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MICROENCAPSULATION OF *L. FERMENTUM* ISOLATED FROM TRADITIONAL DAIRY PRODUCTS AND ITS STABILITY ON EXPOSURE TO SIMULATED GASTROINTESTINAL CONDITIONS

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ABSTRACT

The physiological state of food may affect the survival potential of health promoting microorganisms. The current research was thus undertaken for comparing the invitro stability of two similar species of *L. fermentum* isolated from two different dairy products. The isolates were analyzed for their viability after microencapsulation in sodium alginate and were also compared using the non-encapsulated strain in simulated gastric and intestinal conditions. Viability of the cultures were also compared against reference standard (i.e) Lactobacillus acidophilus procured from MTCC Chandigarh. The percentage log reduction of non- encapsulated cultures i.e curd, raw milk and MTCC was 58.32%,58.28%,58.43% while that of encapsulated cultures was 10.19%, 10.03% and 11.18% as observed in gastric juice. The log reduction of non -encapsulated cultures as observed was 3.80%, 3.10% and 2.23% for curd raw milk and MTCC cultures respectively while that of encapsulated cultures was 1.54%, 1.52% and 1.16%in simulated intestinal conditions. The raw milk isolate was found with slightly better adaptation in response to the viability both in case of gastric and intestinal juice. The result thus justifies the physiological state of food which may affect the osmotic response and stress of similar microflora although isolated from two different food consortiums.

Keywords: -*Lactobacillus*, microencapsulation, sodium alginate, adaptation

INTRODUCTION

Lactobacillus is one of the frequently used probiotics in food products. The lactic acid bacteria (LAB) are widely used in many traditional preparations across the globe for its desirable flavor and aroma (Lanxin, 2019). LAB found in these products have distinct inherent characteristics as they synthesize different types of metabolites like bacteriocins, lactic acid, hydrogen peroxide, diacetyl, and carbon dioxide (Vieco *et al.,* 2019). By virtue of these properties, they have been reported to have health benefits in the management of weight, type 2 diabetes, hypertension, cholesterol reduction and diarrheal diseases (Mathur *et al.,* 2020). The current state of evidence suggests that probiotic effects are strain specific (Mcfarland *et al.,* 2018). The food consortium often referred with probiotic benefits like curd, buttermilk, shrikhand constitutes the major domain of Indian market (Roy and Kumar, 2018). These traditional fermented products are source of precious LAB and now these have been replaced by commercial strain due to industrial production of fermented foods. The study was thus prompted to isolate LAB strains from two conventional dairy sources and analyzed for 16sRNA and their probiotic properties were also compared with the reference strain of *Lactobacillus acidophilus*. Till now the reported literature has little evidence to support the functional variance of two similar species isolated from two different food consortiums. The current study was thus undertaken for comparing the survival potential and functional variance of two similar species of lactic acid bacteria isolated from different food consortium consumed by the major population.

The viability of these bacteria is often challenged by the environmental stress factors such as presence of oxygen, mechanical damage, high temperatures of storage and processing; interaction with foods or their non-compatibility with fermentation medium and sometimes due to high acidic foods (Lopez *et al.,* 2015). For metabolic stability and proper biological significance, probiotic viable count must be around 10^6cfu/g and hence microencapsulation can serve as a relevant technique (Chavarri *et al.,* 2010). The exploration of sodium alginate as a coating matrix has been found effective for the protection and functional properties of core material, and probiotics (*Lactobaccilus bulgaricus*) against simulated GIT conditions (Pan *et al.,* 2013).In present study the survival of lactic acid bacteria in simulated gastric and intestinal juice was also analyzed after microencapsulation in sodium alginate matrix while their viability was also compared to analyze the impact of physiological state of food that can have an impact on the survival potential of the two similar species.

MATERIALS AND METHODS

Isolation of lactic acid bacteria and biochemical characterization of obtained isolates; Selection of similar species

The lactic acid bacteria were isolated from the dairy products like curd and raw milk and buttermilk as the people preference was quite high. Isolation was performed using serial dilution method (Mathialagan *et al.,* 2018) using the selective differential media (i.e) MRS (De Man, Rogosa and Sharpe agar procured from Himedia. Morphologically distinct and well isolated colonies were

Table1. Morphological, physiological and biochemical characteristics of isolated Lactobacilli and identification of species through pibwin software

S.no.	**1.**	**2.**
Food Source	Curd	Raw milk (cow)
Coded isolates	AKCN 1	MAANRCM
Gram staining	G +ve	G +ve
Shape	Bacilli	Bacilli
Motility test	–	–
Catalase test	–	–
Gas production from glucose	+++	+++
Glucose fermentation	+++	+++
Sucrose fermentation	+	+
Lactose fermentation	+	+
Arabinose fermentation	+	+
Sorbitol fermentation	–	–
Mannitol fermentation	+	+
Ribose fermentation	+	+
Nitrate Reduction test	–	–
Ammonia from Arginine	+	–
Indoletest	–	–
Citrate Utilization test	+	+

Table 2. Probiotic potential of isolated *L. fermentum* and MTCC standard on exposure to simulated gastric juice and simulated intestinal juice

Viability in (cfu/ml) of free and microencapsulated lactic acid bacteria on exposure to simulated gastric conditions during exposure

Time	(*L. fermentum*) Curd : Non-encapsulated	(*L. fermentum*):Curd Encapsulated	(*L. fermentum*) Raw milk Non encapsulated	(*L. fermentum*) Raw milk: Encapsulated	(*L. acidpphillus*) MTCC 10307 Non- encapsulated (Standard)	(*L. acidpphillus*) MTCC 10307; (Standard) Encapsulated
0 min	9.55±0.10a	9.61±0.09y	9.59±0.13a	9.67±0.11y	9.63±0.12a	9.66±0.05x
60 min	6.64±0.11b	9.45±0.12y	6.27±0.15b	9.58±0.09y	6.25±0.10b	9.25±0.09y
120 min	3.98±0.13c	8.63±0.11z	4.00±0.12c	8.70±0.12 z	4.02±0.13c	8.58±0.06z
Log reduction in (cfu/ml)	5.57	0.98	5.59	0.97	5.61	1.08

Note. Each value represents the mean value with standard deviation (SD) from the three trials undertaken. Values in lower cases i.e superscripts (a,b,c) and values in alternate columns respectively presented in uppercase i.e superscript (X,Y,Z) are significantly different by Tukey's multiple range test at ($p<0.05$).

Viability (cfu/ml) of free and microencapsulated lactic acid bacteria on exposure to simulated intestinal conditions during exposure

Strain and food source	(Encapsulated/ Non encapsulated)	0 min	60 min	120 min	180min	Log reduction in (cfu/ml)
(*L. fermentum*):Curd	Non-encapsulated	3.94±0.08Z	4.29±0.08Y	4.51±0.05X	3.80±0.08Z	0.15
(*L. fermentum*): Curd	Encapsulated	8.40±0.10bc	8.56±0.08ab	8.66±0.04a	8.27±0.06c	0.13
(*L. fermentum*):Raw milk	Non encapsulated	3.87±0.05X	4.49±0.03Y	4.62±0.07Y	3.93±0.03X	0.12
(*L. fermentum*):Raw milk	Encapsulated	8.5±0.08c	8.63±0.08ab	8.81±0.03a	8.37±0.07c	0.13
(*L. acidophillus*): MTCC 10307	Non encapsulated	4.03±0.07Z	4.39±0.04X	4.24±0.03Y	3.94±0.02Z	0.09
(*L. acidophillus*): MTCC 10307	Encapsulated	8.55±0.10bc	8.69±0.03b	8.73±0.02b	8.45±0.09c	0.10

Note. Each value represents the mean value with standard deviation (SD) observed from the three trials undertaken. Values denoted with alphabets a,b,c in superscripts are significantly different while the values in alternate rows presented in uppercase i.e superscript X,Y,Z are also significantly different by Tukey's multiple range test with ($p<0.05$).

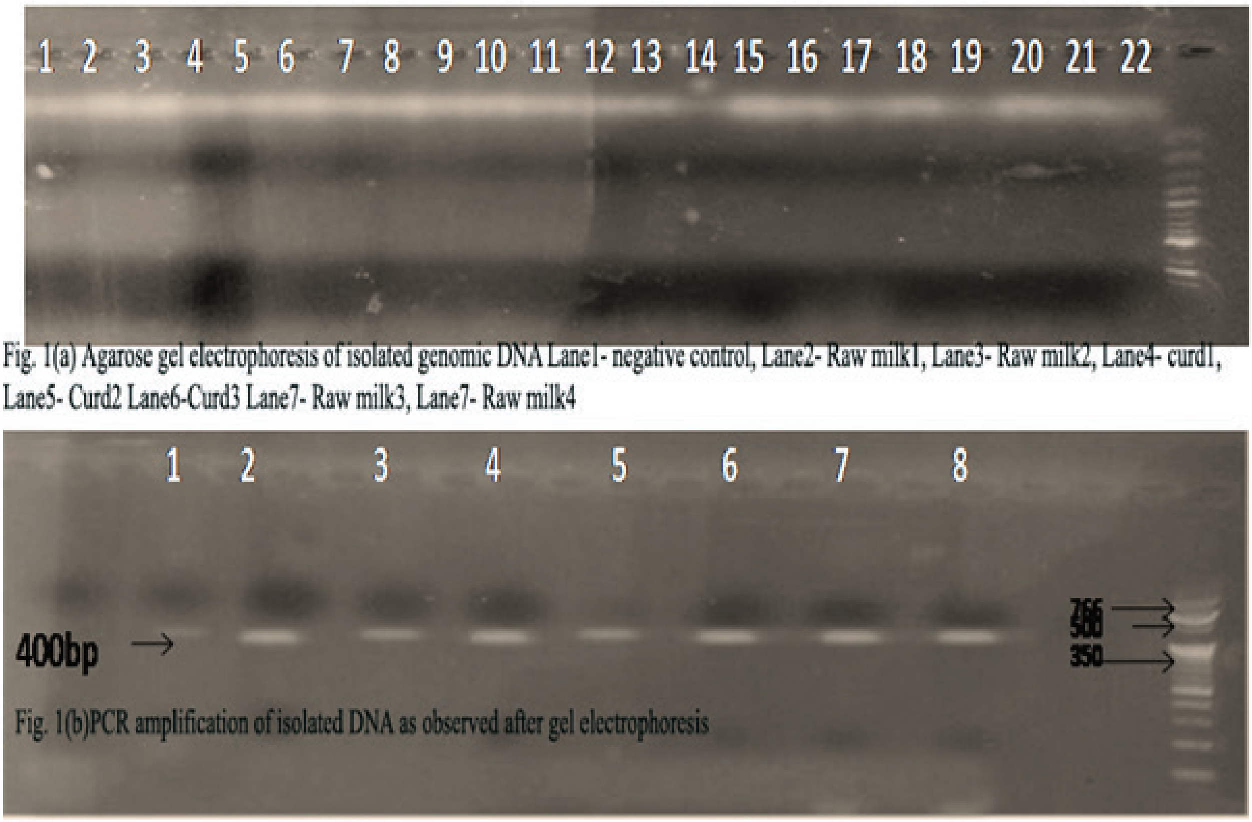

Figure 1 (a) showing the isolated genomic DNA;
(b) showing the PCR amplification of isolated DNA after gel electrophoresis

picked and transferred to new MRS agar plates after further subculturing and testing of biochemical properties. Gram staining was performed as per the method described by (Sharma *et al.,* 2007). The isolates were tested for catalase, motility, gas production from glucose and survival in different salt concentrations as referred by (Yu qian *et al.,* 2018).Also, their ability to survive at elevated temperature was performed. The isolates were further tested for fermentation of different carbohydrates sources like glucose, sucrose, lactose, arabinose, sorbitol, mannitol, and ribose. Identification of the bacteria was performed through comparison of biochemical parameters as referred by Bargey's Manual of Determinative Bacteriology, 8th edition. Although 22 isolates were obtained from 15different dairy samples (data not included) the two similar species were thus selected for understanding their response and variance under similar conditions. All the other isolated cultures were kept away for carrying out some other research works.

Identification of obtained isolate through Pibwin 2007

The identification of obtained LAB was done through Pibwin software (PIB Win, 2007). Results based on the biochemical parameters and carbohydrate fermentation was utilized in expressing the genus of the isolates based on probabilistic identification of bacteria through window programme (Garg *et al.,* 2013). This programme enables the utilization of windows version DOS for identifying bacteria based on selective biochemical tests for identification of any unknown isolate and retrieval of data based on probalistic results.

Molecular identification of isolates

Primer designing

Molecular identification was carried out through the previously designed primer (Rohani *et al.,* 2015). The primers used for the amplification of 16s rRNA gene was forward (5'TGGAAACAGGTGCTAATACCG 3') and reverse (3'CCATTGTGGAAGATTCCC5') respectively based on the conserved region of 16 s rRNA gene. The primers were synthesized from G Biosciences, India.

Isolation of genomic DNA PCR amplification and gel electrophoresis

Isolation of genomic DNA and PCR amplification of isolated genomic DNA was performed by phenol chloroform, method as mentioned by (Parayre *et al.,* 2007) with some suitable modification of steps. The obtained PCR product was analyzed in gel electrophoresis for identification of PCR product size. The integrity and amplification of DNA was observed with help of UV transilluminator (Genetic, India).

Microencapsulation of selected lactic acid bacteria

Microencapsulation was performed using simple extrusion technique as mentioned by (Chavarri *et al.,* 2010). The overgrown cultures were harvested after centrifugation at 5000 rpm and 4 followed by extrusion through a sterilized syringe of 1mm in calcium chloride solution (0.3M) after dissolving in sodium alginate concentration of 2%.The harvested cultures were properly agitated for the deposit of calcium followed by replacement of sodium After the removal of surface moisture from the microcapsules the beads were then transferred in clean LDPE for further studies in refrigeration conditions.

Encapsulation efficiency (%)

Encapsulation efficiency was determined by the ratio of enumeration of plate count as described by (Chavarri *et al.,* 2010) after dissolving the micro-beads in sodium citrate solution of 0.1 M which was followed by vortexing and rupturing of the entire microcapsule and release of live bacteria. The % encapsulation efficiency (EY) was determined as: -

$$EY = \left(\frac{N}{N0}\right) \cdot 100$$

Where N is the number of viable entrapped cells released from the beads and N0 is the number of free cells added to sodium alginate matrix used for encapsulation.

Stability of lactic acid bacteria in simulated gastric and intestinal juice

Simulated gastric juice was prepared using hydrochloric acid (HCl) buffer of pH 2 containing: NaCl-8 g L−1; KCl-0.2 g/sL; Na2HPO4·2H2O-8.25 g/L; NaH2PO4-14.35 g/LL; CaCl2·2H2O-0.1 g L; MgCl2·6H2O-0.18 g/L and pepsin (Sigma-Aldrich)-3 g/L as described by (Sandoval *et al.,* 2010).The microcapsules were exposed in simulated gastric juice (9ml) containing 1 g of microencapsulated lactic acid bacteria. Free cells (1ml) were added in simulated gastric juice (9ml) and were exposed in simulated gastric juice for a period of 120 min at 37°C. Both the encapsulated and non-encapsulated bacteria were exposed in simulated gastric juice for a period of 120 minutes and viability was enumerated at 0 min, 60 min and 120 min, after disintegration of microcapsules. The log reduction attained after exposure of the bacterial cells was thus evaluated.

Simulated intestinal juice (SIJ) was prepared by dissolving bile salts in intestinal solution containing6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$ and 1.386 g/L $NaHCO_3$and pH 7.5 to final concentrations of 3.0 g/L (Chavarri *et al.,* 2010).The disintegrated microcapsules that were previously exposed in simulated gastric juice for 120 min, were then transferred in simulated intestinal juice for a period of 180 min at 37°C (Sagheddu *et al.,* 2018). Enumeration of viable count was performed after serial dilution using colony count method and the results were expressed in log cfu/g after 24 hours of incubation. The loss in probiotic viability was calculated which was the difference between initial log count and the corresponding value after exposurefor 120 minutes in simulated gastric juiceand180 minutes in simulated intestinal juice respectively as referred by (Chavvari *et al.,* 2010).

Statistical Analysis

The results were reported with observations in triplicates corresponding to the mean and standard deviation of the obtained values. The data were analyzed by two-way ANOVA at 95% confidence level. All statistical analyses were performed with IBM® SPSS® Statistics v.20 (IBM Corp. Armonk, New York, USA).

RESULTS AND DISCUSSIONS

Morphological, physiological and biochemical characteristics of isolated *Lactobacilli and* identification of species through pibwin software

The results in (table 1) show that the isolated bacteria were gram positive, catalase negative and non-motile. Morphologically their appearance was bacilli with rod shaped structures. The bacteria were able to produce gas from glucose after fermentation. The isolates were able to ferment lactose, arabinose, sorbitol, mannitol, and ribose as observed. The isolates obtained from curd were able to hydrolyze arginine while partial arginine hydrolyzing characteristics were also observed with raw milk isolate. However, the isolates were able to utilize citrate as a secondary carbon source and were citrate positive. The results were found in close agreement to (Julendra *et al.,* 2017). Based on the biochemical parameters and carbohydrate fermentation pattern both the selected isolates were found as *L. fermentum* when observed through Pibwin software. However, genus identification was also performed subsequently.

Molecular identification of isolated strains of *Lactobacillus* using 16sRNA gene specific PCR

The integrity of isolated genome DNA can be observed in the gelelectrophoresis (fig no.1a). As seen no RNA contamination was observed. Using the isolated genomic DNA and designed primers the PCR amplification was performed. The primers were designed using 16s spacer primer regions. These primers were designed in a way that the amplicon size would be 400bp. The findings in the above results were similar to (Rohani *et al.,* 2015) as amplification was thus reported at 400bp. The genome indication being prokaryote was realized through the single band of DNA with similar amplicon size. The gel electrophoresis (Fig no.1 b) clearly shows the band size of 400 bp without any additional band, which clearly indicates the single site binding of primers. The primers were designed in a way for amplification of only prokaryotic genes. The band clearly shows the source of genomic DNA being prokaryote which is the inherent property of lactic acid bacteria.

Analysis of microcapsule size and encapsulation yield

The mean diameter of microspheres measured as a result of 10 random samples taken for the encapsulated lactic acid bacteria was 2.192±0.015 mm for curd, 2.194±0.018mm for raw milk isolate, and 2.184±0.033mm for MTCC standard strain. The results suggested homogeneity amongst the beads size which was due to the processing parameters and all the components taken being similar. The encapsulation yield of the entire three strain was 93.51% for curd, 92.22% for raw milk and 92.69% for MTCC standard strain. The observed encapsulation efficiency was almost similar in all the three strains taken. This could be due to the initial bacterial load taken being similar. Similar findings were reported by Silva and co-workers (2018) showing that microcapsule size and efficiency depends upon the processing parameters. The bead sizes thus observed were of similar dimensions since the matrix was also homogenous. Microcapsules observed were of homogenous shape and size in the entire three experimented matrices. Krasaekoopt and Watcharapoka, (2014) showed that entrapment efficiency is not dependent on the type and concentration or presence of prebiotics in the matrix.

Probiotic potential of isolated *L. fermentum* and MTCC standard on exposure to simulated gastric juice and simulated intestinal juice

Upon comparison amongst the encapsulated and non –encapsulated LAB isolates it was found that the microencapsulated lactic acid bacteria were found with better retention potential than the non–encapsulated bacteria. After exposure in SGJ the non-encapsulated bacteria were found with a log reduction of 5.57, 5.59 and 5.61 log cfu /ml while the encapsulated bacteria were found having a log reduction of 0.98, 0.97 and 1.08 log cfu/ml respectively as shown in (table 2). Samedi and Charles, (2019) found an average loss of 1-2 log cfu in a number of experiments conducted using maltodextrin and starch. Similar trend was observed in the current study supporting the stature of encapsulatedmode used for sustainable release and the efficacy of sodium alginate. The MTCC standard strain was also found with a loss of 5.61 log cfu. However, the trend of log reduction was found similar amongst the encapsulated bacterium which shows that the rigidity offered by sodium alginate capsules in the proportionate amount (i.e. 2%) taken which was found affective. Although, the non -encapsulated cells were found with an average loss of 4.08 log cfu/ml the application of sodium alginate acting as a protective agent for facilitating targeted release was observed. The disintegration of microcapsules was quite exponential between the latter half of gastric transition which continued till 120min of exposure. Active disintegration and release of lactic acid bacteria was reported by Gunsaekaran *et al.,* (2007) in which *L. bulgaricus* were released within 3 hour and 70% of the cells were reported of having been released in the first 1 hour.

Enumeration of viable count was also performed in simulated intestinal juice (SIJ) after exposure of disintegrated capsules and remaining microflora obtained from simulated gastric juice (SGJ) which were again exposed in (SIJ) for a period of 180 min. It was noticed that the rate of loss of viable count was somehow controlled in simulated intestinal juice than simulated gastric juice as shown in (table 2). This could be due to change of pH which allows the repair of damaged cells in intestinal solution. This was correlated with the findings of Silva and co-workers (2017) who reported an increase in the viability of exposed microcapsules in SIJ but due to the gradual drift of the bacteria from the capsules an eventual reduction was noticed in the late log phase. This may be primarily due to the attained pH (6.5) which adds to the survival and repair of ruptured cells. The log reduction of encapsulated microorganism was found as 0.13, 0.13 and 0.10 log cfu respectively while the log reduction in non-encapsulated microbes was 0.15, 0.12, 0.09 log cfu respectively. The log reduction achieved in case of curd was 0.76 log cfu/g while the log reduction achieved in case of raw milk was 0.8 log cfu/g. It was also observed that after 60 min there was an increase in viable count. Similar findings were also reported by Chavvari and co-workers, (2010) who found an increase of 0.16 log cfu/g in probiotic viability of alginate chitosan microcapsules upon exposure in simulated intestinal juice. The study thus supports the sensitivity and sustained release mechanism of using sodium alginate as a coating material. The raw milk isolate was found with better tolerance potential than the curd.

CONCLUSION

The present study thus justifies the nature and adaptation of similar species isolated from different food consortium. Upon exposure to different physiological conditions the raw milk isolate was correspondingly found better than the curd isolate. The raw milk isolate was found having slightly improved tolerance potential. The variance in viability of the two similar species as observed was suggestive about the physiological state of food that may affect the viability of residing microflora.

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