

EXAMINATION OF NEWLY ISOLATED LACTOBACILLI FOR FORMING OF STARTER CULTURE CONSORTIUM WITH PROBIOTIC POTENTIAL

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ABSTRACT

Starter cultures having probiotic potential have been used in various food fermentation industries. More than 35 cultures isolated from various dairy and non-dairy sources were subjected to basic LAB properties and, subsequently, starter culture properties like acidification, milk clotting, flavour production, and proteolytic activity. Five promising isolates identified by 16s rRNA sequencing are *Streptococcus thermophilus* (CUD2 & CUD3), *Lactobacillus plantarum* (FCW2 & FCW4) and *Lactobacillus fermentum* (FC1). These cultures also displayed probiotic properties such as good tolerance to 0.8% bile salt, 6% NaCl, and viability at pH 3.5, antimicrobial activity (tested against *E. coli*, *M. smegmatis*, *S. typhi*, *S. aureus*, and *P. aeruginosa*). All cultures showed notable probiotic properties; CUD2 and CUD3 displayed milk clotting within 6 hours, *L. plantarum* FCW4 and *L. fermentum* FC1 showed good inhibition against all the indicator strains. The compatibility tests indicated that the cultures don't have any antagonistic activity among them and can be effectively used for suitable consortium development for applications in fermented foods.

Keywords: Isolation, Starter culture, Lactic acid bacteria, Probiotics, Consortia

INTRODUCTION

The food-grade lactobacilli are widely used in the dairy industry to produce fermented dairy products such as kumis, buttermilk, kefir, cheese, and yogurt, as they are generally regarded as safe (Burdock and Carabin 2004, Leroy and De Vuyst 2004). Starter cultures are the microbial community used to obtain controlled and predicted fermentation types and are applied in several food industries. The microbiology of starter cultures includes several bacteria, molds, and yeast (Taskila, 2017, Parente et al., 2017) and Probiotics are friendly bacteria with various health benefits; most probiotic bacteria belong to *bifidobacteria* and *lactobacillus.sp* (Isolauri and Ouwehand 2004, Guarner and Malagelada 2003). The selection of starter culture depends upon the final product after fermentation, and each starter differs from the other to exert flavour, colour, texture, and consistency to the product (Buckenhüskes, 1993). For example, cheese flavour is due to the complex mixture of volatile compounds, and it was found that *Propionibacterium freudenreichii* has a significant role in the flavour production in Swiss cheese and *penicillium roqueforti* has a strong effect on the development of the aroma and texture of blue cheese (Thierry et al. 2005, Caron et al. 2021, Coico 2005). Diacetyl production gives a buttery flavour to cheese and other fermented dairy products, and flavour is one of the critical starter culture properties (Keenan and Lindsay 1968, Marshall 1987). The starter culture bacteria *Streptococcus thermophilus* is a gifted strain, and it is found that more than 10²¹ cells are consumed by humans annually, and the worldwide increase in awareness of food and health has promoted the consumption of functional foods (Roberfroid 2000, Siegrist et al., 2008). In the years 2005, 2006 and 2010, the worldwide market share for functional food was found in the progress of 33 billion USD, 73.5 billion USD and 167 billion USD, respectively (Granato et al., 2010, Knowledge et al., 2010) (Kotilainen, Liisa, 2006). It is reported that probiotics have a significant part in the functional food market, i.e., 60-70 % of functional food belongs to probiotics (Goktepe et al., 2005). Starter cultures with probiotic potential will be an added advantage when it is applied to food (Hasler, 2002). A wide range of health advantages have been reported for starter culture bacteria over the years and alleviation of lactose intolerance is one among them. The incapability of digesting lactose in adults has been overcome worldwide. The consumption of milk sugar can cause diarrhoea, bloating and abdominal pain in lactose-intolerant people (Panesar et al., 2006). It is known that these individuals can consume milk products like yogurt that is fermented using lactic acid bacteria (*Lactobacillus acidophilus*), which converts milk sugar lactose into lactic acid (Panesar, 2011). The viable lactic acid bacteria can colonise in gastrointestinal tract and thus, it can inhibit the foodborne pathogens. Starter cultures like *Lactobacillus bulgarius*, *Streptococcus thermophilus* and *Bifidobacterium bifidum* have been reported for protection against gastrointestinal infections and immune-stimulatory effects (Gandhi D, 2000). The health-promoting product like fermented table olives with starter cultures are extensively studied for the cholesterol-lowering activity in blood serum (Bonatsou et al., 2017). Latest studies revealed that fermented foods

have significant anti-cancer effects and it has been proved that bacillus strains isolated from chungkukjang a Korean fermented soy paste, can act against AGS human gastric carcinoma cells (Seo et al 2009). A study revealed that probiotic bacteria could reduce allergic reactions, modify the antigen, and cause less immunogenicity. It also strengthens the gut mucosal lining and diminishes intestinal permeability (Licciardi et al. 2013, Furrrie 2005, Prescott and Björkstén 2007). The human gut microflora is not the same from one region to other. A recent study on healthy Indian people living at sea level and high altitude areas revealed that firmicutes dominate their gut microflora. The community living at low altitudes had a unique microbiome with higher diversity and homogeneity (Daset et al., 2018). This study aims to isolate indigenous starter culture bacteria with probiotic potential for industrial applications, especially in the dairy industry. The consumption of probiotic functional foods is better to restore gut microbiota in people, mainly seniors. The development of industrial starters with probiotic potential would be an added plus to food supplements like improved quality and shelf life.

MATERIALS AND METHODS

Sources and Isolation

Various samples like curd, fermented coconut water, fresh curd, raw milk, sourdough, and homemade yogurt were collected to isolate Lactic acid bacteria and stored in a sterile condition until used. These samples were serially diluted (10⁻¹ to 10⁻⁸) using 0.81% of normal saline. 100 µL of sample from each dilution were spread aseptically on MRS Agar medium (Himedia laboratories, India) supplemented with 8 g/L calcium carbonate, and the culture plates were incubated at 37°C for 48h (Coico, 2005). The bacterial colonies that show a sizeable clear zone was picked and named then sub-cultured in MRS agar media. *Streptococcus thermophilus* (ST) and *Lactobacillus rhamnosus* (WB) were used as positive control and glycerol stocks of all isolated cultures were made in 20% glycerol and stored at -80°C.

Screening for biological properties of Lactic acid bacteria

The primary identification tests of lactic acid bacteria comprise gram staining (Coico, 2005) and catalase test. Catalase test was done by using the slide method (Reiner, 2010). The fermentation type of LAB was identified based on lowering the pH by acid production and gas generation. In order to identify the fermentative type, MRS broth with 0.05 g/L bromocresol green was taken in test tubes and sterilized by autoclaving, each culture was aseptically inoculated and kept for incubation at 37°C, and a tube with no culture was taken as control. The culture with the presence of gas and colour change was taken as heterofermentative and those with colour change only identified as homofermentative (Kostinek et al., 2005)

Starter culture properties

Acidification: Acid production of the isolates was identified by inoculating each isolate to MRS broth (pH 7) and incubated for 12 hrs. The reduction in pH is monitored by a pH meter (Oakton pH700) and those cultures bring down the pH in the range 2.5-3 were considered a good acid producer. **Milk clotting:** It was done by inoculating overnight grown culture (10ml/L) to sterile 50 g/L skim milk and incubated at 37°C for up to 24h. The cultures that showed milk clotting within 12h were considered potent for milk clotting. **Proteolytic activity:** To check the proteolytic activity MRS medium supplemented 0.05 g/L skim milk was prepared and to the skim milk MRS agar plates, 10 µL of overnight cultures were spotted and the plates were dried then incubated at 37°C for 24 – 48h and checked for the clear zone. **Di-acetyl or acetoin production:** The Production of diacetyl or acetoin by cultures indicates the butter flavour development ability. To ensure this, 1mL supernatant of each culture added 0.5mL alpha naphthol and 0.2 mL of KOH/Creatine reagent. The formation of a red ring indicated a positive reaction in a few seconds and from 5 to 10 minutes (Chen et al., 2017)

DNA isolation and 16s PCR for genotypic identification

Genomic DNA of isolates are extracted and purified by using a DNA isolation kit (QIAGEN, United States) and the purified DNA samples were subjected to 16S PCR by using universal primers (Forward primer 16S F', Reverse primer 1429 R') and then the Purified PCR product was then sequenced, and BLAST analysis was done.

Probiotic Properties

Bile salt tolerance: In order to check the tolerance to bile salt, 100 µL of overnight grown cultures were inoculated to 10 mL MRS medium containing 8 g/L of bile salt and incubated for 24h at 37°C, MRS broth without bile salt was used as control. The survival percentage was calculated by the plate count method with respect to the CFU formed in control. Each experiment was done in duplicates (Walker & Gilliland, 1993). **pH tolerance:** A volume of 100 µL overnight grown cultures were inoculated to 10 ml MRS broth, adjusted to pH 3.5 by adding concentrated HCl. MRS broth with pH seven was used as a control. The inoculated cultures were incubated at 37°C for 24h and the survival percentage was calculated by the plate counting method concerning the control plate. Each experiment was done in duplicates (Bao et al., 2010). **NaCl tolerance study:** 100 µL of the pre-incubated culture was inoculated to 10 ml MRS broth containing 60 g/L sodium chloride and incubated at 37°C for 24h. The inoculated MRS broth without NaCl is used as a control and the survival percentage was calculated by using the plate count method. Each experiment was done in duplicate. **Cell surface hydrophobicity:** The hydrophobic nature of cell surface of cultures was identified by the method described by Tamang et al. (2000). Overnight grown cells were pelleted by centrifugation and were added an equal volume of Ringer solution and mixed well. The initial OD of the cell suspension was measured at 600nm, then an equal

amount of Hexadecane was added to the cell suspension and mixed well for 2 minutes and kept aside to separate the two layers. The absorbance of the lower layer was measured at 600nm. The percentage of hydrophobicity was calculated by the difference of initial and final OD with respect to the initial OD. **β-Galactosidase activity:** MRS agar plates were spread with 100µl of X-gal and then dried. The dried plates were streaked with each culture and incubated at 37°C for 48h. The blue/green colour formation of colonies indicates the presence of the β galactosidase enzyme (Karasová et al., 2018). **Anti Microbial activity:** Each culture was spotted in MRS agar plates and then incubated at 37°C for 24h. *E.coli*, *S.aureus*, *S.typhi*, *M.smegmatis*, *P.aeruginosa* cultures were obtained from Microbial Processes and Technology Division, CSIR IIIT Thiruvananthapuram and used as indicator strains, these cultures were seeded in Muller Hinton Agar at bearable temperature and 20 mL each culture were poured aseptically over the incubated isolates on MRS agar. After solidifying, the culture plates were kept at 37°C for 48h and checked for the development of the inhibition zone. The zone diameter inhibition (ZDI) formed was measured and interpreted according to (Shokryazdan et al., 2014) that is ZDI >20mm, 20-10mm, <10mm considered as strong, intermediate and weak inhibitions, respectively. **Cross streak method for consortia formulation:** MRS agar plates were streaked with isolates so that each culture could contact each other, and then the plates were incubated at 37°C for 48h. The inhibited growth of isolates indicates negative results for consortia development (Barefoot & Klaenhammer, 1983).

RESULT AND DISCUSSION

Typical starter cultures may not have probiotic potential, so the isolation of microorganisms with both probiotic and starter will be very significant for industrial applications. A total of 35 isolates were obtained from various sources. Among them, 15 isolates showed the primary biological characteristics of lactic acid bacteria like gram-positive catalase-negative, homofermentative and heterofermentative nature (Table 1) and then screened for their starter culture properties. Five isolates, namely FCW2, FCW4, FC1, CUD2 and CUD3, showed the best acidification by reducing pH to 3.5. CUD2 and CUD3 showed the best milk clotting activity within 6h. The other two cultures, FCW2 and FCW4, showed proteolytic activity by producing a clear zone in MRS agar plates supplemented with skim milk. After screening for diacetyl or acetoin production, which gives buttery flavour to the fermented foods FCW2, FCW4, CUD2 and CUD3 showed the best activity by producing red coloured ring within five minutes and it shows these cultures can produce flavour compounds. Based on the biological tests for lactic acid bacteria and starter culture properties, the best five isolates were selected for identification by 16S rRNA sequencing. The identified cultures are *Lactobacillus plantarum* FCW2, *Lactobacillus plantarum* FCW4, *Lactobacillus fermentum* FC1, *Streptococcus thermophilus* CUD2 and *Streptococcus thermophilus* CUD3. The 16s rRNA sequences were submitted into the NCBI database and the accession numbers are MT176609, MT180563, MT180729, MT176488, MT176494 (Table 2).

Table 1 Biological and Starter culture properties of lactic acid bacteria isolated from various sources

SOURCE	Isolate code	gram staining	catalase test	Fermentation type	acid production	Proteolytic activity	Milk clotting within six hours	Milk clotting after 18-24 h	Di acetyl or acetoin production
Sourdough	SDO1	+	-	Homo	++	-	-	++	-
Sour curd	CUD2	+	-	Homo	+	-	+	+++	+++
	CUD3	+	-	Homo	+	-	+	+++	+++
Fermented Coconut water	FCW1	+	-	Hetero	++	-	-	-	++
	FCW2	+	-	Hetero	+++	+	-	+	+++
	FCW3	+	-	Hetero	++	-	-	-	++
Raw Milk	FCW4	+	-	Hetero	+++	+	-	+	+++
	RM1	+	-	Homo	+	-	-	+	++
	RM2	+	-	Homo	+	-	-	+	++
Fresh curd	RM3	+	-	Homo	+	-	-	+	+
	FC1	+	-	Hetero	+++	-	-	+++	+
Homemade yogurt	HY1	+	-	Homo	+	-	-	+++	+
	HY2	+	-	Homo	++	-	-	++	+
	HY3	+	-	Homo	+	-	-	++	+
	HY4	+	-	Homo	+	-	-	++	+

Legend:+++ sign indicates high production, ++ sign indicates medium production, + sign indicates low production, and - sign indicates no production

Table 2 species identification of lactic acid bacteria with starter culture properties based on 16s rRNA gene

Culture code	Isolation Source	Name of the isolate	NCBI Accession No
FCW2	Fermented coconut water	<i>L.plantarum</i>	MT176609
FCW4	Fermented coconut water	<i>L.plantarum</i>	MT180563
FC1	Fresh curd	<i>L.fermentum</i>	MT180729
CUD2	Sour Curd	<i>S.thermophilus</i>	MT176488
CUD3	Sour curd	<i>S.thermophilus</i>	MT176494

The probiotic potentials of the five selected starters were done and among five isolates, *L.plantarum* FCW4 and *L.plantarum* FCW2 showed a survival rate of 79.87% and 79.29%, respectively the presence of 8 g/L bile salt. *L.fermentum* FC1 bared a survival rate of 88.87% and only 35.53% of *S.thermophilus* CUD3 could survive and the culture CUD2 was inhibited. One of the positive control cultures, *S.thermophilus* ST, showed 29.82 percent of viability and *L.rhamnosus* WB could not grow at this concentration of bile(Figure 1A)The pH tolerance study of five isolates was identified (Figure 1B). In this study, all the isolates showed more than sixty percent survival in the pH 3.5 and *L.fermentum* FC1 was found to be the best culture with a tolerance of 99.74 percent.The Survival capacity of the isolated in the presence of 60 g/L NaCl was checked and found 60% tolerance in all cultures.*L.plantarum*FCW4 showed top tolerance ability (Figure1C). For cell surface hydrophobicity, only *L. plantarum* strain FCW2 showed 88.9 % of hydrophobicity; other strains like FCW4, FC1, CUD2, CUD3 are significantly less hydrophobic compared to the control strains ST and WB (Figure 1D).It was interesting to see the β -galactosidase activity of the lactic acid starter bacteria,all of them can produce the β -galactosidase enzyme, which is visible by the blue colour around the colonies *L.plantarum* FC1 showed the best β -Galactosidase activity. Inhibition of foodborne pathogens has been checked and it was found the *L.plantarum* FCW2,*L.plantarum* FCW4, and *L.fermentum* FC1 exhibited significant inhibition against all the indicator strains. The starter culture's compatibility waschecked by cross streak assay in MRS agar plate and it was found that all five cultures did not show any inhibition of each other.

CUD2 and CUD3 showed best milk clotting within 6h and they are appropriate for cheese and yogurt. Production of proteolytic enzymes in lactic acid bacteria is extensively studied and it is crucial for cheese ripening, better growth during fermentation and maintaining viability during fermentation (Onkoret et al., 2007). *L.plantarum* FCW2 and *L. plantarum* FCW4 showed well proteolytic activity by forming clear zone like the LAB isolates from different fruits with proteolytic activity by demonstrating a clear zone in skim milk agar (Abubakr et al., 2017). Diacetyl or acetoin gives buttery flavour to the fermented milk products.In a study by Franciosi et al.,(2009), wild lactic acid bacteria can produce diacetyl, and it is a technological property of LAB. All five isolates are good producers of these flavour compounds.

The following are the primary characteristics for lactic acid bacteria to be a probiotic strain (1)they should be under GRASS status (2) they should be capable of tolerating bile salt, sodium chloride salt, pH (3) they should show antagonism against pathogenic bacteria (4) they should be viable during processing and storage (Roberfroid 2000, Lin et al., 2006, Lonkar et al., 2005). Exo polysaccharide-producing strains of yogurt starter bacteria can tolerate low pH and bile salts (0.15 to 0.3%) (Bokeet et al., 2010) and avital ability of a probiotic strain to survive the presence of gastric bile salt. It was interesting to see that our isolates can tolerate upto 0.8% of bile salt concentration. In order to establish probiotic effects, the bacteria need to go through the stomach and the inner wall of the intestine .usually, the pH of the human stomach ranges from 1.5 to 3.5, so those bacteria should have the ability of pH tolerance (Jacobsenet al., 1999)*L.fermentum* expressed 99.9% tolerance of pH 3.5 and (Zoumpopoulou et al., 2008) also reported that *L.fermentum* ACA-DC 179 could survive in a pH of 2.5. Tolerance to NaCl is an essential technological property of lactic acid bacteria to preserve and ferment vegetables, sausages, and cheese making. As Ge et al. (2011) reported, when LAB is exposed to osmotic tolerance due to salts and sugars, it will inhibit growth and fermentation, so the selection of osmotic tolerant LAB would be the promising strains for the application in various food fermentation and preservation industries. The five starter cultures isolated exhibited more than 60% of survival capacity in 60 g/L of NaCl.The aggregation of bacterial cells to the mucus layer of the intestine is one of the critical probiotic characters and it can be studied by measuring cell surface hydrophobicity. A previous report explained the hydrophobicity of bacteria's outermost surface and their attachment to the epithelial surface. So the higher value of hydrophobicity is proportional to the adhesion of bacterial cells toward the epithelial layer of the intestine (Rosenberg et al.,1980). In our work, the highest value of hydrophobicity was expressed by *L.plantarum* FCW2 and it is found to be greater than the value of hydrophobicity reported by Vinderola and Reinheimer (2003).

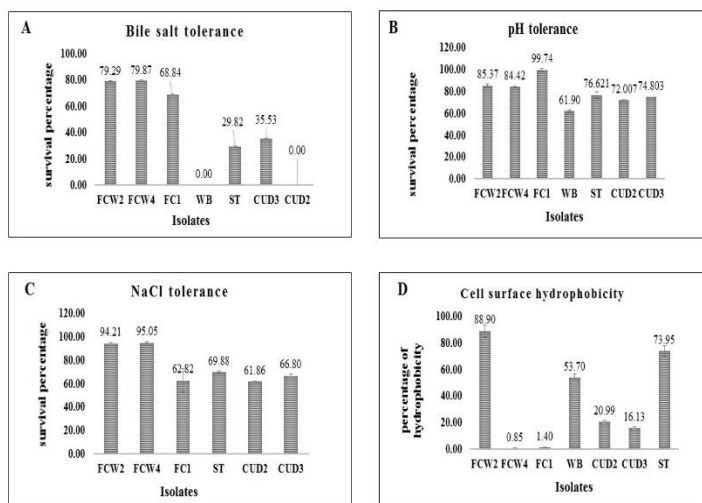


Figure 1 Probiotic properties of isolates, **A** tolerance against 0.8% of bile salt, **B** tolerance against 3.5 pH, **C** tolerance against 6% NaCl, **D** percentage of cell surface hydrophobicity

The cultures in the developed consortia satisfy various starter culture and probiotic properties for successful application in the food industry. The three lactobacilli *L.plantarum* FCW2,*L.plantarum* FCW4 and *L.fermentum* FC1 have good acidification potential by producing lactic acid, which is an essential feature for making pickles and milk products. Acidification is an crucial property for the production of nunu a fermented milk product in Ghana (Akabanda et al., 2014). *Streptococcus thermophilus* is a probiotic mesophilic bacteria that is used in various fermented milk products like yogurt and cheese (Michel et al., 2001) reported that; the use of *S.thermophilus* and other bacteria showed the disappearance of leftover galactose in cheddar cheese. The *S.thermophilus* stains

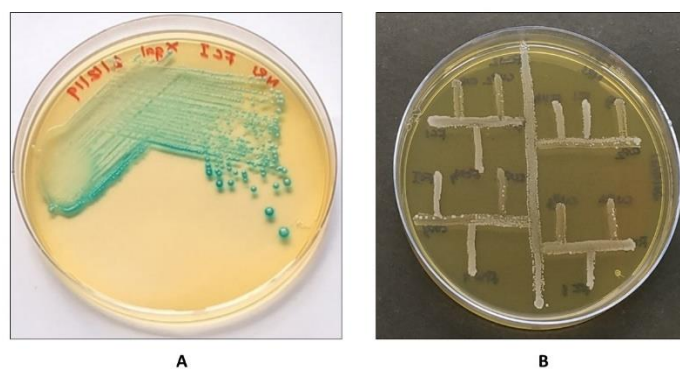


Figure 2 A β -Galactosidase activity of *L. Fermentum* in MRS agar supplemented with X-gal, **B** Cross streak assay showing compatibility of Five potent isolates

The β -Galactosidase enzyme is widely used in the dairy industry and helps treat lactose intolerance (Gheyntchi et al. 2015, Vasiljevic and Jelen 2001). So the starter cultures which can produce β -galactosidase enzyme will be an added advantage when it is used for dairy foods. All isolated starter bacteria were found to produce this enzyme, particularly *L.fermentum* FC1 (Figure 2A). The integration of probiotics has been proved it is efficient in preventing or countering multidrug-resistant biofilm-forming bacterial pathogens, and probiotic bacterial effects are strain-specific, so this aspect makes them vital in health and food preservation (Vuottoet al., 2014, Eid et al., 2016). In our study, the three lactobacillus strains, *L.plantarum* FCW2, *L.plantarum* FCW4 and *L.fermentum* FC1, the strain *L.*

plantarum FCW4 exhibited potent inhibition against all the indicator pathogen, which is similar to the earlier report (Divya et al., 2012) and the two streptococcal strains did not show any inhibition (Table 3). An earlier report revealed that indigenous Lactobacilli have the ability to act like antibiotics against potentially pathogenic bacteria (Halder et al., 2017) and we obtained similar results. A Cross streak assay was done (Sharma et al., 2017) for checking the compatibility of the probiotic bacterial strains and found that five starter bacteria are compatible with each other (Figure 2B) and they are promising cultures to be used together in various food formulations.

Table 3 zone inhibition diameter of starter cultures against indicator strains

Zone inhibition diameter (mm)					
Cultures	<i>E.coli</i>	<i>S.aureus</i>	<i>M.smegmatis</i>	<i>S.typhi</i>	<i>P.aeruginosa</i>
<i>L.plantarum</i> FCW2	40.5±0.35	38±2.12	36.5±2.47	27±2.12	20.5±0.35
<i>L.plantarum</i> FCW4	57±0.71	51.5±0.35	40±1.41	32±0.0	29.5±0.35
<i>L.fermentum</i> FC1	32.5±1.77	27.5±1.77	38.5±1.60	37.5±1.77	33±0.71
<i>S.thermophilus</i> CUD2	0	0	0	0	0
<i>S.thermophilus</i> CUD3	0	0	0	0	0

CONCLUSION

The findings of the work denote that the indigenous culture consortia formulated are valid candidates of starter cultures and probiotics. The five isolates, including two streptococci and three lactobacilli, have both starter culture and probiotic potential and each strain is different in exerting the effects. The probiotic characteristics may be variable among species and even among the same strains. So an adequate consortia formation is recommended for specific food applications and the formulated consortia would be hopeful for dairy food applications and other fermented foods.

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