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Isolation of *Leuconostoc mesenteroides* from Traditional South Indian Fame Idly Batter and Preparation of Sucrose-Free Synbiotic Chocolate

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Abstract

To isolate and identify *Leuconostoc mesenteroides* from fermented Idly batter and its application in preparing sucrose-free synbiotic chocolate. The probiotic potential of seven different bacterial isolates were obtained from two samples of idly batter collected. Further bacterial isolates were identified and belong to the genus *Leuconostoc,* After 16S rRNA gene sequencing analysis, confirmed that the isolated Lactobacillus (LAB) strain was *Leuconostoc mesenteroides.* Also this strain was confirmed to produce bacteriocin. Among all, One isolate shown highest growth inhibitory activity against *E.coli* and to evaluate the survival % of them under adverse environmental conditions of gastro-intestinal conditions. The survival % was determined to be 67.2 %, 93 %, 70%, and 96 % under low pH 2.5, 0.3% bile salt, pepsin at pH 2.5 and pancreatin at pH 6. They were microencapsulated with inulin to maintain the stability of LAB in chocolate and inulin incorporation will also stimulate the population of intestinal flora that potentially enhances digestion and metabolism. The sucrose-free synbiotic chocolate was thus formulated using lyophilisate inorder to commercialize a functional food. The viability of LAB in synbiotic chocolate was also estimated by demonstrating a gastro-intestinal fluid model. The survival % of LAB in synbiotic chocolate under simulated gastric and intestinal condition The idea of combining both probiotic and prebiotic to make a product with the goodness of both, when consumed, it gives the synergistic effect.

Article Info

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Keywords

Idly batter, Leuconostoc mesenteroides, E.coli, Aspartame, inulin, bacteriocin.

Introduction

Significant benefit of metabolic products from lactic acid bacteria such as exopolysaccharides (EPS) and fatty acids beneficial effects on human health (Kumar *et al.,* 2018). Probiotic is derived from two Greek words, live microorganisms which when administered in adequate amounts, confer a health benefit on its host (Breidt, *et al*., 1993; Angel Alegria, *et al*., 2013). This action of probiotics is mainly due to its colonizing ability and pH alteration activity in the gut. The activity of probiotics also depends on the type of probiotic strain selected and the amount of probiotic portion consumed.

Studies on functionality of lactic acid, and its physiological effects of lactic acid gave diverse application of LAB strains. For example, dietary linoleic acid is converted into conjugated linoleic acid by lactic acid bacteria. Then conjugated linoleic acid is known to reduce systemic inflammatory mediators in healthy young adults, and to improve glycemic response, lipid profile, and oxidative stress in obese patients (Kuroda *et al.,* 2018).

Leuconostoc mesenteroides belongs to the order Lactobacillales and Leuconostocaceae family. It has

official recognition as GRAS (Generally Regarded as Safe) and approved by the Qualified Presumption of Safety (QPS) for food production and human consumption. *Leuconostoc mesenteroides* are mesophilic, gram positive, non-motile, non-spore forming, catalase negative, facultative anaerobe, obligatory heterofermentative cocci that are often ellipsoidal usually occur as a single cocci and/or in pairs and chains. It grows well at 30°C for 48hours. The facultative anaerobic nature of *L. mesenteroides* enables it to develop in the absence of oxygen and they produce higher growth rates within a shorter adaption stage. *Leuconostoc spp.* are inherently resistant to vancomycin (Dorota Zyzlewicz, *et al.,* 2010). *Leuconostoc mesenteroides* has been reported to promote health benefits particularly inhibits gram negative bacteria and fungi as it secretes bacteriocin, referred as mesenterocin. Probiotics have the ability to bind aflatoxin to their surfaces and then degrade them (En Yang, et al., 2018).

Ulcers that are induced by *Helicobacter pylori* are prevented by consumption of probiotics (Ramakrishna Chetana, *et al.,* 2013). More effective colonisation of the intestinal tract due to high fat content. It has been reported to show better clinical efficiency in enhancing Th1. *Leuconostoc* strains are more potent inducers of Th1 type cytokines like IL-12 and IFN-gamma than *lactobacillus spp. Leuconostoc mesenteroides* are the most potent inducer of TNF-alpha (Eugene Karenzi, *et al.*, 2015).

Prebiotics are defined as non-digestible substances that when consumed provide beneficial physiological effects on the host by selectively stimulating the favourable growth The selectively fermented ingredient allows specific changes, Inulin-type prebiotic compounds include inulin, oligo- fructose (Ji Eun Kim, et al., 2014). They present as natural constituents in root vegetables that include Jerusalem artichokes, burdock, chicory, leeks, and onions are especially rich source. Inulin-type prebiotics are used as functional food ingredients in beverages, yogurts, biscuits, spreads and as dietary supplements. Inulin-type fructans are indigestible and are fermented in the colon (Kim, *et al.,* 2012; Francieli Dalcanton, *et al*., 2018).

Dairy products have been considered as the best matrices to deliver probiotics. On the other hand, there is a growing interest in the development of non-dairy based probiotic products due to the drawbacks related to the consumption of dairy products, including lactose intolerance and the unfavourable cholesterol content (Rakesh Somashekaraiah, *et al.,* 2019). The chocolate is a food stuff that has been consumed by wide range of people in the world. Chocolate is a source of polyphenols (mainly flavonoids), biogenic amines (tyramine and phenylethylene), purine alkaloids (caffeine and theobromine) and mineral salts (i.e. magnesium) (Clorinda Malmo, *et al.,* 2013).

Cocoa phenolics are bioactive compounds possessing anti-oxidant, anti-radical, antiplatelet, anti-inflammatory, anti-carcinogenic products and are also considered to be prebiotics (Wankhade *et al.,* 2017). Cocoa polyphenol and ω-3 fatty acids are effective in decreasing the risk of cardiovascular diseases, insulin resistance, amelioration of skin-aging (Kuroda *et al.,* 2018). The greatest advantage of chocolate is its sensorial properties and consumer perception.

Encapsulation of probiotic with a prebiotic was a new concept and a notable parameter applied in this aspect to develop a synbiotic product as a chocolate. Since human body cannot digest prebiotics, it goes through the small intestine undigested because mammals lack the necessary machinery to degrade complex polysaccharides such as starch, glycan, xylan etc. Therefore it goes through small intestine undigested and move to large intestine. Synergistic action of intestinal microflora degrade these complex molecules into simpler ones that are then readily absorbed thereby releasing probiotic, in the large intestine. In this manner, the target strain supplemented in chocolate reaches its destination where it flourishes and provides all the essential health benefits.

Materials and Methods

Collection of Samples

Freshly grounded idly batter samples were collected from 2 different households locally. Samples were collected in sterile containers and taken to the laboratory for further analysis.

Isolation and identification of *L.mesenteroides*

In order to isolate *L.mesenteroides* from india fame idly batter, One gram of batter sample was homogenised in sterile phosphate buffer saline solution (pH-7.2) to get a uniform suspension. They were then serially diluted and uniformly spread on MRS agar and incubated at 30°C for 24 hours. Morphologically different colonies were selected and purified by repeated streaking on MRS agar

media supplemented with 50mg/ml of Vancomycin. The gram positive isolates were inoculated in MRS broth and incubated at 30°C for 48 hours. After 48 hours, all the isolated gram positive LAB were subjected to agar well diffusion method with the supplement of cotrimazole to prevent fungal growth. Seven isolates were selected from sample and were purified by subculturing on MRS agar plates followed by microscopic examination, selection by vancomycin resistant and bacteriocin production for further identification

Assessment of probiotic properties

Acid, bile, pepsin and pancreatin tolerance Assay

The selected probiotic cells were tested for viability and bile condition following the protocol (Abekhti *et al.,* 2014) The collected pellets were suspended in sterile PBS adjusted to pH 2.5 to the initial volume. The mixture was then incubated at 37°C for 4 hours then were serially diluted in (0.85% NaCl) and MRS broth containing 0.3% (w/v) bile salt (oxgall) was inoculated with active LAB cultures. 3 mg/mL of pepsin was suspended in sterile saline solution and adjusted to pH 2.5. 0.3% bile salt and 1 mg/mL of pancreatin were dissolved in sterile saline solution and adjusted to pH 8.0 with *L.mesenteroides* incubated at 37° C for 6 hour on MRS. The viable cell population was determined by the spread plate method using MRS agar plates were then incubated at 37° C for 48 hours (Felsenstein *et al.,* 1985).

Physiological characterization

Temperature tolerance and Milk coagulation assay

L.mesenteroides was inoculated in 3 MRS and 9 ml of 10% skimmed milk supplemented with 0.5% yeast extract broth tubes and were incubated at 30° C for 48 hours. The viable cell population after 1 hour of heat treatments at 60° C and at 70° C by spread plate method on MRS Agar plates then were then incubated at 30° C for 48 hours. The survival percentage of heat treated broth culture was determined by comparing the viable cell population of control.

Phenotypic characterization

Gram staining, catalase test, oxidase test, indole test, methyl red test, Voges-Proskauer test, citrate utilization, nitrate reduction, urease test, TSI test, oxidative and fermentative test, gelatin hydrolysis test, carbohydrate fermentation test were performed. All tests were performed with 48 hours old culture of *L.mesenteroides*.

Safety evaluation of LAB strain-Hemolysis assay

An overnight culture of the *L.mesenteroides* strains was streaked on blood agar medium and incubated for 48 hours at 30° C.

Microbial Growth Curve

Inoculate a loopful of culture of *L.mesenteroides* in MRS broth medium and biomass study was done for 48 hours with readings being taken at an interval of 8 hours. The growth curve was determined by measuring the OD value at 600 nm using a spectrophotometer.

Growth pattern of LAB strain in presence of inulin

A loopful of culture of *L.mesenteroides* was inoculated in MRS broth supplemented with 1% inulin and incubated at 30°C for 48 hours. The growth pattern was determined by measuring the OD value at 600 nm using a spectrophotometer.

Production of sucrose-free synbiotic chocolate

Lyophilisation of LAB strain

48 hours old culture of *L.mesenteroides* was added to 250 ml of MRS broth that is supplemented with 1% of inulin and the culture was incubated at 30°C for 48 hours. The cells were harvested by centrifuging at 2750 rpm for 30 minutes, washed twice with distilled water, mixed with the same amount of inulin. Frozen at -20°C and lyophilised for 24 hours in a lyophiliser under the following conditions:

- Temperature of heating plates -15°C, pressure 50 MPa.
- Temperature of condenser -45°C.
- Ultimate temperature of drying + 20°C.

Preparation of sucrose-free synbiotic chocolate

- 25 g Cocoa butter was melted in water bath.
- To the melted butter, cocoa powder and finely powdered sucrose-free sweetener or aspartame were mixed until homogenous mixture was formed.
- To this mixture, the lyophilised prebiotic encapsulated probiotic bacteria were added at 40°C in the proportion of 3.33 g/100 g that provided the functional level of LAB (at least 10^6-10^9 CFU/g).

- It was then poured into chocolate mould of suitable shape and cooled at 4° C for 15 minutes to harden the chocolate.

Demonstration of gastro-intestinal fluid model

One gram of synbiotic chocolate mixed into 10 ml of PBS at pH 2.5 was used to simulate gastric fluid. The viable cell population was determined at 0 hour and 3 hours of incubation on MRS Agar plates at 37° C for 48 hours.

Results and Discussion

Isolation of *Leuconostoc* sp

Molecular identification of LAB strain

Isolation of genomic DNA and Identification of bacteria by 16S r RNA gene sequencing

Sequences of the sample

>Isolate 3

>03_16SF_S17178

CAGTCGACGCACAGCGAAGCGTGCATGCACCTT
TCGAGTGAGTGGCGAACGGGTGAGTAACACGTG
GACAACCTGCGTCAAGGCTGGGGATAACATTTG
GAAACAGATGCTAATACCGAATAAAACTTAGTG
TCGCATGACAAAAAGTTAAAAGGGGCTTCGGCG
TCACCTAGAGATGGATCCGCGGTGCATTAGTTA
GTTGGTGGGGTAAAGGCCTACCAAGACAATGGT
GCATAGCCGAGTTGAGAGACTGATCGGCCACAT
TGGGACTGAGACACGGCCCAAACTCCTACGGGA
GGCTGCAGTAGGGAATCTTCCACAATGGGCGAA
GGCCTGATGGAGCAACGCCGCGTGTGTGATGAA
GGCTTTCGGGTCGTAAAGCACTGTTGTATGGGA
AGAACAGCTAGAATAGGAAATGATTTTAGTTTG
ACGGTACCATACCAGAAAGGGACGGCTAAATAC
GTGCCAGCAGCCGCGGTAATACGTATGTCCCGA
GCGTTATCCGGATTTATTGGGCGTAAAGCGAGC
GCAGACGGTTTATTAAGTCTGATGTGAAAGCCC
GGAGCTCAACTCCGGAATGGCATTGGAAACTGG
TTAACTTGAGTGCAGTAGAGGTAAGTGGAACTC
CATGTGTAGCGGTGGAATGCGTAGATATATGGA
AGAACACCAGTGGCGAAGGCGGCTTACTGGACT
GCAACTGACGTTGAGGCTCGAAAGTGTGGGTAG
CAAACAGGATTAGATACCCTGGTAGTCCACACC
GTAAACGATGAACACTAGGTGTTAGGAGGTTTC
CGCCTCTTAGTGCCGAAGCTAACGCATTAAGTG
TTCCGCCTGGGGAGTACGACCGCAAGGTTGAAA
CTCAAAGGAATTGACGGGGACCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTTTGAAGCTT
TTAGAGATAGAAGTGTTCTCTTCGGAGACAAAG
TGACAGGTGGTGCATGGTCGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCC

Phylogeny tree analysis

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). Based on the BLAST analysis in the NCBI, RDB taxonomy analysis and phylogeny tree clearly revealed that that the given sample was belog to the taxa is *Leuconostoc mesenteroides*.

Assessment of Probiotic properties

Physiological characterisation

In light of growing interest in functional food rich in nutrients and health-promoting compounds as well as considering the demand from the consumers, primarily from children, probiotic chocolate is an interesting product meeting all these criteria (Cisowska *et al.,* 2019).

Since there is only few study prepared synbiotic chocolate using *Leconostoc mesenterodes* from fame idly batter.

Seven different bacterial isolates were obtained from two samples of idly batter collected from two different households locally. Based on gram character and vancomycin resistant property, 4 isolates were found to belong to the genus *Leuconostoc*, which are bacteriocin producing LAB.

Among four, One *Leuconostoc* isolate was selected that exhibited greater growth inhibitory activity against *E.coli,* which is a predominant enteric bacteria. Further reports provided by 16S rRNA gene sequencing, confirmed that the isolated LAB was *Leuconostoc mesenteroides.*

The invitro evaluation of probiotic properties of *Leuconostoc mesenteroides* showed 67.2%, 93%, 70%, and 96% survival by tolerating low pH 2.5, 0.3% bile salt, pepsin at pH 2.5 and pancreatin at pH 8 which are the common gastro intestinal conditions of mammals.

Table.1 Phenotypic characterisation of *L.mesenteroides*

S. No.	Features	Observation
1	**Colony morphology**	
	Shape	Round
	Size	Less than 1 mm
	Colour	White
	Consistency	Slimy
2	**Preliminary tests**	
	Gram staining	Gram-positive cocci
	Motility	Non-motile
	Catalase	Negative
	Oxidase	Positive
3	**Biochemical characteristics**	
	Indole	Negative
	Methyl red	Negative
	Voges proskauer	Negative
	Citrate utilization	Negative
	Nitrate reduction	Negative
	Urease	Negative
	TSI	K/A
	Oxidative and fermentative	Positive
4	**Carbohydrate fermentation tests**	
	Glucose	Acid and gas production
	Fructose	Acid and gas production
	Lactose	Acid production only
	Sucrose	Acid production only

Table.2 Probiotic properties of *L.mesenteroides*

Assays	Initial counts of *Leuconostoc mesenteroides* (log cfu/ ml)	Survival of *Leuconostoc mesenteroides* after a recommended period of incubation at appropriate conditions (log cfu/ ml)	Survival percentage
Acid tolerance	2.47	1.66	67.2%
Bile salt tolerance	2.78	2.65	93%
Pepsin tolerance	2.45	1.71	70%
Pancreatin tolerance	2.80	2.71	96%

Table.3 Temperature tolerance of *L.mesenteroides*

Temperature (at ^{o}C)	Thermo-tolerants count (cfu/mL)	Thermo-tolerance rate (%)
30^{o}C	2.74	95.6
60^{o}C	2.62	

Table.4 Growth parameters of *L.mesenteroides*

Time (in hours)	Optical density (at 600 nm)
0 hour	0.494
after 8 hours	0.598
after 16 hours	0.612
after 24 hours	0.703
after 32 hours	1.039
after 40 hours	0.934
after 48 hours	0.802

Table.5 Growth parameter of *L.mesenteroides* in the presence of inulin on MRS broth

Time (in hours)	Optical density (at 600 nm)
0	0.457
24	0.729

Table.6 In MRS Broth supplemented with 1% inulin

Time (in hours)	Optical density (at 600 nm)
0	0.927
24	1.532

Table.7 Survivability of *Leuconostoc mesenteroides* under gastric condition

Initial counts (log cfu/ ml)	2.77
Survival after 3 hours at pH 2.5 (log cfu/ ml)	2.57
Survival %	93

Table.8 Survivability of *Leuconostoc mesenteroides* under intestinal condition

Initial counts (log cfu/ ml)	2.79
Survival after 4 hours at pH 2.5 (log cfu/ ml)	2.71
Survival %	97

Figure.1 Bacteriocin extracted from four vancomycin resistant *Leuconostoc sp.*

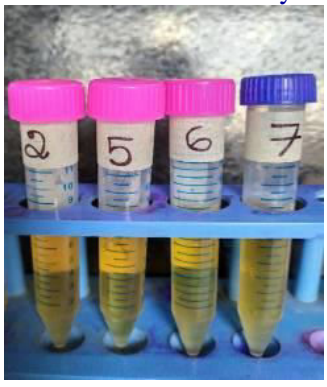


Figure.2 Invitro stomach model

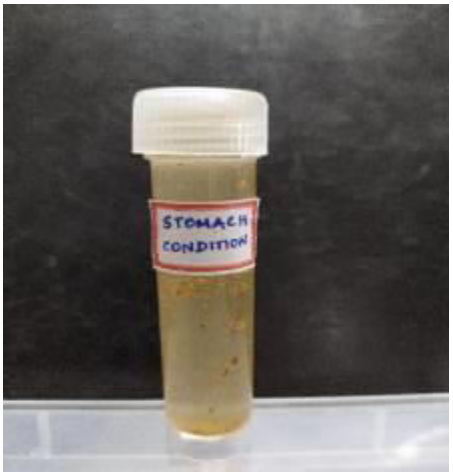
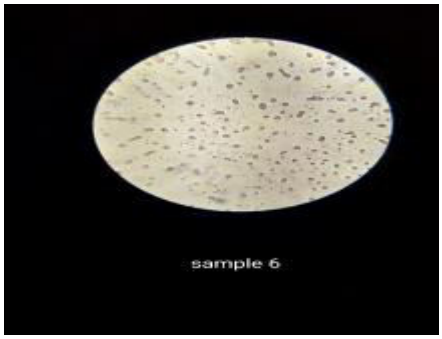


Figure.3 Invitro intestine model

Figure.4 *L.mesenteroides*



(a) Pure culture of *L.mesenteroides* **(b) Microscopic appearance of *L.mesenteroides***

Figure.5 Cultural appearance of *L.mesenteroides* on blood agar

Figure.6

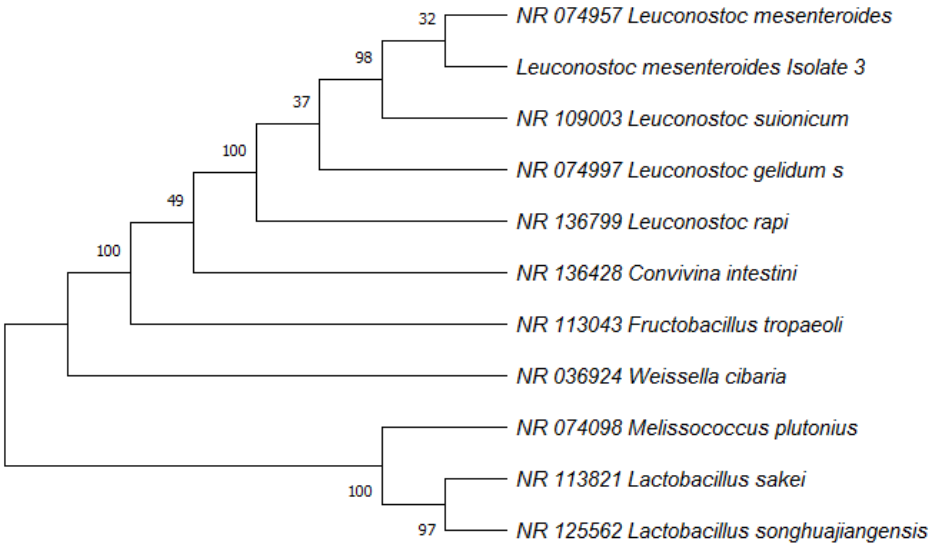
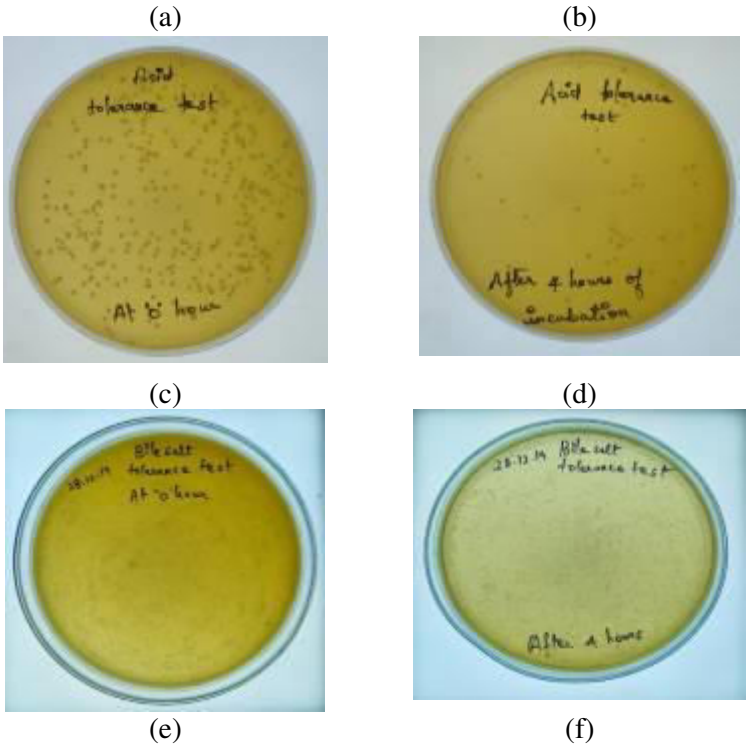


Figure.7 (a)-(f).Temperature tolerance of *Leuconostoc mesenteroides* at different conditions



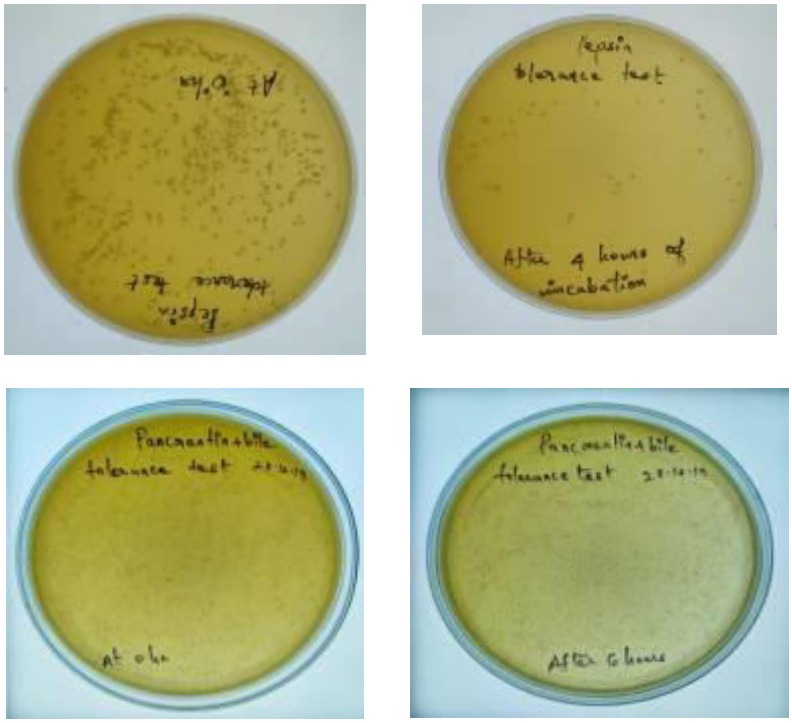


Figure.8 Viable number of colonies at (a) 30°C (b) 60°C and (c) 70°C

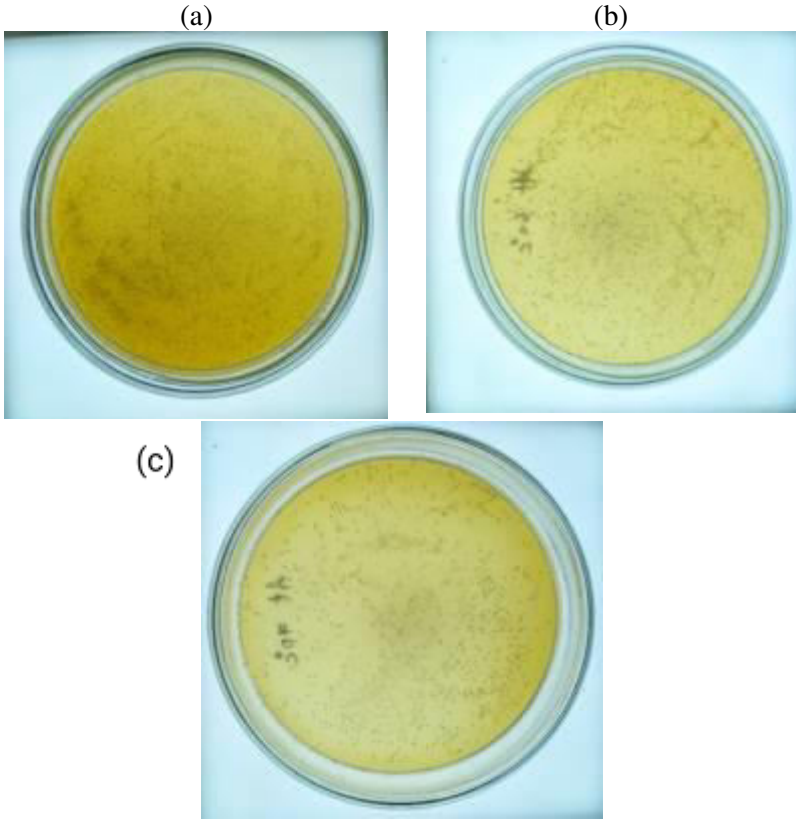


Figure.9 Milk coagulation activity of *L.mesenteroides*

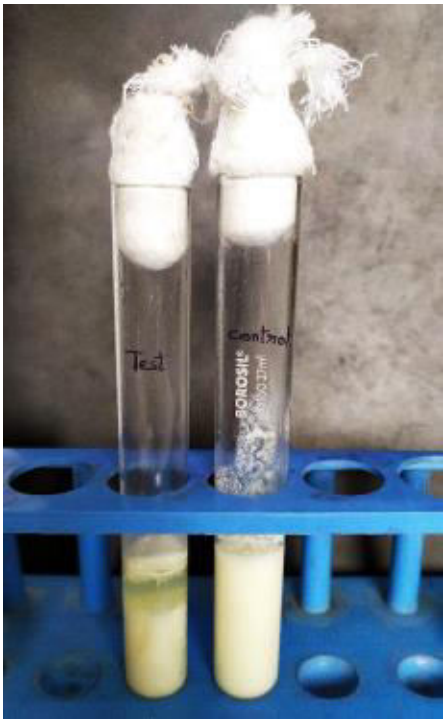


Figure.10 Lyophilised culture of *L.mesenteroides*

Figure.11 Preparation of sucrose-free synbiotic chocolate

Figure.12 (a) and (b) viable colonies of *L.mesenteroides* under gastric condition

(a) **(b)**

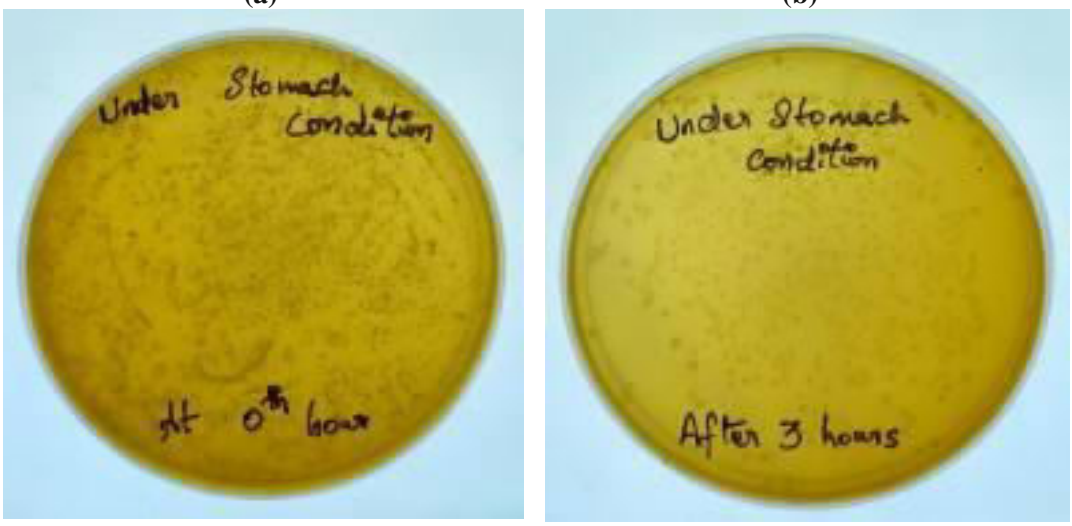
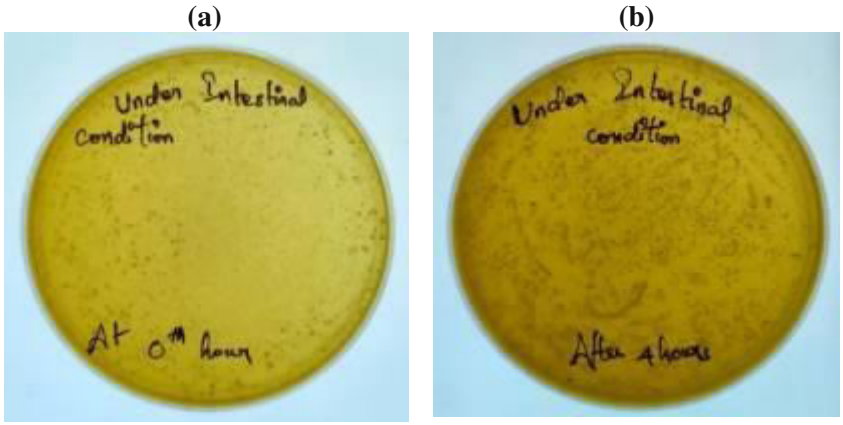
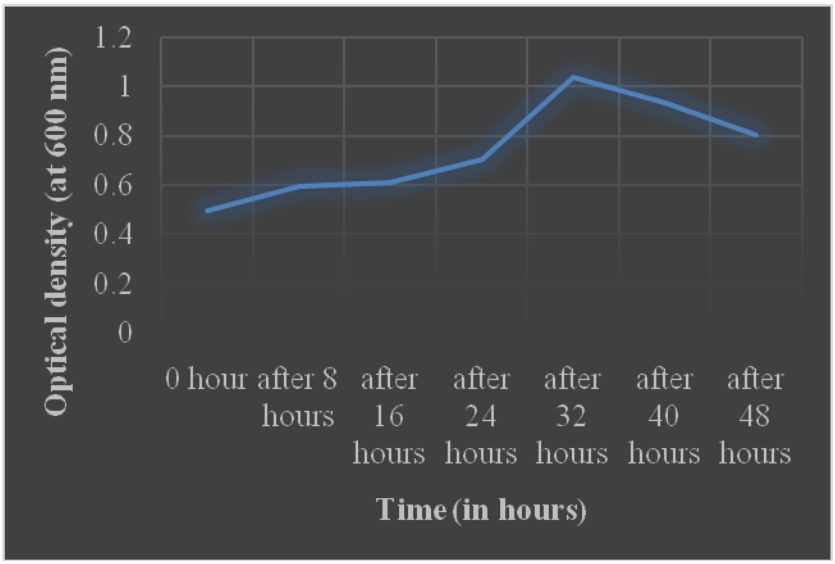


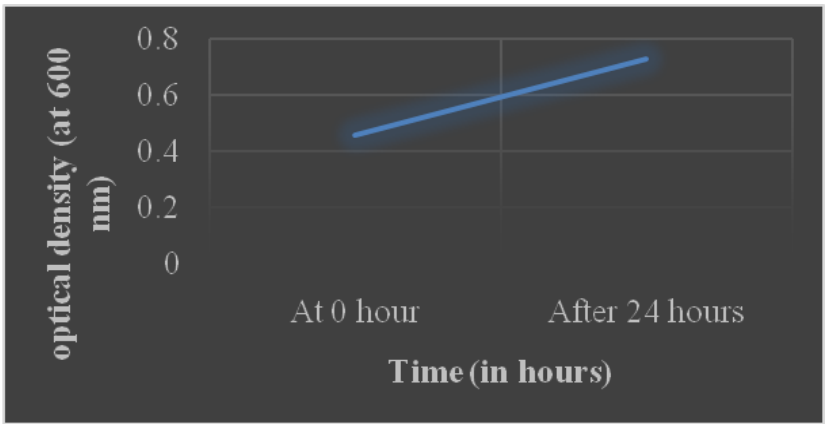
Figure.13 (a) and (b) viable colonies of *L.mesenteroides under intestinal condition*



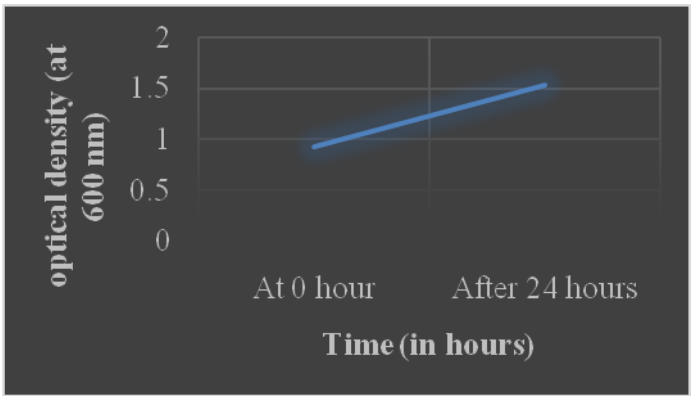
Graph.1 Growth curve of *L.mesenteroides*



Graph.2 Growth rate of *L.mesenteroides* in MRS broth



Graph.3 Growth rate of *L.mesenteroides* in MRS broth containing 1% inulin



The survival% of *Leuconostoc mesenteroides* at 60°C and 70°C were found to be 95.6% and 93%. This result affirmed its stability during manufacture of chocolate and thus it was elected to be supplemented via a chocolate as it includes many essential health benefits when consumed, it will afford enough haleness.

Leuconostoc mesenteroides are non-hemolytic bacteria and that further proving the non-pathogenic status of them. To be incorporated into food products, the probiotic cells should maintain their viability and functionality, they also need to survive the adverse environmental conditions of the upper gastrointestinal tract and should be able to colonize the gut. Unfortunately, after cell ingestion, the number of live microorganisms reaching the gut is too low to exert their action, making cell protection necessary

Growth kinetics of *Leuconostoc mesenteroides* were also evaluated in presence of 1% inulin by measuring the optical density (OD) value at 600 nm. The OD value of this culture grown in presence of inulin evidenced more progressive probiotic population when compared to OD value of culture grown in absence of inulin. On that account inulin was incorporated as an encapsulating material for probiotic, *Leuconostoc mesenteroides* and then lyophilised. A sucrose-free synbiotic chocolate was prepared by implementing this lyophilisate. Other authors have observed a greater influence of pH on this same parameter for *Leuconostoc mesenteroides* ATCC 8293 cells

The present study aimed to produce a synbiotic merchandise in the form of chocolate, considering inulin as prebiotic. Because chocolate is criticized for its saturated fatty acids and high calorific content, aspartame, a sucrose-free sweetener was added to eliminate these disadvantages. In contrast (Jung-Ae Kim *et al.,* 2019)

A gastro-intestinal fluid model demonstrated to assess the viability of lyophilised LAB in synbiotic chocolate. It displayed 93% and 97% survival of lyophilised *Leuconostoc mesenteroides* under simulated gastric and intestinal conditions. These results were in accordance (Mohammad Ali Khosravi Zanjani *et al.,* 2018) the microencapsulated technique can be fruitfully used in the food technology to obtain potential probiotic food products. The idea of combining both probiotic and prebiotic to make a product with the goodness of both, when consumed, it gives the synergistic effect.

Conclusion

In the present study, we have isolated *Leuconostoc mesenteroides* from a traditional South Indian fame idly batter and a sucrose-free synbiotic chocolate was formulated since the consumer interest to functional foods has been increasing every passing day. Aspartame, a sucrose free sweetener was added to avoid negative aspects of chocolate that include existence of saturated fat. Although the LAB were found to possess desirable in vitro properties to a greater extent, they are microencapsulated to make the LAB more resistant to heat stress and simulated gastrointestinal conditions compared to free cells. Inulin was a significant compound, a prebiotic substance that was used to encapsulate *Leuconostoc mesenteroides*. The data presented here show that the microencapsulated technique can be fruitfully used in the food technology to obtain potential probiotic food products. The idea of combining both probiotic and prebiotic to make a product with the goodness of both, when consumed, it gives the synergistic effect.

Conflict of Interest

We declare that we have no conflict of interest

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