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Development of Vegan Probiotic Chocolate

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ABSTRACT

Background: Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. However, options for lactose-intolerant individuals are limited due to the predominant use of dairy-based carriers. The present study was undertaken to isolate and identify potential probiotic strains and develop a novel dairy-free probiotic chocolate using microencapsulated *Lactobacillus* spp. with fruit pulp as a natural prebiotic source.

Methods: Fifteen bacterial isolates were obtained from yogurt samples and subjected to biochemical and sugar fermentation tests, leading to the identification of five *Lactobacillus* isolates (LC1 to LC5). These were evaluated for probiotic potential under varied pH (2 to 7) and NaCl concentrations (2%, 4%, 6% and 8%). Antimicrobial activity was assessed *via* the well diffusion method and antibiotic susceptibility was determined by the disc diffusion assay. The most promising isolate, LC1, was microencapsulated and incorporated into a dairy-free chocolate matrix using coconut pulp as a prebiotic. The encapsulated beads were added before setting the chocolate at 2-3°C and the final product was analyzed for texture properties.

Result: Optimal bacterial growth was observed at pH 6. Isolate LC1 demonstrated the highest salt tolerance and significant antimicrobial activity with a 3 mm inhibition zone. It also showed minimal susceptibility to antibiotics, indicating probiotic robustness. The incorporation of microencapsulated LC1 into chocolate slightly increased hardness compared to the control but retained desirable sensory properties. The developed product offers a functional and palatable probiotic food option for lactose-intolerant individuals.

Key words: Antimicrobial activity, Coconut pulp, Lactose intolerance, Microencapsulation, Probiotics, Salt tolerance.

INTRODUCTION

Probiotics, whether consumed as dietary supplements or through functional food products, have emerged as key components in the realm of health-promoting nutrition. They have long been recognized as vital agents offering a range of potential health benefits and are a focal point in commercial health food development (Sanz *et al*., 2016; Hamad *et al*., 2022). The term probiotic was first introduced by Werner Kollath in 1953, derived from the Latin pro and the Greek Bio, meaning "for life." Kollath described probiotics as active substances with essential roles in supporting various aspects of health (Gasbarrini *et al*., 2016). Later, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Microorganisms from several genera, including *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Propionibacterium* and *Bacillus*, have been identified as promising candidates for probiotic application (De Brito Alves *et al*., 2016; Hamad *et al*., 2022). Probiotics not only aid the gut by increasing the number of beneficial bacteria and inhibiting harmful bacteria but also strengthen the body's immune response. Prebiotics, act as 'food' for these beneficial probiotic bacteria, promoting their activity. The combinations of pre-and probiotics are called symbiotics as both work together in a synergistic manner to enhance the probiotic benefits (Sugandhi *et al*., 2018; Gehlot *et al*., 2018).

Probiotics can be consumed through various means, including their incorporation into dairy and non-dairy food products or as dietary supplements (Fenster *et al*., 2019). Many fermented foods naturally contain active microbial strains that are genetically similar to those recognized as probiotics. These fermented products not only contribute beneficial microbes but also improve the functional and nutritional profile of foods by transforming raw substrates into bioactive and bioavailable compounds (Marco *et al*., 2017). An effective daily dose of probiotics has been identified as approximately 10y colony-forming units (CFU) (Hill *et al*., 2014). To meet this effective dosage, the food industry has introduced probiotics into a wide range of products such as beverages, yogurt, ice cream, bread and more. However, a major challenge in probiotic application is their vulnerability to harsh processing conditions and

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gastrointestinal (GI) stresses, which can compromise their viability. Despite these limitations, consumer interest in probiotic-enriched foods remains strong due to their associated health benefits (Konuray and Erginkaya, 2018).

Lactose intolerance (LI) is a condition characterized by the inability to digest lactose due to low levels of lactase enzyme activity. Symptoms of LI include bloating, nausea, abdominal cramping and diarrhoea. Lactase enzyme activity is affected by several factors such as age, race, integrity of the small intestinal membrane and small intestinal transit time. Prevalence of LI in adult populations varies from 5 per cent to 15 per cent in European and North American countries, and 50 per cent to 90 per cent in African, Asian, and South American countries, highest rates of LI are found in the Asian populations (Misselwitz *et al.,* 2013).

Prebiotics are non-digestible, selectively fermented dietary fibers that promote the growth and activity of specific bacterial genera in the gastrointestinal tract, thereby offering health benefits to the host. They can feed the intestinal microbiota, and their degradation products are short-chain fatty acids that are released into blood circulation, consequently, affecting not only the gastrointestinal tracts but also other distant organs. Fructo-oligosaccharides and galacto-oligosaccharides are the two important groups of prebiotics with beneficial effects on human health (Davani-Davari *et al.*, 2019)

The most widely studied prebiotics are inulin-type fructans and galacto-oligosaccharides. The selective nature of prebiotics is driven by species-specific gene clusters found in saccharolytic bacteria, which are regulated by substrate-responsive signaling mechanisms. The beneficial effects of prebiotics are largely attributed to immune modulation and the production of bacterial metabolites. In humans, prebiotic supplementation has been shown to increase the abundance of beneficial gut microbiota, such as Bifidobacteria, enhance immune function and, depending on the microbial response, stimulate the production of short-chain fatty acids (SCFAs). Gastrointestinal disorders like irritable bowel syndrome (IBS) and Crohn's disease are commonly associated with a decline in beneficial gut bacteria and heightened mucosal inflammation (Wilson and Whelan, 2017).

Encapsulation is a mechanical or physicochemical process that traps a potentially sensitive material and provide a protective barrier between it and the external conditions (Katke *et al*., 2022). Vegan chocolate is made of fine solid sugar and cocoa particles suspended in a continuous fat phase. Chocolate solidifies between 20 and 25°C and melts at body temperature (37°C), resulting in a smooth suspension of solid particles and a pleasant sensation in the mouth. The continuous phase affects sensory qualities like melt in the mouth or mouth feel. There are plenty of commercially accessible probiotic-infused dairy-based items on the market, including frozen treats, dairy products, baby food, chocolate and puddings (Abbasiliasi *et al*., 2019). The development of probiotic chocolate with fruit pulp added for prebiotic benefits is suggested by the current investigation.

The main objective of this study is to develop a vegan probiotic chocolate, along with fruit pulp as prebiotic source, using microencapsulated *Lactobacillus* strains which were isolated, identified and screened for their potential probiotic properties.

MATERIALS AND METHODS

Isolation of lactobacillus

The lactic acid bacteria were isolated from yoghurt collected from the local market. A loop full of yogurt was inoculated into 20 ml sterile MRS broth under aseptic conditions and incubated at 37°C for 24 h in a rotary orbital shaker. A loop full of the inoculated broth was streak plated on MRS agar and incubated at 37°C up to 18 h. Bacterial colonies grown were purified by repeated streaking. The bacterial isolates were examined for their colony morphology, gram staining character, biochemical tests (Mannan *et al.,* 2017). Identified pure cultures were stored in MRS agar slant for further study.

Screening of bacterial isolates for probiotic properties

Acid tolerance test

Test tubes containing MRS broth were adjusted to pH 2 to 7 using HCl and NaOH to decrease and increase the pH respectively. After sterilization, each test tube was inoculated with 0.1% fresh overnight grown probiotic culture, incubated at 37°C for 24 h and the growth was determined by Spectrophotometer at 600nm absorbance (Reda *et al*., 2018).

Salt tolerance test

Test tubes containing MRS broth with different concentrations of NaCl (2%, 4%, 6% and 8%) were prepared. After sterilization, each test tube was inoculated with 0.1% fresh overnight culture, incubated at 37°C for 24 h and the growth was determined by Spectrophotometer at 600 nm absorbance (Reda *et al.,* 2018).

Antimicrobial activity

The *Lactobacillus* isolates were examined for their antimicrobial activity against the indicator microorganism *Staphylococcus aureus* by well diffusion method. Antibacterial activity was estimated as the diameter of the inhibitory zone formed around the wells (Forhad *et al.,* 2015).

Antibiotic susceptibility test

Antibiotic susceptibility test is widely performed using disc diffusion method. MRS agar plate containing 10 µL of bacterial isolate was prepared in this present study. Antibiotic discs containing Meropenem, Ciproflaxin, Tobramycin, Moxiflaxin, Ofloxacin, Sparfloxacin, Levoflaxin, Impenem were placed on MRS agar plates containing bacterial isolate and zone of inhibition were noted after incubation at 37°C for 24 hr (Hoque *et al*., 2010).

Encapsulation of probiotic bacteria

Procedure

Sodium alginate solution was prepared by dissolving 2 g (w/v) of sodium alginate powder with 100 ml distilled water in 250 ml sterilized beaker. Afterward, it was heated until boiling for proper mixing. Calcium chloride solution was prepared by dissolving 5 g (w/v) calcium chloride granules in 500 ml beaker with simultaneous gentle stirring. Both the reagents were sterilized before use. The cell biomass was added to a 2.0% (w/v) strength sodium alginate solution with gentle stirring. Under aseptic conditions, the bacterial alginate suspension was packed into the sanitized container of spray gun (make pilot spray gun P-5) having nozzle size of 1.6 mm and this was connected to a laboratory air compressor (range: 14-58 psi). The filled alginate cell biomass mix was then sprayed into prepared 1% (w/v) calcium chloride solution. This allowed the formation of microcapsules, and these beads were allowed to harden for 5 min. The probiotic ca-alginate beads were then harvested with the help of sanitized nylon sieve (size <100 µm) and were air-dried at the normal temperature (Mandal *et al.,* 2012).

Preparation of probiotic chocolates

Preparation of chocolate

By double boiler method, over low heat, add 50 gm-coconut oil, 10gm-cocoa powder, 45 gm-icing sugar. Stir constantly until well combined and a pourable consistency is achieved. Add in the vanilla essence and whisk until just combined. The prepared chocolate was allowed to cool to room temperature (Fig 1).

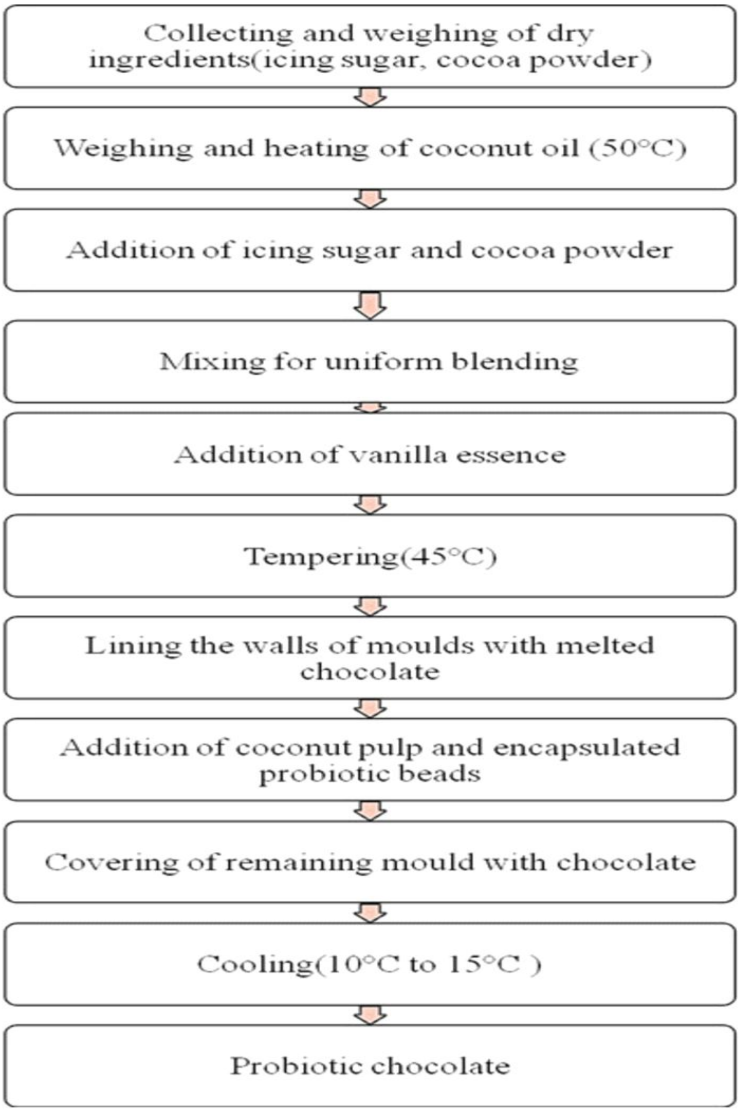

Fig 1: Flowchart for preparation of probiotic chocolate.

Preparation of fruit pulp

Collect tender coconut flesh and shred into fine pieces. Blend the coconut shreds with equal amount of water. Strain the mixture to separate coconut milk. The coconut pulp is obtained.

Texture analysis

Chocolate samples in block shape were analysed for their textural properties using Brookfield CT3-texture analyser with TA44 type penetration probe. Hardness and fracturability were determined by penetration analysis with test speed 1mm/s, pre-test speed 1 mm/s in 2 cycle penetration. Analysis was done in ambient temperature at 30°C, where each sample was frozen in first cycle and entered melting phase in second cycle (Limbardo *et al.*, 2017).

RESULTS AND DISCUSSION

This chapter contains the results obtained for isolation, identification of lactobacillus bacteria and results of screening test for potential probiotic bacteria. The obtained results are represented in suitable forms and discussed.

Isolation of lactobacillus bacteria

A total of 15 *lactobacillus* isolates were cultured on MRS agar plate by streak plate method and initially subjected to morphological and biochemical tests.

Identification of lactobacillus bacteria

Based on the morphological characteristics of the bacteria, among 15 isolates five isolates were found to be Gram-positive, non-sporeforming, rod shaped as shown in Table 1.

Screening of bacterial isolates for probiotic properties

Acid tolerance

The acid tolerance test helps in studying the survival of the strains under low pH gastric juice conditions.In the present studies, it was observed that as pH increased from 2 to 7, there was a gradual increase in turbidity and reached maximum at pH 6 as shown in the Fig 2. This indicates that the probiotic isolates are tolerant to acidic environment. Table 4 indicates that LC1 isolate showed maximum growth (OD_{580nm}=0.250) at pH 2 followed by LC4 (OD_{580nm}=0.214) and minimum growth is seen in LC5. Pundir *et al*. (2013) reported that the lactobacillus isolated from fresh vegetables and curd survived in pH 3.5 to pH 7. Similar study conducted by Hoque *et al*. (2010) found lactobacillus growth in pH 2.5 to pH 8.5.

The sodium chloride tolerance test was performed to test the organism's ability to tolerate various osmotic conditions. As sodium chloride concentration increases from 2 to 6 percentthe growth was decreased as shown in Fig 3. Table 2 indicates the growth of bacteria at different NaCl concentrations. The LC1 showed maximum growth at 8 percent concentration (OD_{580nm}=1.026) followed by LC3 (OD_{580nm}=0.649) and LC5 showed minimum growth (OD_{580nm}=0.198). Mannan *et al.* (2017) stated that all the isolates were able to grow 2% and 4% of sodium chloride concentration of lactobacillus species from yoghurt and cheese samples in Dhaka Metropolitan area. Absorbance at 580 nm.

Table 1: Colony morphological characteristics of lactobacillus isolates obtained from yogurt.

Isolates	Size	Colour	Elevation	Texture	Opacity	Gram reaction	Shape and arrangement
LC1	Small	Creamish white	Raised	Smooth	Opaque	+	Rods in chains
LC2	small	Creamish white	Raised	Smooth	Opaque	+	Rods in chains
LC3	small	Creamish white	Raised	Smooth	Opaque	+	Rods in chains
LC4	small	Creamish white	Raised	Smooth	Opaque	+	Rods in chains
LC5	small	Creamish white	Raised	Smooth	Opaque	+	Rods in chains

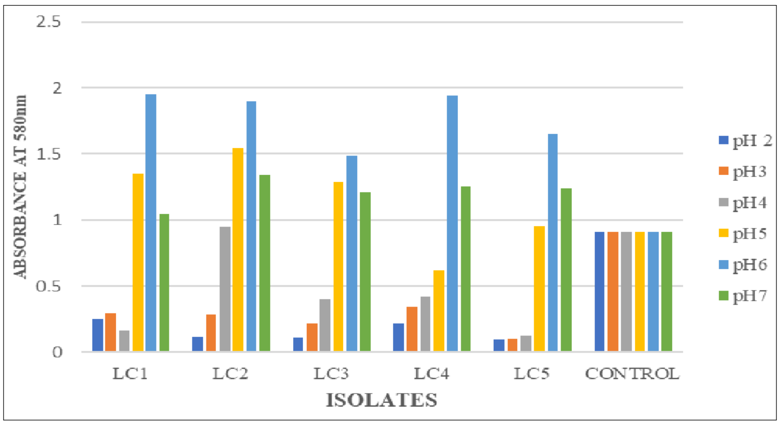

Fig 2: Acid tolerance of lactobacillus isolates.

Table 2: Growth of lactobacillus isolates at different NaCl concentrations.

Isolates	NaCl			
	2%	4%	6%	8%
LC1	1.929±0.054^{a}	1.407±0.021^{b}	1.346±0.012^{b}	1.026±0.015^{a}
LC2	1.866±0.011^{ab}	1.648±0.046^{a}	1.282±0.012^{b}	0.435±0.006^{c}
LC3	1.788±0.002^{b}	1.534±0.014^{a}	1.289±0.003^{b}	0.649±0.004^{b}
LC4	1.828±0.003^{ab}	1.784±0.004^{a}	1.400±0.002^{a}	0.615±0.006^{b}
LC5	1.829±0.002^{ab}	1.516±0.007^{a}	1.323±0.004^{b}	0.198±0.006^{b}
Control	0.0910±0.001^{a}	0.912±0.001^{a}	0.0910±0.001^{a}	0.910±0.001^{a}

Values are mean±SD (n=3).

Different superscript letters within a column differ significantly (p<0.005) according to one-way ANOVA and Tukey's multiple comparison test.

Table 3: Antimicrobial activity of lactobacillus isolates against pathogenic bacteria.

Isolates	Zone of inhibition (mm)
LC1	03
LC2	1.6
LC3	1.2
LC4	00
LC5	00
Control	00

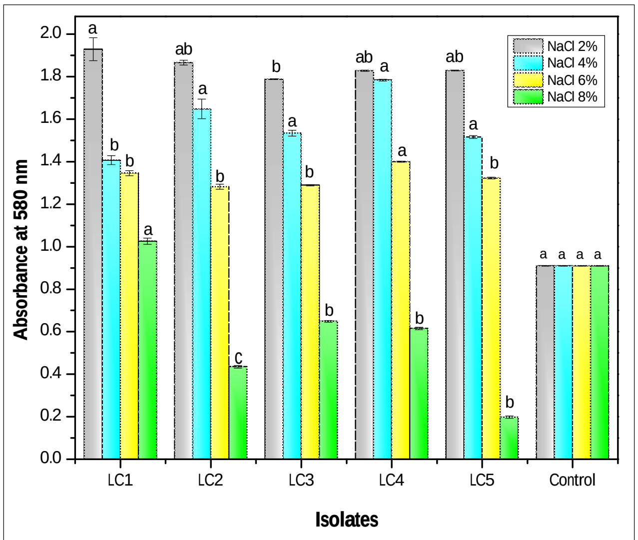

Fig 3: NaCl tolerance of lactobacillus isolates.

Antimicrobial activity

The results of antimicrobial activity of the probiotic culture showed that the probiotic isolate can restrain the growth of pathogenic bacteria. The zone of inhibition produced by the bacteria indicates its antimicrobial activity as shown in Table 3. The antimicrobial effect could be due to the production of acetic acid or lactic acids that lowers the overall pH. The isolate LC1 clearly exhibited the zone of inhibition (3 mm) for antimicrobial activity against *S. aureus*as as shown in Fig 4. The isolates LC2 and LC3 produced a inhibition zone of 1.6 and 1.2 mm respectively. Whereas LC4 and LC5 isolates did not exhibit zone of inhibition against the pathogenic bacteria.

Sharma and Bisht (2017) reported the effects of lactobacillus isolates of curd against food borne and human pathogens. It was observed that none of the lactobacilli cellfree supernatant (CFS) exhibited inhibitory activity against four pathogens, namely *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Klebsiella pneumoniae*. *Bacillus cereus*, *Salmonella enterica serovar Typhi* and *Shigella flexneri* were moderately inhibited by majority of CFSs, whereas, weak activity was observed against Pseudomonas aeruginosa and Proteus mirabilis. CFS of some of the curd isolates displayed antagonistic activity against Streptococcus mutans; however, human milk lactobacilli did not displayed any inhibitory activity against them. Kang *et al*. (2017) also reported that lactobacillus spp. had bactericidal effect against planktonic and biofilm *S.aureus*. It was observed that Cell-free supernatant that was pH neutralized and heat inactivated or proteinase K treated had significantly reduced killing of *L. salivarius* than with pH-neutralized supernatant alone.

Antibiotic susceptibility

The zones of inhibition observed against the eight antibiotics indicated that probiotic culture isolated in the present study is sensitive to Meropenem, Moxifloxacin and Impenem as shown in Fig 5. Among all the isolates it was observed that LC1 showed lesser zone of inhibition to antibiotics and no zone of inhibition to two antibiotics (Ofloxacin, Levoflaxin), whereas LC5 isolate was found to be more susceptible to all the antibioticsas indicated in Table 4.

Sharma and Bisht (2017) reported LAB isolates of curdorigin showed sensitivity towards Ampicillin, Imipenem, Meropenem, Chloramphenicol and Erythromycin while investigating the antibiotic sensitivity pattern of indigenous Lactobacilli isolated from curd and human milk samples.

Preparation of probiotic chocolates

Several studies in recent years have shown the therapeutic benefits derived from the ingestion of probiotic foods. Chocolate is a more effective delivery system than capsules and tablets for probiotics and could be an attractive alternative for consumers. Chocolate has a distinct taste, flavour and texture and is also a source of biologically active substances, like polyphenols that show substantial antioxidant properties and have a positive impact on human health, principally on the cardiovascular system (Homayouni *et al.*, 2016). An *in vitro* model of the human digestive tract (Simulator of the Human Intestinal Microbial Ecosystem, SHIME) shows that chocolate can indeed represent an ideal carrier for the intestinal delivery of probiotics.

In this context, in the present study steps are taken up to develop a probiotic chocolate with the isolated probiotic culture and test its viability and consumers satisfaction survey. Incorporation of the immobilised LAB in chocolate is an excellent solution to protect them from environmental stress conditions and for optimal delivery. In this work, chocolate has been evaluated as a potential protective carrier for oral delivery of a microencapsulated mixture of *Lactobacillus* sp. The Lactobacillus strains were encapsulated as micro-beads by using sodium alginate and calcium chloride and these beads were incorporated into chocolate suspension. Polysaccharides in the coconut pulp constitute an effective growth substrate for enhancement of *Lactobacillus* spp. proliferation. (Abbasiliasi *et al.*, 2019) reported that the polysaccharides show stronger activity such as better resistance to human

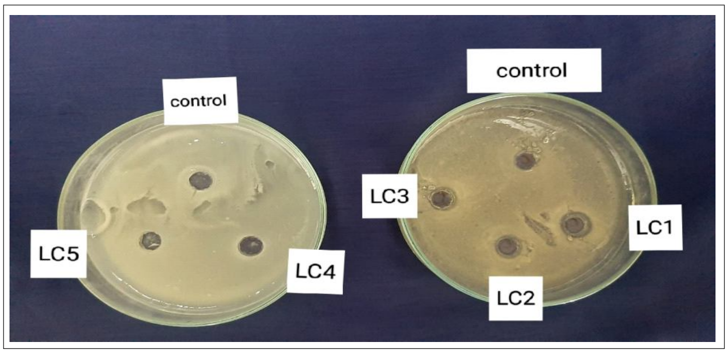

Fig 4: Zone of inhibition produced by lactobacillus bacteria.

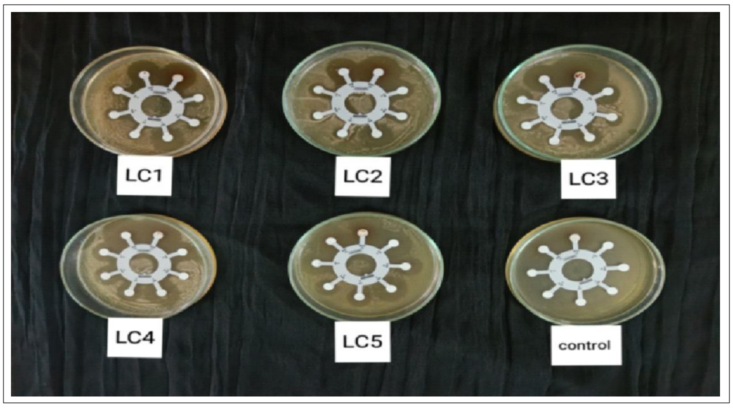

Fig 5: Effects of different antibiotics on lactobacillus isolates.

Table 4: Effects of different antibiotics on lactobacillus isolates.

Antibiotic	Zone of inhibition (in mm)					
	LC1	LC2	LC3	LC4	LC5	Control
Meropenem	10.0	11.0	14.0	14.0	14.0	00
Ciproflaxin	2.0	4.0	7.0	4.0	7.0	00
Tobramycin	2.0	3.0	4.0	00	6.0	00
Moxifloxacin	8.0	7.0	4.0	6.0	8.0	00
Ofloxacin	00	00	00	4.0	5.0	00
Sparfloxacin	3.0	1.0	1.0	5.0	4.0	00
Levoflaxin	00	2.0	5.0	5.0	6.0	00
Impenem	8.0	8.0	9.0	13.0	14.0	00

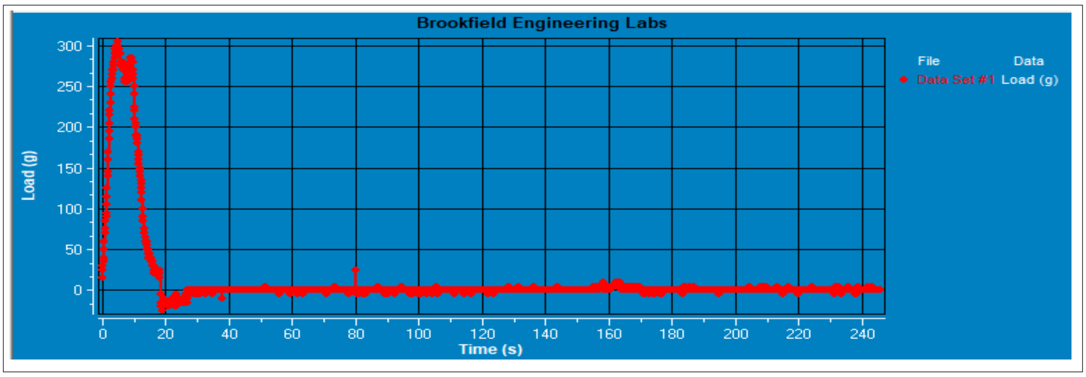

Fig 6: TPA graph for probiotic chocolate.

gastric juice and a-amylase as compared to that of commercial prebiotic-FOS, which ensures their reaching the colon to effectively stimulate the growth of probiotic bacteria. A chocolate with encapsulated probiotic bacteria and coconut pulp was prepared.

Texture analysis

The hardness and fracturability of probiotic chocolate determined by the texture analyzer at ambient temperature 30°C was found to be 305 g and 207 g respectively as shown in Fig 6. The hardness of the prepared chocolate was found to be slightly greater than a normal dark chocolate made with coconut oil which may be due to the presence of coconut pulp and microencapsulated beads. (Limbardo *et al*., 2017) reported that the substitution of coconut oil for cocoa butter reduced the hardness of chocolate bar from 152.5 to 74.75 g.

CONCLUSION

A total of 15 isolates were isolated from yogurt. Among these 15 isolates five were identified as lactobacillus spp. (LC1, LC2, LC3, LC4, LC5). These were screened for their probiotic properties. In the acid tolerance test, LC1 isolate showed maximum growth (OD_{580nm}=0.250) at p^H 2. In the NaCl tolerance test, at 8 per cent NaCl concentration, LC1 isolate showed maximum growth (OD_{580nm}=1.013). Among all the isolates LC1 showeda clear zone of inhibition of 3 mm against pathogen bacteria. In antibiotic susceptibility test, LC1 isolate showed lesser zone of inhibition to antibiotics.

Hence, LC1 isolate was found to be efficient and have potential probiotic properties among the five isolates and was used to prepare the encapsulated probiotic beads. The probiotic chocolate was formulated using fruit pulp, probiotic beads and chocolate is a functional food since it has the beneficial effects of fruit, probiotic and dairy free chocolate. From texture analysis, the hardness of the prepared chocolate was found to be slightly greater than a normal dairy free chocolate which may be due to the presence of coconut pulp and microencapsulated beads. The idea of combining all these dairy free ingredients and making a product with the goodness of each of the ingredients gives the combined effect in the functional food and serves as a probiotic source for lactose intolerant people.

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Conflict of interest

Authors declare that there is no conflict of interest.

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