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Probiotic Characterization of Indigenous Lactic Strains using Foldscope and Development of Functional Yogurt

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ABSTRACT

The study is on isolation and characterization of lactic acid bacteria from fermented food samples of Garo Hills through Foldscope and thereafter study of the probiotic potentiality of the strains and finally production of value-added functional yogurt. The microbiological analysis was done using foldscope leading to isolation of 51 strains from a total of 15 fermented food samples. Biochemical characterization of the isolates for the confirmation of genus and species through sugar fermentation pattern using API kit led to the isolation of four strains- *Lactobacillus brevis* FJWJi, *Lactobacillus pentosus* RBCi, *Lactobacillus fermentum* FRWGv and *Lactobacillus delbrueckii* RBWGii, selected on the basis of their coagulating efficiency in milk medium. Probiotic attributes of the selected isolates were determined. The isolates could survive high pH conditions of pH2 and pH3 with viability of log 4.47 CFU/ml by *Lactobacillus pentosus* RBCi and tolerate 0.5-2% of bile salt with viability of log 6.51 CFU/ml by *Lactobacillus brevis* FJWJi. The isolates could hydrolyse bile salt but zone of precipitation was absent possibly due to the source of isolation. The isolates were susceptible to most of the antibiotics except for Nalidixic acid and Vancomycin. The cellular auto aggregation percentage for the isolates ranged from 63% to 27% and *Lactobacillus brevis* FJWJi showed the highest cell surface hydrophobicity of 26%. Therefore, the isolates possess the potentiality of probiotics. The four isolates were used to develop functional cow milk yogurt and storage study was done thereafter. Samples of Treatment 3 had the highest acceptance, especially on Day 3 based on the sensory analysis.

Keywords: *Lactobacillus*, probiotics, yogurt, fermented foods, Garo Hills

Microscopes play a crucial role in the field of biology, especially in researches. It is a ubiquitous tool which provides a connection between the largely known macro-world and the obscure micro-world. Among the different types of microscopes used to delve into the miniature world, a new addition is the Foldscope. Foldscope, named so, because it is made of paper and can be folded to make a fully functional microscope using simple origami techniques. This DIY (Do It Yourself) cost efficient and rustic tool is of at-most convenience in the field of biological research and medical screening. With a magnification of 2000X, the foldscope ameliorates the trouble faced by field researchers (Cybulski *et al.* 2014).

Fermented food products are of importance to human health as they provide great health benefits. Meghalaya, in the north eastern region of India, is known for eclectic fermented food products which include fermented soybean (*Kinema, Hawaijhar, Akhone* and *Tungrymbai*) and non-soybean legume foods, alcoholic beverages (*Ghanta, Jaan, Chubitchi*), bamboo shoot foods (*Soibum*, *Mesu*), fermented cereal and pulse foods, fermented vegetables (*Gundruk, Sinke*), fermented smoked fish products (*Ngare, Hentak, Nakham*), preserved meat products and milk beverages (Tamang *et al.* 2012).

Among the numerous microorganisms responsible for the fermentation of the food products, lactic

acid bacteria play a major role. *Lactobacillus* and *Bifidobacterium* are given priority to be used as probiotic, however, *Lactobacillus* is of more importance (Archer and Halami, 2015). Probiotics, as defined by the FAO/WHO are "live microorganisms that, when administered in adequate amounts, confer health benefits on the host" (Joint F.A.O, 2002). Probiotics mean "for life" and are currently in high demand for its beneficial properties. The probiotic attributes of *Lactobacillus* include stabilization of the intestinal microbiota and prevention of intestinal infection. Some strains of *Lactobacillus* are responsible for reduction of cholesterol level, enhancement of lactose intolerance and immune system. In order to function as a probiotic, the *Lactobacillus* must survive the acidic environment and high salt level of the gastrointestinal tract. It should have good adhesion properties and should be persistent enough to resist pathogens by various means (Jiménez *et al.* 2010; Morita *et al.* 2008; Park *et al.* 2005; Zoumpopoulou *et al.* 2008). Studies on the probiotic attributes of *Lactobacillus* are being currently explored widely in different laboratories in the world as it has the potential to be a novel biological therapeutic agent.

In this study, lactic acid bacteria were isolated from different fermented food products of the Garo Hills of Meghalaya, with the help of Foldscope. The isolated strains were identified and characterized and its probiotic potentiality was evaluated. Furthermore, the selected strains were used to prepare functional cow milk yogurt followed by shelf life study. The pH and acidity were checked every other day and the viable cell count was noted as well. Sensory evaluation based on attributes like aroma, appearance and texture, mouthfeel, aftertaste and overall acceptability was done.

MATERIALS AND METHODS

Collection of ethnic fermented food samples from North East region

A total of 15 traditional fermented food products were collected from different regions of the Garo Hills, Meghalaya, India which includes fermented smoked fish (*Nakham*), bamboo shoots (*Me'akri*), rice (*Wanti*) and rice beverages (*Mi'bitchi*). Table 1 represents all the fermented food collected from the Garo Hills.

Enrichment, screening and isolation of lactic cultures

1 ml of the diluted samples were inoculated into tubes of MRS and M17 broth and incubated at 25°C and 37°C for 24 and 48 hrs. Sterile immersion oil was layered on the top to prevent aerobic growth.

Gram staining, Catalase test and Motility Test

The lactic isolates were gram stained to differentiate the gram-positive bacteria from gram negative bacteria (Collins *et al.* 2004). Catalase test was done on the gram-positive isolates (Nelson *et al.* 1995). The hanging drop experiment was performed (MacFaddin *et al.* 2000) and the motility of the bacteria was checked under the light microscope at a magnification of 40X. The growth of the isolates against 6.5% NaCl medium was tested where light inoculum of the isolates was inoculated in the NaCl medium and incubated at 37°C for 24-48 hrs (Hajna and Perry, 1943).

Biochemical tests of screened lactic isolates through API 50CH kit

Few lactic isolates were screened and identified based on the biochemical tests done by using API 50CH kit. Among the 12 isolates, 4 lactic isolates were selected based on their efficiency in curdling of milk. The results obtained from the tested isolates were compared with information from the database, apiweb™ and the fermentation profiles were interpreted (https://apiweb.biomerieux.com) as given in Table 3.

Determination of Probiotic attributes of the selected Lactobacillus isolates

pH

pH conditions of 2.0, 3.0 and 7.0 at time intervals of 0 hr, 2 hr and 4 hr, respectively, was assayed in MRS broth and the survival of *Lactobacillus* isolates in acidic medium was calculated in terms of log CFU/mL (Hati *et al.* 2014).

Table 1: Collected fermented food samples from different places of Garo Hills, Meghalaya

Sl. No.	Sample Name	Place	Isolate Code
	SAMPLE- Fermented fish (*Nakham*) (05 samples)		
1	Fermented fish - 5 types	Jengjal, West Garo Hills	FJWG
2	Fermented fish - 5 types	Rombagre, West Garo Hills	FRG
3	Fermented fish - 3 types	Asanang, West Garo Hills	FAWG
4	Fermented fish - 5 types	Garobadha, South-west Garo Hills	FGSG
5	Fermented fish - 5 types	Rongram, West Garo Hills	FRWG
	SAMPLE- Fermented bamboo shoots (*Me'akri*) (02 samples)		
6	Fermented bamboo shoots- 5 types	Jengjal, West Garo Hills	BJWG
7	Fermented bamboo shoots- 3 types	Songsak, East Garo Hills	BSEG
	SAMPLE- Fermented rice (*Wanti*) (04 samples)		
8	Fermented rice - 2 types	Resubelpara, North Garo Hills	RRNG
9	Fermented rice - 3 types	Adokgre, East Garo Hills	RAEG
10	Fermented rice - 2 types	Selbalgre, West Garo Hills	RSWG
11	Fermented rice- 3 types	Babadam, West Garo Hills	RBWG
	SAMPLE- Fermented rice beverage (*Mi'bitchi*) (04 samples)		
12	Fermented rice beverage - 3 types	Selbalgre, West Garo Hills	RBS
13	Fermented rice beverage - 2 types	Babadam, West Garo Hills	RBB
14	Fermented rice beverage - 2 types	Chandigre, East Garo Hills	RBC
15	Fermented rice beverage - 3 types	Boldoka, West Garo Hills	RBG

Antibiotic Susceptibility test

The antibiotic disk diffusion (HiMedia, Mumbai, India) method was used to check the antibiotic susceptibility of the LAB isolates towards various antibiotics (Agaliya *et al.* 2012). The diameter (mm) of zone of inhibition around the antibiotic discs were measured using antibiotic zone scale and results were expressed in terms of resistance or susceptibility by comparing with the interpretative zone diameters given by Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2011).

Bile Tolerance

Bile salt solution was prepared using oxgall powder (HiMedia, India). The Bile Tolerance test was conducted by the method followed by Hati *et al.* (2014). The survival of Lactobacillus isolates in bile was calculated in terms of log CFU/mL.

Bile Salt Hydrolase Activity

Direct plate assay method was employed for detection of BSH activity. The active cultures were streaked on previously solidified MRS agar plates containing 0.5% (w/v) bile, sodium taurodeoxycholate and sodium taurocholate and 0.37 g/L of $CaCl_2$. The petri plates were incubated at 37°C anaerobically for 3 days in GasPak jar. The activity will be indicated when the hydrolysed product of the salt precipitated in the agar medium in and around the colony (Lee *et al.* 2011).

Cellular auto-aggregation

The aggregation rate of the *Lactobacillus* isolates was calculated as per method of Del *et al.* (2000) with some modification. The percentage difference between the initial and final absorbance has given an index of cellular auto-aggregation that can be expressed as: Agg. % = $100 \times (A_{initial} - A_{final})/Ab_{initial}$; Where, $A_{initial}$: initial absorbance at 600nm; A_{final}: final absorbance at 600nm; Agg%: Aggregation index.

Cell Surface Hydrophobicity

The percentage of cell surface hydrophobicity

Table 2: Morphological and Physiological characters of isolates from different fermented food samples obtained from various regions of Garo Hills, Meghalaya

Sl. No.	Isolates	Medium and Temperature (°C)	Colony Characteristics	Shape	Catalase Test	Motility Test	Growth at 6.5% NaCl	Gram Reaction
Sample: Fermented fish (FJWJ); Jengjal, West Garo Hills								
1	FJWJ i	MRS, 37°C	Small, entire, rough, creamish white	Cocci	-ve	-ve	+ve	+ve
2	FJWJ ii	MRS, 37°C	Small, pinpoint	Cocci	-ve	-ve	+ve	+ve
3	FJWJ iii	M17, 27°C	Medium, slightly raised from centre, creamish white	Cocci	+ve	-ve	-ve	+ve
4	FJWJ iv	M17, 27°C	Medium, flat, rough, translucent	Bacilli	+ve	-ve	-ve	+ve
5	FJWJ v	MRS, 25°C	Pinpoint	Cocci	+ve	-ve	-ve	+ve
Sample: Fermented fish (FRG); Rombagre, West Garo Hills								
6	FRG i	M17, 37°C	Pinpoint, off white	Cocci	-ve	-ve	+ve	+ve
7	FRG ii	MRS, 25°C	Medium, slightly raised, entire, off white	Cocci	-ve	-ve	+ve	+ve
8	FRG iii	M17, 37°C	Medium, slightly raised, entire, off white	Cocci	-ve	-ve	+ve	+ve
9	FRG iv	MRS, 25°C	Medium, entire, translucent	Cocci	+ve	-ve	+ve	+ve
10	FRG v	MRS, 37°C	Pinpoint, translucent	Cocci	-ve	-ve	+ve	+ve
Sample: Fermented Fish (FAWG); Asanang, West Garo Hills								
11	FAWG i	MRS,37°C	Small, translucent	Bacilli	-ve	-ve	-ve	+ve
12	FAWG ii	M17, 37°C	Pinpoint, entire, translucent	Cocci	-ve	-ve	+ve	+ve
13	FAWG iii	M17, 25°C	Medium, translucent	Cocci	-ve	-ve	-ve	+ve
Sample: Fermented Borali Fish (FRWG); Rongram, West Garo Hills								
14	FRWG i	MRS, 25°C	Medium, flat, entire, translucent	Cocci	-ve	-ve	+ve	+ve
15	FRWG ii	MRS, 25°C	Medium, raised from center, off white	Cocci	-ve	-ve	+ve	+ve
16	FRWG iii	M17, 37°C	Pinpoint, spindle	Cocci	-ve	-ve	-ve	+ve
17	FRWG iv	MRS, 25°C	Small spindle, entire	Cocci	-ve	-ve	-ve	+ve
18	FRWG v	MRS, 37°C	Small, entire, translucent	Bacilli	-ve	-ve	-ve	+ve
Sample: Fermented Fish (FGSG); Garobadha, Southwest Garo Hills								
19	FGSG i	MRS, 37°C	Small, entire, translucent	Bacilli	-ve	-ve	-ve	+ve
20	FGSG ii	M17, 25°C	Small, off white,	Cocci	-ve	-ve	+ve	+ve

21	FGSG iii	MRS, 25°C	Medium	Cocci	-ve	-ve	+ve	+ve
22	FGSG iv	M17, 25°C	Medium	Cocci	-ve	-ve	+ve	+ve
23	FGSG v	MRS, 37°C	Larger	Cocci	+ve	-ve	–ve	+ve
Sample: Fermented bamboo shoots (BJWG); Jengjal, West Garo Hills								
24	BJWG i	MRS, 37°C	Small, entire, rough, translucent	Bacilli	-ve	-ve	-ve	**+ve**
25	BJWG ii	MRS, 37°C	Small, off white,	Cocci	-ve	-ve	+ve	+ve
26	BJWG iii	MRS, 25°C	Medium	Cocci	-ve	-ve	+ve	+ve
27	BJWG iv	M17, 37°C	Medium	Cocci	-ve	-ve	+ve	+ve
28	BJWG v	MRS, 25°C	Larger	Cocci	+ve	-ve	-ve	+ve
Sample: Fermented bamboo shoots (BJWG) ; Songsak, East Garo Hills								
29	BJWG i	MRS, 37°C	Medium, entire, rough translucent	Short rods in chins	-ve	-ve	-ve	+ve
30	BJWG ii	M17, 37°C	Small, entire, off white	Cocci	-ve	-ve	+ve	+ve
31	BJWG iii	MRS, 25°C	Medium, entire, rough, trannslucent	Cocci	-ve	-ve	+ve	+ve
Sample: Fermented rice (RRNG); Resubelpara, North Garo Hills								
32	RRNG i	MRS, 37°C	Pinpoint, transparent	Bacilli	-ve	-ve	-ve	+ve
33	RRNG ii	MRS, 37°C	Pinpoint, transparent	Bacilli	-ve	-ve	-ve	+ve
Sample: Fermented rice (RAEG); Adokgre, East Garo Hills								
34	RAEG i	MRS,37°C	Pinpoint, transparent	Bacilli	-ve	-ve	-ve	+ve
35	RAEG ii	MRS,37°C	Medium, translucent	Short thick bacilli	+ve	-ve	-ve	+ve
36	RAEG iii	MRS,37°C	Pinpoint, transparent	Bacilli	-ve	-ve	-ve	+ve
Sample: Fermented rice (RSWG); Selbalgre, West Garo Hills								
37	RSWG i	MRS, 25°C	Pinpoint, transparent	Cocci	-ve	-ve	-ve	+ve
38	RSWG ii	MRS, 37°C	Pinpoint, transparent	Cocco bacilli	-ve	-ve	-ve	+ve
Sample: Fermented rice (RBWG); Babadam, West Garo Hills								
39	RBWG i	MRS, 37°C	Pinpoint, transparent	Cocco bacilli	-ve	-ve	-ve	+ve
40	RBWG ii	MRS, 37°C	Small, translucent	Thick rods in chains	-ve	-ve	-ve	+ve
41	RBWG iii	MRS,25°C	Small, transparent	Cocci	-ve	-ve	+ve	+ve
Sample: Fermented rice beverage (RBS): Selbalgre, West Garo Hills								
42	RBS i	MRS, 37°C	Pinpoint, translucent	Thin bacilli	-ve	-ve	-ve	+ve
43	RBS ii	MRS,25°C	Small, round, chalky white	Large oval shaped bacilli	+ve	-ve	-ve	+ve
44	RBS iii	MRS, 37°C	Pinpoint, transparent	Bacilli with curved end	-ve	-ve	-ve	+ve

Sample: Fermented rice beverage (RBB); Babadam, West Garo Hills								
45	RBB i	MRS, 37°C	Pinpoint, transparent	Short thick Bacilli	-ve	-ve	-ve	+ve
46	RBB ii	MRS,25°C	Pinpoint, translucent	Cocci	-ve	-ve	-ve	+ve
Sample: Fermented rice beverage (RBC); Chandigre, East Garo Hills								
47	RBC i	MRS, 37°C	Pinpoint, transparent	Long bacilli in chains with curved ended	-ve	-ve	-ve	+ve
48	RBC ii	MRS,25°C	Pinpoint, translucent	Thin bacilli	-ve	-ve	-ve	+ve
Sample: Fermented rice beverage (RBG); Boldoka, West Garo Hills								
49	RBG i	MRS,25°C	Pinpoint, translucent	Thin bacilli	-ve	-ve	-ve	+ve
50	RBG ii	MRS, 37°C	Pinpoint, transparent	Thick rods in pairs	-ve	-ve	-ve	+ve
51	RBG iii	MRS, 37°C	Small, off white	cocci	-ve	-ve	-ve	+ve

was determined by following Hati *et al.* 2014. The percentage hydrophobicity (%H) is measured based on the following formulae:

$$\% \text{ H} = \frac{A0 - A1}{A0} \times 100$$

Where, A0: Initial O.D600, A1: Final O.D600, %H: Percentage of hydrophobicity

Preparation of starter inoculum for yogurt production

The four lactic isolates obtained (FJWJi, RBCi, FRWGv and RBWGii) were revived in MRS broth individually and were transferred into sterile rehydrated skim milk followed by preservation in glycerol stocks by placing them in 1 ml aliquots in cryovials and further storing at −40°C. Each of the activated *Lactobacillus* cultures were inoculated into skimmed milk medium and incubated at 37°C for another 18hrs after two consecutive transfers of the lactic strains in MRS broth (HiMedia, India) which were incubated at 37°C for 24hrs. Finally, the working lactic strains were inoculated into cow milk medium in order to check their activity (Hati *et al.* 2015).

Yogurt preparation

Cow milk was pasteurized at 65°C for 30 mins to which 7% of skimmed milk and 15% sugar was added. The medium was let to cool down to 40°C to which 0.7ml of respective food grade flavour was added and mixed well. Subsequently, 5-6% of the starter culture from the skimmed milk medium was added to the milk medium and mixed thoroughly. Equal volume of the content was then transferred into food grade sterile cups and incubated at 37°C.

The following treatments were done for each sample:

- Treatment 1- 1000ml Cow Milk + 7% Skimmed Milk + 15% Sugar + 0.7ml flavour (Raspberry) + 5% Culture
- Treatment 2- 1000ml Cow Milk + 7% Skimmed Milk + 15% Sugar + 0.7ml flavour (Kala Khatta) + 5% Culture
- Treatment 3- 1000ml Cow Milk + 7% Skimmed Milk + 15% Sugar + 0.7ml flavour (Elaichi) + 5% Culture
- Treatment 4- 1000ml Cow Milk + 7% Skimmed Milk + 15% Sugar + 0.7ml flavour (Rose White) + 5% Culture

Shelf Life Study

After an incubation time of 12-18 hours, the cups containing the yogurt was kept in refrigeration conditions and shelf-life study was conducted on Day 0, 3, 5 and 7. Organoleptic evaluation, pH and titratable acidity and microbiological analysis of the yogurt was done for Day 0, 3, 5 and 7.

Determination of titratable acidity of yogurt

The method described by Dave and Shah (1997) was followed to determine the titratable acidity of the yogurt.

pH determination

A digital pH meter was used to measure the pH of yogurt at 25°C after being calibrated with freshly prepared pH 7.0 and 4.0 standard buffers.

Microbiological analysis

Enumeration of total number of aerobes, lactic acid bacteria, yeast, molds and coliforms was done by standard plating methods in MRS (deMan Rogosa Sharpe), EMB (Eosin Methylene Blue) and SCA (Sabourand Chloramphenicol Agar) agar plates for lactic acid bacteria and aerobes, coliforms and yeast and molds respectively (Marshall, 1993).

Organoleptic Evaluation

A nine-point hedonic scale was used to evaluate the prepared functional yogurt, based on qualitative attributes like taste, texture, colour, aroma, flavour and overall acceptability (Peryam *et al.* 1952).

Statistical Analysis

All data obtained during the period of shelf life study was analysed statistically using SPSS version 20 (IBM SPSS Statistics 20). P value ≤ 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Isolation and Characterization of the isolates from different fermented food samples

A total of 110 colonies were picked up from 15 ethnic fermented food samples and 51 isolates were screened based on gram reaction. All the isolates were gram positive, having rod or cocci shape. They were all catalase negative. Few isolates also showed growth against 6.5% NaCl medium and all the isolates were non-motile in nature based on the hanging drop experiment. *Lactobacillus* isolates were screened from the 51 isolates through API 50 CH Kit and it was found that all the isolates are Glycerol, Erythritol, D-Arabinose, Xylitol, Inulin and Glycogen negative. Some isolates are D-Galactose positive but all the isolates were D-Glucose positive. Except for 3 strains, all the isolates were D-Lactose positive (bovine origin). Based on the results from the API kit the 12 isolates were identified. These isolates were subjected to curdling of skimmed milk and based on the curdling efficiency of the isolates, 4 *Lactobacillus* namely *Lactobacillus brevis* FJWJi, *Lactobacillus pentosus* RBCi, *Lactobacillus fermentum* FRWGv and *Lactobacillus delbrueckii* RBWGii were selected and further analysis of the probiotic attributes were done. Table 2 represents the morphological and physiological characters of the isolates from different fermented food products.

Determination of Probiotic Attributes of the selected isolates

pH tolerance

Table 3 represents the viability of different *Lactobacillus* isolates at different pH conditions. In comparison to control (i.e. pH 7), the viability of cell in acidic pH conditions decremented. *Lactobacillus pentosus* RBCi and *Lactobacillus brevis* FJWJi showed highest survival rate at pH 2 and pH 3. The survival rate of *Lactobacillus fermentum* FRWGv fell steep down to log 3.74 CFU/ml at pH 2 for 4hours. There has been 3 log reduction from the control at pH 2 and pH 3 for most of the isolates except for *Lactobacillus fermentum* FRWGv which showed 5 log reduction in cell viability.

Bile Tolerance

Lactobacillus brevis FJWJi could survive the given bile concentration of 0.5% the most whereas it was found that *Lactobacillus delbrueckii* RBWGii showed decrementing viability at the same concentration. In comparison to control, there was 1-2 log reduction in the viable cell count. The Fig. 1 represents the bile tolerance index of the isolates. *Lactobacillus brevis* FJWJi had a viable cell count of 6.51 log CFU/ml s

Table 3: Identified isolates through API kit

Sl. No.	Strains	Identified Organisms	Sl. No.	Strains	Identified Organisms
A	FJWJ i	*Lactobacillus brevis*	E	RRNG ii	*Lactobacillus plantarum*
B	RBC i	*Lactobacillus pentosus*	F	RBWG ii	*Lactobacillus delbruekii*
C	FRWG v	*Lactobacillus fermentum*	G	BJWG i	*Lactobacillus plantarum*
D	FAWG i	*Lactobacillus fermentum*	H	FGSG i	*Lactobacillus plantarum*
I	RAEG i	*Lactobacillus acidophilus*	J	RBS iii	*Lactobacillus delbrueckii*
K	FJWJ v	*Lactococcus lactis*	L	FRG ii	*Lactococcus lactis*

***L. delbrueckii* (RBS iii)** ***L. lactis* (FJWJ v)** ***L. brevis* (FJWJ i)** ***L. pentosus* (RBC i)** ***L. acidophilus* (RAEG i)**

Fig. 1: Identified *Lactobacillus* isolates through API 50 CH kit

whereas *Lactobacillus delbrueckii* RBWGv had a viable cell count of 5.25 log CFU/ml after an incubation period of 4hrs.

Bile salt Hydrolase test

The efficacy of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection and similarly survival rate of these organisms in various bile salts (sodium taurodeoxycholate and sodium taurocholate) have been depicted in this study. Although zone of salt precipitation was not clearly visualized in the plates but the growth of cultures shows partial deconjugation of bile salts. Fig. 3 shows the plates on which the isolates grew but with no zone of salt precipitation.

Antibiotic Susceptibility

Table 4 represents the zone of inhibition of the isolates against various antibiotics. The *Lactobacillus* isolates were found to be susceptible to most of the antibiotics except for Nalidixic acid and Vancomycin. *Lactobacillus fermentum* FRWGv is the most susceptible strain of all the isolates. *Lactobacillus delbrueckii* RBWGii and *Lactobacillus pentosus* RBCi showed resistance to Oxacillin and Methicillin whereas *Lactobacillus brevis* FJWJi is susceptible to both the antibiotics. However, it is seen that *Lactobacillus fermentum* FRWGv showed very less susceptibility to Oxacillin but is resistant to Methicillin.

Cellular auto-aggregation

Lactobacillus pentosus RBCi showed the highest percentage (63%) of cellular auto aggregation whereas *Lactobacillus brevis* FJWJi showed the lowest percentage (27%) of cellular auto aggregation. The other two isolates also showed good percentage of cellular auto aggregation, where *Lactobacillus fermentum* FRWGv showed 58% of aggregation and *Lactobacillus delbrueckii* RBWGii showed 55% of aggregation. Figure 4 represents the Cellular Auto-Aggregation percentage of each isolate.

Table 4: Viable cell count in acidic pH conditions

Strains	2.0 pH			3.0 pH			Control (pH 7.0)
	0 h	2 h	4 h	0 h	2 h	4 h	
FJWJ i	8.04 ± 0.061	5.20 ± 0.074	4.28 ± 0.12	8.01 ± 0.07	5.84 ± 0.070	5.11 ± 0.088	8.40 ± 0.010
RBC i	8.33 ± 0.080	5.11 ± 0.025	4.47 ± 0.044	8.12 ± 0.011	5.60 ± 0.020	5.00 ± 0.077	8.61 ±0.050
RBWG ii	8.25 ± 0.0101	5.18 ± 0.041	4.08 ± 0.087	7.47 ± 0.09	5.17 ± 0.017	4.38 ± 0.053	8.44 ± 0.066
FRWG v	8.17 ± 0.072	5.05 ± 0.077	3.74 ± 0.041	7.58 ± 0.020	5.36 ± 0.055	4.24 ± 0.065	8.39 ± 0.079

Values are mean ± SD of three independent determinations (n = 3) of each sample.

Table 5: Antibiotic Susceptibility of the selected *Lactobacillus* isolates

Antibiotics (Conc.)	Lactobacillus isolates (ZOI in mm)			
	FJWJi	FRWGv	RBWGii	RBCi
AZM15	17	22	19	20
S10	10	13	14	14
OX1	10	10	R	R
MET15	10	R	R	R
TE30	16	22	18	19
E15	23	27	27	23
RIF	20	28	24	20
VA30	R	R	R	R
NX10	R	R	R	R
K30	R	11	11	11

*__AZM15__- *Azithromycin;* __S10__- *Streptomycin;* __OX1__- *Oxacillin;* __MET15__- *Methicillin;* __TE30__- *Tetracycline;* __E15__- *Erythromycin;* __RIF__- *Rifampicin;* __VA30__- *Vancomycin;* __NX10__- *Nalidixic acid;* __K30__- *Kanamycin;* __ZOI__- *zone of inhibition;* __R__- *resistance of isolates to the respective antibiotics.*

Cell surface Hydrophobicity

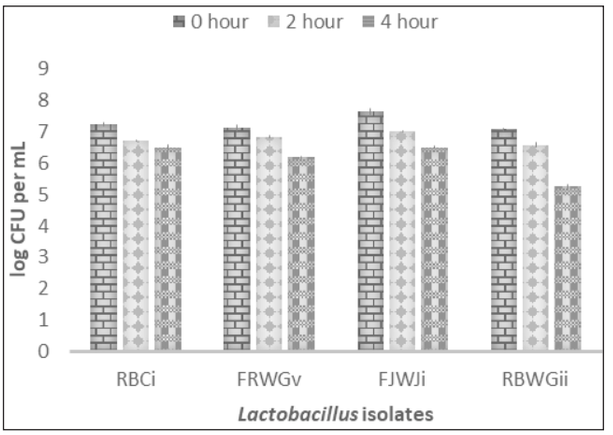
Fig. 5 represents the cell surface hydrophobicity percentage of each lactic acid isolate. In this study, *Lactobacillus brevis* FJWJi shows the highest cell surface hydrophobicity of 26% followed by *Lactobacillus delbrueckii* RBWGii with a hydrophobicity of 23%. *Lactobacillus fermentum* FRWGv has a hydrophobicity of 21% and *Lactobacillus pentosus* RBCi has the least percentage of hydrophobicity (15%).

Shelf-Life Study of Yogurt

pH and Titratable Acidity of Yogurt

As depicted in figure 6 below, an increase in acidity was observed for each treatment till Day 5 to a value of 4.293 from 2.420 in Treatment 2 on Day 5. For Treatment 3 the acidity was 2.358 on Day 0 which increased to 4.149 on Day 5. In case of Treatment 4, the acidity decreased on Day 5. The acidity decreased significantly for all the treatments on Day 7. In case of Treatment 1, the acidity decreased from 3.618 on Day 5 to 2.169 on Day 7. Under refrigeration conditions, the pH of yogurt for Day 0,3,5 and 7 as depicted in the graph below was found to be decreasing from Day 0 to Day 5. The pH of Treatment 3 decreased from 4.60 on Day 0 to 4.28 on Day 5 and pH of Treatment 1 decreased from 4.43 on Day 0 to 4.12 on Day 5. It was observed that on Day 7 the pH increased in all the treatments to a value of 4.50 in Treatment 2. In case of Treatment 4, it was seen that the pH increased on Day 5 and then significant decrease was seen on Day 7. In all the treatment, it was seen that the acidity

decreased and pH increased significantly on Day 7 indicating decrease in viable cell count.



** Values are mean ± S.D. of triplicate determinations (n=3)*

Fig. 2: Representation of Bile tolerance level of the isolates

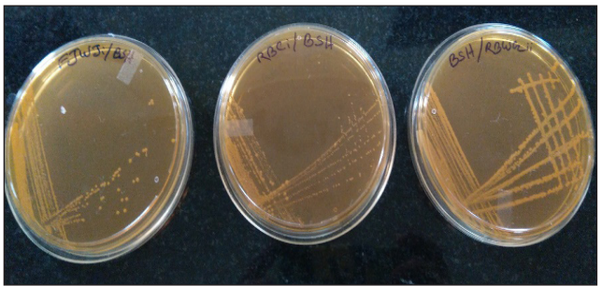
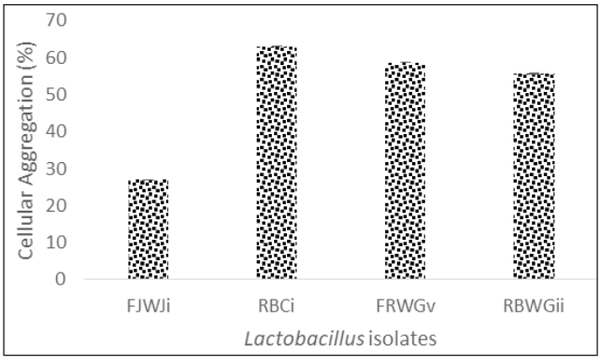


Fig. 3: Plates showing growth of the isolates in presence of bile salt

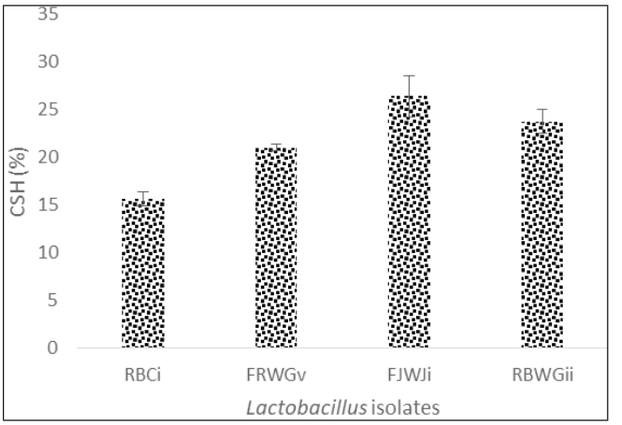
Microbiological Analysis

The result of viable cell count of the probiotic culture during the shelf life study is shown in Fig. 7. Under refrigeration condition, 1 log value increase was seen in viable cell count from Day 0 to Day 3 and subsequently the cell count increased by 5 log value on Day 5. In case of Treatment 3, the viable cell count increased from log 2.28 CFU/mL on Day 0 to log 3.17 CFU/mL on Day 3 and subsequently to log 4.25 CFU/mL on Day 5. The viable cell count decreased significantly for all the treatments on Day 7 to log 4.20 CFU/mL for Treatment 3. In treatment 3, on Day 5 growth of yeast and mold was observed.



Values are mean ± S.D. of triplicate determinations (n=3)

Fig. 4: Cellular aggregation efficacy of the selected *Lactobacillus* isolates



** Values are mean ± S.D. of triplicate determinations (n=3)*

Fig. 5: Cell surface hydrophobicity of the selected *Lactobacillus* isolates

Organoleptic Evaluation

The Fig. 8 depicts the results of the organoleptic evaluation of the functional yogurt. A panel of 5 members, mostly acquainted with dairy products evaluated the samples based on aroma, appearance and texture, flavour, mouthfeel and aftertaste. The scores were given in accordance to hedonic scale ranging from 1 to 9, where 1 represents largely dislike and 9 represents like extremely. The scores given for the first 5 days of storage of the sample show overall high acceptability. Treatment 3 was largely liked by the panellist whereas there was slight dislike for

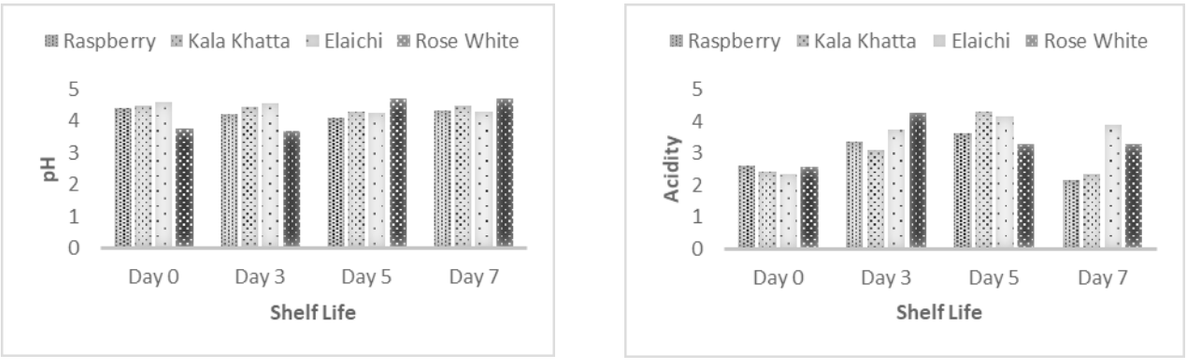


Fig. 6: Representation of the pH and acidity change during storage study of functional yogurt

Treatment 2 and 4. Treatment 1 was moderately liked based on its appearance and texture, flavour and mouthfeel. No significant appreciation for aroma was noted for the samples. Treatment 1 and 3 was scored high for appearance and texture, mouthfeel and aftertaste. The aftertaste for all the treatment on Day 7 was slightly disliked by the panellist. The taste acceptability for Day 7 declined significantly which could possibly be because of low pH and high acidity. The slight acceptance of the sample on Day 7 combined with decline in microbial count for all the samples led to the ending of the shelf life study.

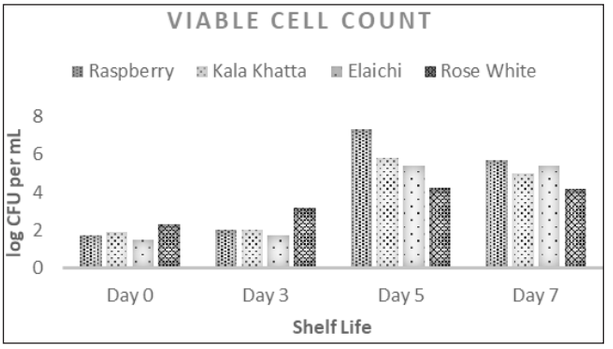


Fig. 7: Representation of the viable cell count in log CFU/mL during the storage study of yogurt

The overall acceptability of the functional yogurt on appearance and texture, flavour, mouthfeel and aftertaste were the highest for Treatment 3 when compared to the other treatments. The statistical analysis of the data obtained during sensory analysis was done using SPSS. The results show that the functional yogurt was the most acceptable on Day 3 for all the treatments with the highest mean of 7.96 for Treatment 3. Similarly, results suggest that the yogurt was only slightly acceptable on Day 7. In comparison to all other treatments Day 3 of Treatment 3 had the highest acceptance ($p \leq 0.05$). In the present study, no specific variation was found between Treatment 1 and Treatment 2 and had moderate acceptance whereas Treatment 4 had slight acceptance.

In this study, the probiotic attributes of the lactic isolates from various fermented food products of the Garo Hills were determined. The lactic isolates in this study could withstand the high pH conditions as shown in the results. Similar results were reported by Kathiriya *et al.* (2015) where it was found that LAB isolates acquired from fermented milk showed relatively more resistance at pH 3 than pH 2. A reduction in 3 and 4 log value was found at pH 2 and pH 3 respectively in viability after 3 hours in *Lactobacillus plantarum* C6 isolated from fermented cheese (Hati *et al.* 2014). The lethality of acidic pH i.e., pH 2 was noted after an incubation period of 4 hours. A reduction of 5 log value was observed in the viable count (Ng *et al.* 2015). The probable reason as stated by Van De Guchte *et al.* 2002, might be failure by the isolates to pump out excess acid thereby not being able to maintain their intracellular pH. This led to acidification, which in turn damaged the DNA and certain proteins causing the death of the cell. The Acid Tolerance Response (ATR) to acid treatment induce pH homeostasis, protection and repair mechanisms

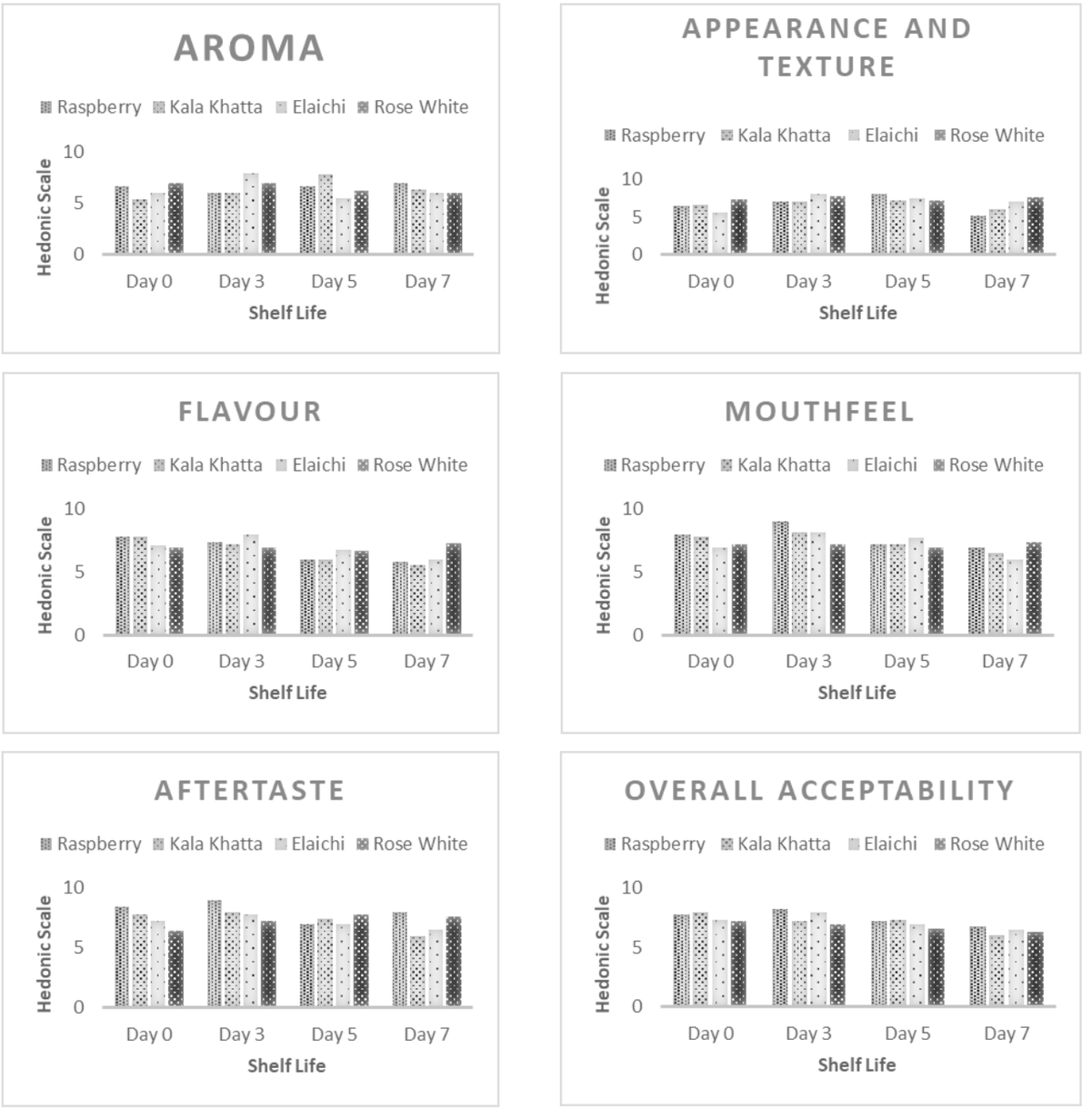


Fig. 8: Representation of the hedonic scale score for Aroma, Appearance and Texture, Flavour, Texture, Aftertaste and Overall Acceptability of the functional yogurt during organoleptic evaluation

in the LAB cells (Maria *et al.* 2001). *Lactobacillus pentosus* and *Lactobacillus brevis* are more liable to resist acidic condition due to higher capability of initiating the ATR. In studies on *Lactobacillus pentosus*, isolated from fermented sap of palm tree, the strain could not tolerate pH 1, but at pH 2 the percentage of survival was 70% at the first hour and then gradually the value decremented to 55% and to 0% at the third and the fourth hour respectively. At pH 3, the survival percentage was 84%, 60%, 52% and 48% at the first, second, third and fourth hour respectively (Fossi *et al.* 2015). Tolerance to acid is

a prerequisite for the isolates to exert its probiotic benefits as after ingestion, they should survive the acidic environment of the gastrointestinal tract before colonising in the hindgut. The fasting pH of human stomach is 1.5 and post feeding the pH decreases to around 3 to 5 (Cotter and Hill, 2003). The findings of this study are in accordance with the reports given in studies conducted on different LAB strains isolated in different laboratories.

Surpassing the effect of bile present in the intestine is also a prerequisite a probiotic must possess to exert its beneficial effects. The human liver secretes about a litre of bile juice into the small intestine every day and the conc. ranges from 0.3-0.5% avg. (Begley *et al.* 2005). Lebeer *et al.* 2008 reported that the property of tolerance to bile has caused the production of bile salt hydrolases, ATPases and other systems. Literatures report that the LAB isolates could retain their viability even after 12-hour exposure to different concentration (0.5-2%) of bile salt. The possible reason could be the ability of the *Lactobacillaire* to hydrolyse toxic bile salts aided by the bile hydrolase enzyme. (Erkkilä *et al.* 2000). Study on *Lactobacillus plantarum* C6 isolated from fermented cheese showed its ability to tolerate bile salts (1-2%) effectively. At a conc. of 1% bile, the viable cell count was 6.98 log counts after an incubation period of 3 hours, however, it was reported that viability decremented significantly when compared to the control. When the concentration was increased to 2% bile, *Lactobacillus plantarum* C6 had shown 3 log reduction after an incubation period of 3hour (Hati *et al.* 2014). Kumari *et al.* in the year 2016 reported that all of the LAB isolates from fermented food were able to tolerate bile salt (0.5-2%), even after an incubation period of 12hours. Similar studies on *Lactobacillus fermentum* strains say they could tolerate different concentration of bile salts after an incubation period of 4hour, with a survival rate of >80% at 0.3% bile salt concentration. However, it was reported that the resistance decreased significantly as the concentration of bile salt was increased (Owusu-Kwarteng *et al.* 2015). The antimicrobial activity of bile salts being dissolution of the bacterial membrane, it is important that the LAB isolates survive in the gastrointestinal tract to exert its probiotic effects (Begley M, 2005).

The absence of zone of precipitation by the isolates in the bile hydrolase activity test can be presumed to be their isolation source since activity is generally higher in lactic strains obtained from faecal matter that possesses rich conjugated as well as unconjugated bile acids (Kathiriya *et al.* 2015). The hydrolase enzyme aids in the catalysis of the amide bond between the steroid moiety and amino acid side chain of the bile acids (Lebeer *et al.* 2008). The exact function of BSH is unknown, but it is thought to play a role in bile detoxification into nutritional form of deconjugated products (Begley *et al.* 2006). The deconjugated bile salts co-precipitate cholesterol which bind to bacterial cells thus augmenting their excretion (Mathara *et al.* 2008). Kaushik *et al.* (2009) reported that the bacteria in the gastrointestinal tract acquire the bsh gene by horizontal gene transfer. The LAB isolated from faecal and diary sources could degrade and grow in TDCA which signifies their ability to produce the hydrolase enzyme (Archer and Halami, 2005). It was also reported by Archer and Halami, (2005) that the highest cholesterol reduction was displayed by the isolates having positive BSH activity. The abundance of bsh genes in *Lactobacillus plantarum* as indicated by genome analysis proves its efficiency in mitigating hypercholesteremia and cardiovascular diseases (Lambert *et al.* 2008). Studies conducted by Owusu-Kwarteng *et al.* (2015) on *Lactobacillus fermentum* strains showed that although the isolates were able to grow in the presence of bile salt, the ability to hydrolyse TDSA was demonstrated only by four strains. However, data suggest that detoxification of bile salts by the microbial BSH-activity aid to the beneficial effects of the probiotic isolates (Begley *et al.* 2005, 2006).

Susceptibility to antibiotics by the probiotics is also considered to be an essential attribute. The isolates in this study are susceptible to various antibiotics like Azithromycin, Streptomycin, Oxacillin, Methicillin, Tetracyclin, Erythromycin, Rifampicin and Kanamycin. Lactobacillus *plantarum* C6 isolated from fermented cheese showed very small zone

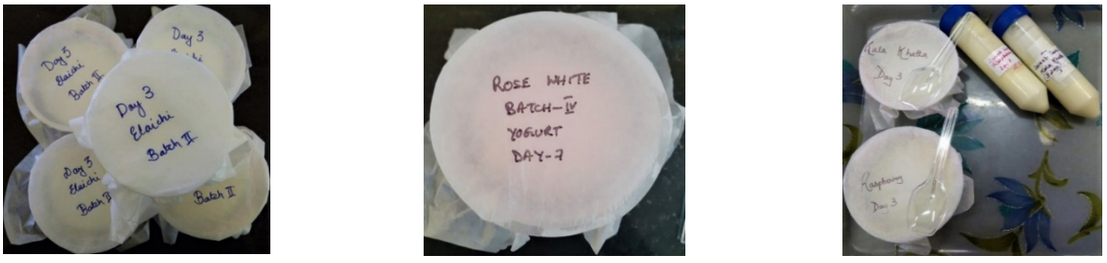


Fig. 9: Food Grade cups containing the functional yogurt

of inhibition in case of Kanamycin and is totally resistant to Vancomycin (Hati *et al.* 2014). Some strains if *Lactobacillus fermentum* isolated from West African fermented millet dough showed resistance, mostly at low levels, to the family of protein synthesis inhibitors which includes Streptomycin, Kanamycin and Gentamycin, as reported by Owusu-Kwarteng *et al.* (2015).

Similar results are reported by Klayraung *et al.* 2008, where LAB isolated from Thai Traditional food is resistant to protein synthesis inhibitors like Chloramphenicol, Quinipristine, Erythromycin, Kanamycin, Rifampicin, Tetracycline and Streptomycin. *Lactobacillus fermentum* isolated from human gut showed resistance to Erythromycin (Fons *et al.* 1997) and also Ahn *et al.* 1992 reported resistance of *Lactobacillus* spp., isolated from diary to Chloramphenicol. The presence of D-Ala-D-lactate in place of normal dipeptide D-Ala-D-Ala in their peptidoglycan has been learned to be the reason behind the resistance of *Lactobacillus* to Vancomycin, which targets the peptidoglycan (Coppola *et al.* 2005). The probiotic cultures could influence our intestinal gut microflora, so it is important that they don't carry genes for multidrug resistance. The hindgut microflora will acquire the MDR genes from the probiotic cultures by horizontal gene transfer. This might help the opportunistic pathogens present in the host gut to survive even after administration of antibiotics by the host. The European Union (EU) Scientific Committee on Animal Nutrition (SCAN) has given out guidelines stating that bacteria containing acquired antibiotic resistant genes must not be used in feeds (SCAN, 2012).

In accordance with above mentioned, the *Lactobacillus* isolates in our study does not contain antibiotic resistance genes and thus can be considered safe for intake. In contrast to the fact that probiotic cultures should not confer antibiotic resistance to the host hindgut microflora, some studies also state that resistance to antibiotics is a requisite for the probiotic to exert its beneficial effects. The literature states that the probiotics should not be susceptible to the antibiotics ingested by the host for other ailments. It must survive and colonise in the gastrointestinal tract.

The auto-aggregation capability of the isolates shows their ability to clump to its own strains which is a positive attribute for the probiotics as this ability of clumping prevents colonization by the pathogens by releasing antimicrobial substances in the intestines. Kathiriya *et al.* (2016) reported that the rate of auto aggregation increases with increased incubation time. Studies say that the percentage of auto aggregation increased from 14.57-36.40% after 2 hours of incubation period to 29.57-59.54% during the fifth hour. A complete strain dependent aggregation to a maximum of 74% was found in *Lactobacillus acidophilius* M92 over an incubation period of five hours. Some strains show that *Lactobacillus fermentum* adhere efficiently to fibronectin and mucin. Human faeces isolates showed better adherence in comparison to the isolates obtained from dairy products. (Archer and Halim, 2015). Reports state that the proteinaceous strains (S-Layer) on the cell surface mediate auto-aggregation and adhesiveness property in *Lactobacillus rhamnosus* (Kos *et al.* 2003). Moreover, it was also demonstrated that spent

culture supernatants of *Lactobacillus* with the ability to auto-aggregate could help in aggregation of other lactic acid bacteria apart from itself (Schachtsiek *et al.* 2004). So, the isolates in the study qualifies to be used as a probiotic based on the positive attribute of cellular auto aggregation.

Cell surface hydrophobicity plays a major role in adhesion of the probiotic to the GI tract and thereafter colonisation. Adhesion to the host gut epithelial and mucus enhances the probiotic to competitively exclude pathogens and colonize in the gastrointestinal tract. The hydrophobicity test where the hydrophobicity to different hydrocarbons is considered as a quantitative phenomenological approach to test the potential of the organism to colonize the GI tract of the host (Rosenberg *et al.* 1980; Kiely and Olson, 2000). The hydrophobicity percentage of some LAB isolates i.e., *Lactobacillus pentosus* AJ15 (JX683267) was reported to be 19% whereas that of *Lactobacillus pentosus* AJ82 (JX683268) was reported to be as high as 90%. (Dubey and Jeevaratnam, 2015). N-hexadecane, n-octane and xylene was used to evaluate the hydrophobicity of *Lactobacillus plantarum* C6 isolated from fermented cheese. *Lactobacillus plantarum* C6 showed 23.60%, 23.38% and 39.24% of affinity for n-hexadecane, n-octane and o-xylene respectively (Hati *et al.* 2014). Thus, higher adhesion rate helps the probiotic to adhere well and therefore prevent it from being washed-out, especially from the small intestine. The ability of adhesion and the variation in hydrophobicity to solvents depends on the origin of the strains (Ambrosini *et al.* 1998). Kaushik *et al.* (2009) reported the hydrophobicity percentage ranging between 57-58% of *Lactobacillus casei* in n-hexadecane or xylene whereas strains of *Lactobacillus rhamnosus* has shown very low hydrophobicity of 2-5% (Schillinger *et al.* 2005).

In shelf life study conducted by Ertem and Çakmakçi (2017) throughout the storage period, the pH decreased and acidity increased. Similar finding in drinking yogurt containing oat fibre and inulin were reported (Güler-Akın *et al.* 2016). The mean acidity values on the 1st, 7th 14th and 21st days were 0.93%, 0.98%, 0.97% and 1.06% as lactic acid during storage period and Gobdin was believed to increase the acidity of the yogurt samples (Ertem and Çakmakçi, 2017). Increasing the storage temperature increased the titratable acidity and decreased the pH of the sample which indicates an increase in acid producing microorganisms (Al-Kadamang 2002). Reports claim that the degree of syneresis increase in cottage cheese (Schmidt and Bouma, 1992) and yogurt (Richmond *et al.* 1985) as storage temperature is increased and rate of acid production increases. Decrease in viable cell count would nullify the purpose of addition of probiotics to the yogurt and thus it signifies the end of the shelf life of yogurt. Fig. 9 shows the sample yogurt of different treatments in food grade cups.

Studies on yogurt samples with Gobdin, dried white mulberry and walnut have shown that storage period significantly affected the viable cell count of *L. acidophilus*, *L. bulgaricus* and *S. thermophilus.* The addition of Gobdin improved the viability of *L. acidophilius*. The prebiotic effect of mulberry and walnut components on probiotic bacteria was an addition to the value of yogurt (Ertem and Cakmakci, 2017). In some studies, reduction of 1 log CFU/mL in viable cell count was reported (Daneshi *et al.* 2013) after 6 days. The viability of *L. acidophilus, B. lactis* BB 12, *L. rhamnosis* and *L. plantarum* in milk/carrot juice drink ranged from 98.8% to 88% (Daneshi *et al.* 2013). The total viable count of the probiotic product during consumption must be at least 10^5/g as per international standard (Robinson, 1987). Viability of the probiotic in drinks is important till the product is consumed for proper deliverance of sufficient microorganisms. The bacterial population cease to grow after 3 to 5 days due to limitation of nutrients and increase in acidity. The growth of yeast and molds in the yogurt sample after 5 days of storage may be due to contamination after pasteurization. Growth of yeast, mold or coliforms after certain number of days is the indication that the product cannot be consumed thereafter. After a storage period of 5 days the viable cell count was acceptable, but thereafter the cell count decreased, thus diminishing the value of the product. Sensory failure led to the ultimate ending of the storage study.

Studies conducted by Guggisberg *et al.* (2009) says that the attributes like mouthfeel and viscosity increases with increased number of prebiotics, especially inulin. Also, difference in sensory attributes was seen on addition of probiotics into yogurt already containing high level of prebiotics (Allgeyer *et al.* 2010). The overall acceptability of flavoured milk decreased after 6 days of storage to an average of 6.2 ± 0.2 (Junaid *et al.* 2013). In studies conducted by Januário *et al.* (2017), the overall acceptability of beet with carrot, cassava and sweet potato yogurt was higher ($p \leq 0.05$) when compared to corn yogurt. Longevity of the shelf life is important for better consumption of the products. Studies have brought up several methods to increase the viability of the probiotics in yogurt. Addition of certain ingredients like cysteine, casein hydrolysates and whey powder (Dave and Shah, 1998; Gomes *et al.* 1998; Adhikari *et al.* 2003) have shown to increase the shelf life of yogurt. Other methodology includes microencapsulation. Sensory characteristics of the yogurt, especially texture might be affected on addition of viability enhancing ingredients (Kailasapathy, 2006).

In this study, four lactic acid bacteria namely *Lactobacillus brevis* (FJWJi), *Lactobacillus fermentum* (FRWGv), *Lactobacillus pentosus* (RBCi) and *Lactobacillus delbruckii* (RBWGii) isolated from fermented food have shown ability to ferment cow milk faster and have given positive results to certain probiotic attributes. These isolates can therefore be considered as potential probiotics for development of functional food. Among the various treatments conducted for product development, it was noted that Treatment 3, especially on Day 3 was highly acceptable for consumption. During the storage study for 7 days, with the decrease in pH, the acidity increased and viable cell count of the yogurt was acceptable till Day 5. Further, addition of certain ingredients to increase the viability and thus the shelf life of the product is recommended. Study on certain methodologies like microencapsulation of the probiotics can be done further to increase the storage life of the product and thereafter sensory analysis can be done to check the quality of the product.

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