The culture was placed on a slide using inoculation needle and drop of N, N, N′, N′-tetramethyl-p-phenylenediamine solution was added on to the culture and observed for the oxidized purple colour end product which indicates a positive result.

Gas from glucose

Table 1: Morphology, physiology and biochemical characterization of isolates

Characteristics	Lactobacillus sp. (NCIM-2056)	Avant bact	Enteroplus
Morphology characterization			
Colour	White	White	White
Shape	Rod	Cocci	Rod
Size	0.6μm	0.3μm	0.8μ
Form	Circular	Circular	Circular
Motility	Non- motile	Non -motile	Non- motile
Grams reaction	Positive	Positive	Positive
Physiological characterization			
Growth at different temperature			
10°C	-	-	-
31°C	+	+	+
42°C	-	+	+
Oxygen requirement			
Aerobic	+	+	+
Anaerobic	-	-	-
Microaerophilic	+	+	+
Growth at different Bile Salt concentration			
0.1%	++	++	++
0.3%	+	+	+
0.5%	-	-	-
Growth at different NaCl concentration			
2%	+++	++	+++
4%	++	+	++
6%	+	-	+
PH			
4	+	+	+
4.5	+	+	+
5	++	++	++
5.5	++	++	++
Biochemical characterization			
Catalase	-	-	-
Oxidase	-	-	-
Gas from glucose	+	-	-
Arginine hydrolysis	-	+	-
Aesculine hydrolysis	-	-	-
Nitrate reduction	-	-	-
Citrate utilization	-	-	-
Indole test	-	-	-
MR test	-	-	-
VP test	-	-	-

Table 2: Carbohydrate fermentation by isolates

Fermentable sugars	Lactobacillus sp. (NCIM-2056)	Avant bact	Enteroplus
Amygadalin	+	+	+
Cellobiose	+	+	+
Mannose	+	+	+
Mannitol	+	+	+
Sucrose	+	+	+
Arabinose	+	+	+
Lactose	+	+	+
Xylose	+	+	+

Table 3: Estimates of Evolutionary Divergence between Sequence

No.	Distance Matrix	1	2	3	4	5	6	
1	Lactobacillus_rhamnosus_		0.000	0.001	0.001	0.001	0.001	1
2	MG827277.2	0.000		0.001	0.001	0.001	0.001	2
3	MK329243.1	0.001	0.001		0.000	0.000	0.000	3
4	MN173898.1	0.001	0.001	0.000		0.000	0.000	4
5	MN134433.1	0.001	0.001	0.000	0.000		0.000	5
6	MN134431.1	0.001	0.001	0.000	0.000	0.000		6
		1	2	3	4	5	6	

Table 4: Estimates of Evolutionary Divergence between Sequence

No.	Distance Matrix	1	2	3	4	
1	Pediococcus acidilactici strain		0.000	0.000	0.000	1
2	MK329208.1	0.000		0.000	0.000	2
3	MN166312.1	0.000	0.000		0.000	3
4	MN160241.1	0.000	0.000	0.000		4
		1	2	3	4	

Table 5: Estimates of Evolutionary Divergence between Sequence

No.	Distance Matrix	1	2	3	4	5	6	7
1	Lactobacillus_delbrueck		0.000	0.000	0.000	0.003	0.004	1
2	CP002342.1	0.000		0.000	0.000	0.003	0.004	2
3	EF196094.1	0.000	0.000		0.000	0.003	0.004	3
4	EF196093.1	0.000	0.000	0.000		0.003	0.004	4
5	MF191707.1	0.044	0.044	0.044	0.044		0.004	5
6	AF236060.1	0.065	0.065	0.065	0.065	0.078		6
7		1	2	3	4	5	6	7
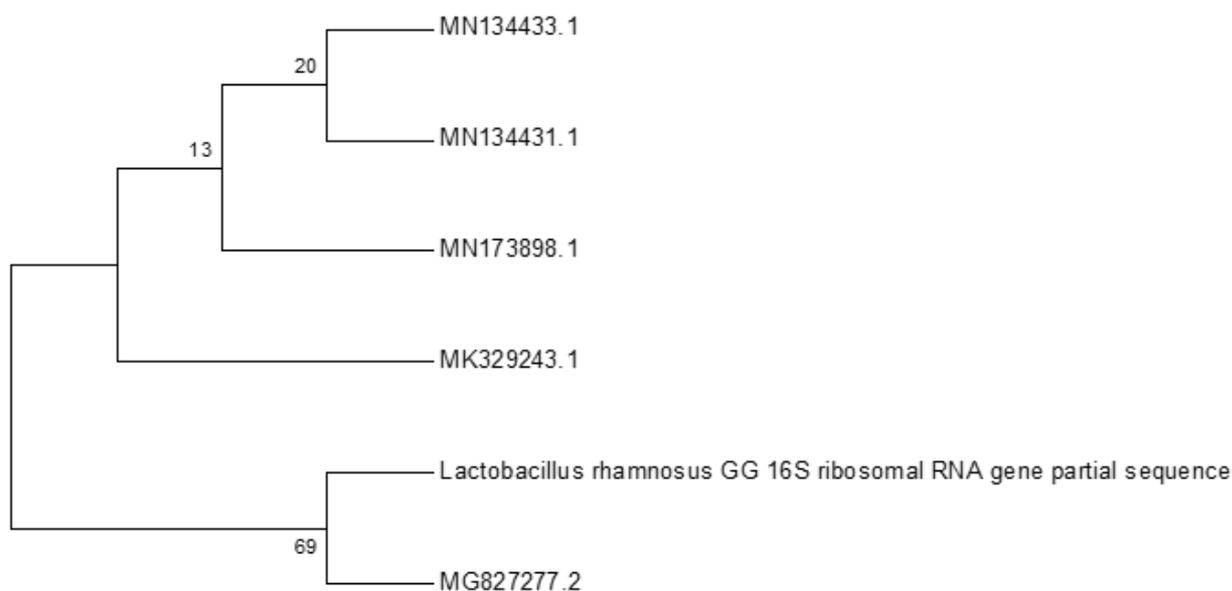
Figure 1: Phylogenetic Tree
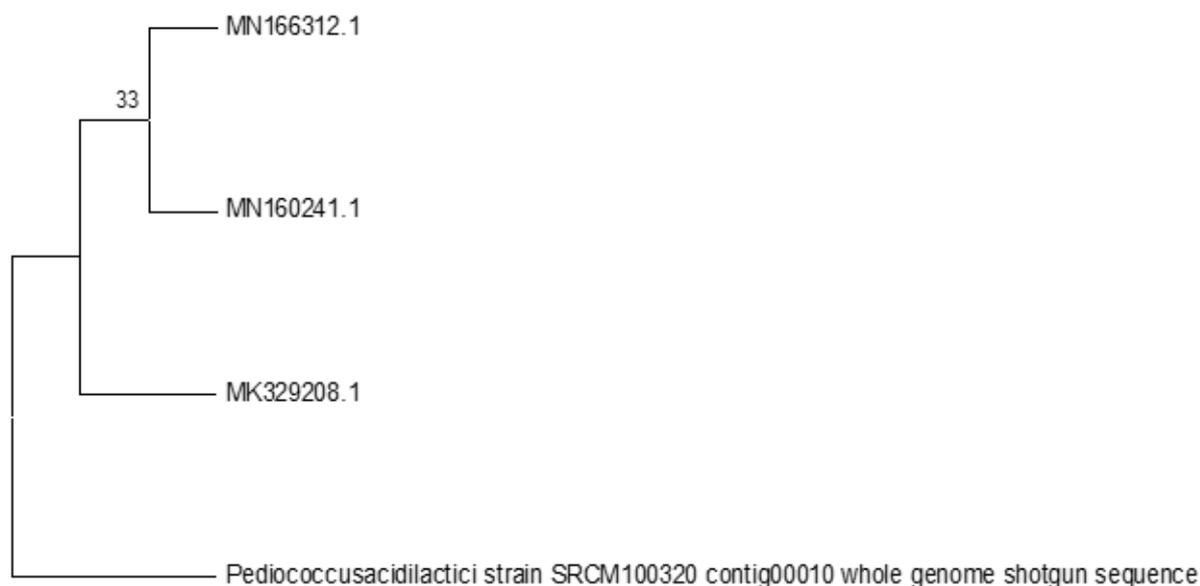
Figure 2: Phylogenetic Tree

Glucose broth added to the test tube and durham's tube inverted in the test tube and were inoculated with culture and incubated at 37°C for 24 - 48h. The gas produced in an inverted Durham tube was recorded as a positive result.

Arginine hydrolysis

Arginine hydrolysis broth were inoculated with isolated cultures and incubation done at 37°C for 48h. After incubation, few drops of Nessler's reagent were added to the test tube and observed for change in colour(yellow to orange), indicating a positive result.

Aesculin hydrolysis

Isolated cultures were streaked on to bile aesculin agar and incubated at 37°C for 24-48h. Isolates were observed for hydrolysis of glycoside aesculin to aesculetin and glucose. Brown to black colour around bacteria growth indicates a positive result.

Nitrate reduction test
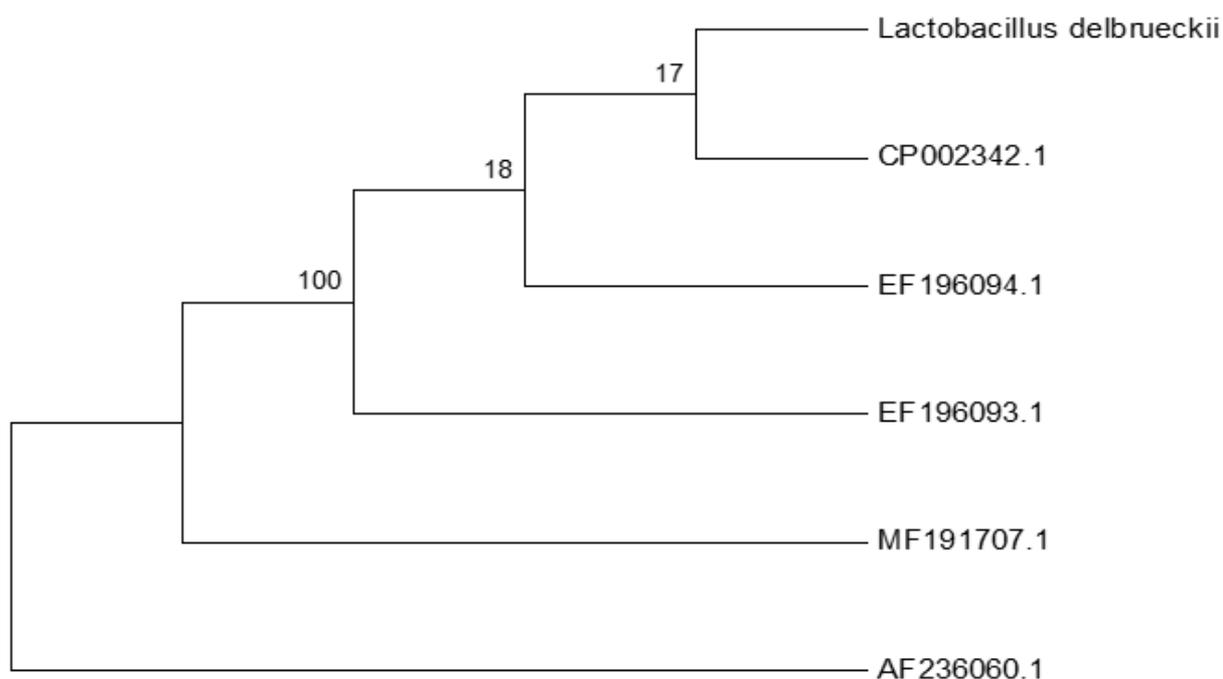
Figure 3: Phylogenetic Tree

Isolates were added in trypticase nitrate broth and incubated at 37°C for 24h.After incubation, 0.5 mL of sulphanilic acid and α-naphthylamine were added into the tubes. Appearance red or pink colour show positive result for nitrate reduction.

Citrate utilization test

Simmons citrate agar is added to isolates incubated at 37°C for 24h. After incubation, the appearance of the blue colour indicates a positive result for citrate utilization.

Indole test

Tryptophan broth added to isolates, incubated at 37°C for 24h. After incubation, 0.5 mL of Kovac's reagent was added. Pink colour ring in the top layer indicates a positive result.

MRVP (Methyl red Voges Proskauer) test

Isolates were added to MRVP broth and incubated for 37°c for 48 h. For the MR test, five drops of methyl red is added to one tube and for the VP test, Barrit's reagent was added to another tube. Red colour indicates MR positive and pink colour indicates VP positive.

Carbohydrates fermentation by isolates

Sugars were used to determine the fermentation profile and further characterization of *Lactobacillus* and *Pediococcus* cultures. Carbohydrates basal media was prepared and pH adjusted to 7- 7.4. Phenol red was added as an indicator. Sugars were mixed to the basal media (500mg of sugar in 50mL of basal media) and autoclaved. Each tube was inoculated with 50μL of inoculum in 250μL of sugar basal media. Control using sterile water was prepared to compare colour change.

Maintenance and propagation of cultures

Isolates of *Lactobacillus* and *Pediococcus* were maintained at -70°C in glycerol stocks in triplicate for use in an experiment at different stages.

Purity of cultures

Lactobacillus and *Pediococcus* species were regularly tested for microscopic examination and catalase tests for confirmation and presence of contamination if any.

Identification of microbial culture using 16s rRNA based molecular technique method

Quantity of isolated Genomic DNA was measured using nanodrop spectrophotometer and quality determined using 2% agarose gel. A DNA single band of high molwt. has been observed. 16S rRNA gene was amplified by 16SrRNAF and 16SrRNAR primers. A single amplified PCR band (1500 bp) was observed after resolution on an agarose gel. The amplified PCR was purified to exclude contaminants. Amplified PCR having forward DNA and reverse DNA sequencing reaction was moved with forward and reverse primers using BDT v3.1. Cycle sequencing kit on ABI 3730xl genetic analyzer. A

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concurrent sequence of the 16S rRNA gene was generated from a forward sequence and reverse sequence. 16S rRNA gene sequence with the NCBI Genbank database was used to carry out BLAST. Based on maximum sameness first ten sequences were selected. Alignment using multiple alignment software programs Clustal W. Distance matrix was generated and the phylogenetic tree was build by using MEGA7.

Phylogenetic tree

The evolutionary history was decided using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with a sum of branch length = 0.09337190 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Positions containing gaps and missing the data were eliminated. There was a total of 5851 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Distance matrix

The no. of base substitutions per site between sequences are shown. Analysis was conducted using the Maximum Composite Likelihood model (Saitou and Nei, 1987). The analysis had 11 nucleotide sequences. The Codon positions included were 1st+2nd+3rd+Noncoding. All the positions containing gaps and missing data were eliminated. There are a total of 5851 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Felsenstein, 1985).

RESULTS AND DISCUSSION

Morphological, physiological and biochemical characterization of NCIM -2056 strain, commercial probiotic (Avant bact and Enteroplus) species are given (Table 1 &Table 2). Identification of microbial culture using 16s rRNA molecular technique was carried out and the results are mentioned (Figures 1, 2 and 3)(Tables 3, 4 and 5)below. Based on sequence homology and phylogenetic analysis, the microbial culture labelled was found to be *Lactobacillus delbrueckii subsp. Bulgaricus. (NCIM-2056 strain) Pediococcus acidilactici (Avant Bact) and Lactobacillus rhamnosus GG. (Enteroplus).*

Commercial probiotics such as enteroplus and avant bact containing different strains of bacteria were isolated and screened for different Physiological Characters, biochemical tests and Identification of probiotic isolates using 16s rRNA based Molecular Technique was carried out to confirm the presence of different bacterial cultures such as *Lactobacillus delbrueckii subsp. Bulgaricus. (NCIM-2056Strain) Pediococcusacidilactici(Avant Bact) and Lactobacillus rhamnosus GG. (Enteroplus).*

In physiological characterization, all strains grew well at a temperature of 31°C, but at 42°C, NCIM culture exhibited no growth. Hence it can be concluded 31°C is ideal for the growth of all strains and few can survive at 42°C. In the oxygen requirement, all strains exhibited microaerophilic and aerobic conditions. It can be stated all strains have a mechanism of an oxidative process for energy generation growth at a bile concentration of 0.1 - 0.3% was observed for all strains. It showed the bile tolerance limit for the microbes. Maximum growth was observed at a concentration of 2% NaCl as concentration increased (4%,6%) growth decreased for all strains. The growth pattern reported wide variation in growth at different concentrations for all strains. The ideal pH for the growth of strains was between 4-5.5. pH increase showed an increase in growth.

In biochemical characterization, catalase and oxidase negative support identification of *Lactobacillus species. Pediococcus species.* Fermentation of glucose was observed in NCIM strain, whereas commercial probiotic strains did not show gas from glucose. *Pediococcus species* showed production of ammonia but *Lactobacillus species* could not hydrolyze arginine. All other biochemical tests were found to be negative, which were characteristic of given microbial samples. All isolates were found to be sugar fermenters and showed survival properties in vitro to be used as an ideal probiotic. This research attempt is to identify commercial probiotic bacteria to be used for specific treatment against diseases.

CONCLUSIONS

In summary, commercial probiotics was successfully isolated and identified as *Lactobacillus* and *Pediococcus* strain from commercial probiotics that exhibited strong survival properties for probiotic application in the gastrointestinal tract. Therefore, the study confirmed the strains showed the identification characteristics of probiotics. However, further research is needed to evaluate them by invitro adhesion assay and evaluate them in animal experiments.

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