CHOCOLATE AS PROBIOTIC DELIVERY VEHICLE

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Abstract
In today's world, food, besides their basic role in nutrition, some additional aspects are becoming increasingly important, which includes the maintenance of health and counteracting diseases. Scientific research refers to health benefits of using probiotics and prebiotics in human nutrition. An increasing demand of consumers for foodstuffs supplemented with live probiotic bacteria, gave rise to studies on the development of foods with these microorganisms. Despite high sugar content, chocolate consumption makes a positive contribution to human nutrition through provision of antioxidants, principally polyphenols. The main objective of this work was to develop a potential probiotic chocolate by using microencapsulated Lactobacillus strains along with fruit pulp as prebiotics and to study the sensory evaluation and shelf-life of probiotic chocolate. The results revealed that the probiotic chocolate prepared with the incorporation of fruit extract like Passion fruit and Strawberry fruit rated the maximum by the consumers through sensory evaluation and the product has shelf-life of twenty days at room temperature without losing any organoleptic properties. The experimental study consent was taken from all the subjects participated in the sensory evaluation of the product prepared in the current research.

Keywords: Chocolate, probiotics, Passion fruit, sensory evaluation, shelf life, Strawberry

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1. INTRODUCTION

Due to the rapid expansion in food industries, several traditional food items are currently being improved to include beneficial components that will deliver advantages beyond mere nourishment. The modification of traditional foods makes it certainly acceptable by the customers than introduction of a new food product (Markowiak and Śliżewska, 2017). Various functional foods are consumed as part of a regular diet and they provide consumers with well-accepted physiological benefits such as probiotic bacteria.

Probiotics are live microorganisms that can be delivered into various types of products like food, drugs and dietary supplements. Consumption of foods accompanied with probiotic strains benefit human health due to their well-known positive role on the function of the gastrointestinal tract and immune system, decrease in cholesterol, and significant anticancer activity. The major sources of these probiotics are fermented dairy products (Somnath et al, 2017).

The health-supporting value of a given foodstuff is appraised based on the amount of live probiotic bacteria exist. The other key criteria to decide the efficacy and the attainment of the product encompassing probiotics are the approval of the product by the consumers and the persistence of probiotic microorganisms in the product. *Lactobacilii, Bifido* bacteria and several other lactic acid bacteria are regarded as probiotics. In recent times, there are several probiotic products such as ice cream, cheese, infant formulas, breakfast cereals, sausages, chocolate and puddings are escalating in the food market. Similarly non - dairy food has been manufactured with the addition of probiotic microorganisms (De et al, 2016).

The distinctive taste and flavor of chocolate is much tempting to all age group. Incorporation of probiotics into chocolate gives a worthy alternative to the routine dairy products (Fenster et al, 2019; Homayouni et al, 2016).

Probiotics can only exert their function after they reach the intestines. But before reaching the intestines, they have to cross through a

multiple harsh environment. So to ensure a good amount of probiotic cells reach the intestine, it is essential to guard the probiotic cells from the tough environment. This can be attained by a number of methods. Encapsulation of cells in a hydrocolloid matrix is one such method. The use of biopolymers such as alginate, k-carrageenan, xanthan gum, and Gellan gum to trap cells is a simple technique to protect cells (Manasouripour et al, 2013).

The conception of making probiotics chocolate is not entirely new as many food companies have appealed to create probiotic chocolate with all the goodness of chocolate and benefits of probiotics. But the present work proposes the production of probiotic chocolate with added fruit pulp to serve as prebiotics that is comparatively new. In the present study, an attempt was made to make a potential probiotic chocolate by using microencapsulated *Lactobacillus* strains fortified with fruit pulp, to study the sensory evaluation and shelf life of the product prepared.

2. MATERIALS AND METHODS

Raw Materials

The raw materials such as cocoa powder, skimmed milk powder, butter, sugar etc. were procured from the local market.

Methods

1) Screening & Identification of probiotic bacteria

A loop full of household yoghurt was inoculated into 20mL sterile MRS broth (De Man-Rogasa-Sharpe) in aseptic condition and incubated at 37℃ for 24h on a rotary orbital shaker. 10µL of the inoculated broth was spread plated on MRS agar plate and incubated at 37℃ up to 48h. Bacterial colonies grown were purified by repeated streaking. The bacterial isolates were examined for their colony morphology, gram staining character, motility, biochemical tests as per the standard procedure ( ).

2) Confirmatory tests for probiotic properties

a) Acid tolerance test

To determine the tolerance of the isolated probiotic strain in acidic environment, growth of the bacterial strains at various pH (2, 3 and 4) were studied. Test tubes containing MRS broth were adjusted to pH 2, 3 and 4. 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 colony count (Hoque et al, 2010).

b) Sodium Chloride Tolerance Test

For the determination of NaCl tolerance of isolated probiotic culture, test tubes containing MRS broth were adjusted with different concentrations of NaCl (2%, 4%, 6% and 8%). 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 colony count (Hoque *et al* 2010).

c) Milk Coagulation Test

To determine the ability of the probiotic bacterial culture to coagulate milk, 0.1% (v/v) culture was inoculated into 100 ml of sterilized cow's milk, incubated for 24 hr and observed for the coagulation of milk (Forhad et al, 2015).

d) Antimicrobial Activity

The bacterial isolates were examined for their antimicrobial activity against the indicator microorganisms' viz. *Escherichia coli*, *Streptococcus sp a*nd *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).

e) Antibiotic Susceptibility Test

Antibiotic susceptibility test is widely performed using disk diffusion method. MRS agar plate containing 10 µL of probiotic bacterial isolate was prepared in this present study. Disc impregnated with the two antibiotics namely Azithromycin and Loperamide Hydrochloride were placed on the agar plates and the zones of inhibition were noted after incubation at 37 °C for 24 h count (Hoque et al, 2010).

3) Encapsulation of probiotic bacteria

The isolated probiotic bacterial culture were immobilized using 3% Sodium alginate and saturated Calcium Chloride and the beads were refrigerated in a sterile condition for further use (Panda, 2014).

4) Preparation of probiotic chocolates

a) Preparation of chocolates

Four table spoons of oil, 1¼ table spoon cocoa powder and two table spoons milk powder was added in sequence in a double boiler and kept on the flame for thorough mixing for 2 minutes. Then powdered sugar was added according to taste, mixed and poured into chocolate moulds. The prepared chocolate was allowed to cool (Ramakrishna et al, 2013).

b) Preparation of fruit extract/ pulp

In the present study, Passion fruit and Strawberry fruit were used as prebiotics. For the preparation of the fruit extract/pulp, a semi ripe/ripe fruit was used. After the removal of outer covering and seeds, juice was extracted from the pulp of using a blender, filter sterilized and used as a source of pre-biotic for the chocolate preparation

c) Incorporation of immobilized probiotic culture and fruit extract to the chocolate

One gram of melted chocolate was used to line the walls of the suitable moulds. Two grams of thickened fruit extract was added to the mould. Depending on the size of the mould around six probiotic beads were added to the mould and it was covered with a layer of melted chocolate. The chocolate was allowed to freeze in fridge for 2h to set. After the chocolate sets, it was carefully removed from the mould to obtain the probiotic product.

B. Sensory evaluation

The product development will be effective only when it is accepted by the consumer. Sensory evaluation was undertaken with fifty members of age group 20-60 years. The experimental study consent was taken from all the subjects participated in the sensory evaluation of the product. Developed products were evaluated for sensory attributes such as color, texture, flavour, taste and overall acceptability. Sensory attributes (organoleptic study) like colour, texture, firmness, and flavour were assessed (Deshpande et al, 2019). The overall acceptability of each product was done on a 9-point hedonic scale (Kaur and Kaur, 2019).

C. Shelf-life study

The shelf life study of the probiotic chocolate was performed as per the procedure given by Sarvari et al, 2014. Briefly 0.01 gm of Chocolate was diluted in sterile saline solution, 0.01 ml aliquots were spread plated into MRS agar plates, incubated at 37°C for 48 h and the colonies were counted. The shelf life study was done at the interval of two days up to 22 days at room temperature (28 ± 2^0C).

3. RESULTS AND DISCUSSION

De Man, Rogosa and Sharpe agar, often abbreviated to MRS, is a selective culture medium designed to favour the luxuriant growth of *Lactobacilli* for lab study. A total of 178 bacterial colonies were isolated on MRS agar plate and initially subjected to morphological and biochemical tests. Through morphological and biochemical tests, the isolated bacterial colonies were found to be Gram-positive, non-spore-forming and catalase-negative, able to ferment all the tested sugars and were considered as presumptive probiotic bacterial isolates (Table 1).

Table 1. Identification of the isolates

Colony Characteristics	Observation
Shape	Rod
Margin	Undulated
Elevation	Convex
Size	Moderate
Texture	Smooth
Appearance	Shiny
Pigmentation	Non-pigmented
Optical property	Translucent
Gram Staining	positive
Motility	Non motile
Indole test	Negative
Biochemical tests	
Methyl Red test	Negative
VP test	Negative
Citrate utilization	Negative
Catalase test	Negative
Sugar fermentation (Glucose/Sucrose/Lactose)	Positive

A. Confirmatory tests for probiotic properties

1) Acid Tolerance

The acid tolerance test helps in studying the survival of strains under low pH gastric juice conditions. In the present study, it was observed that as the pH increased from 1 to 3, there was a gradual increase in number of colonies and it reached maximum at pH 3 beyond which no growth was seen indicating acidic tolerance nature of the isolated probiotic culture (Fig 1). Menconi et al, 2014 also reported similar results and showed that *Lactobacillus* strains were resistant up to pH of 3.0 while working with the identification and characterization of Lactic acid bacteria in a commercial probiotic culture.

2) Sodium Chloride Tolerance Test

The sodium chloride tolerance test was performed to test the organism's ability to tolerate various osmotic conditions. As sodium chloride concentration increased from 2 to 6% growth also increased and no growth was observed at 8% proving the tolerance against sodium chloride up to 6%. Mannan et al, 2017 stated that all the isolates were able to grow at 2% and 4% of sodium chloride concentration but did not grow at 8% sodium chloride while studying the isolation and characterization of *Lactobacillus* species from yogurt and cheese samples in Dhaka Metropolitan area. The present finding was in tune with this report.

3) Milk Coagulation Test

The addition of isolated probiotic culture to the milk resulted in its coagulation after 24h which indicates the production of an enzyme accountable for the production of curd (Fig 2).The present result is in agreement with Forhad et al, 2015 who experienced similar results while analyzing the probiotic properties of the lactic acid bacteria isolated from buffalo milk.

4) Antimicrobial Activity

The results of antimicrobial activity of the probiotic culture (Fig 3) revealed that the isolated probiotic strains can produce antimicrobial product which can restrain the growth of pathogenic bacteria. The antimicrobial effect of probiotics could be due to the production of acetic and lactic acids that lowered the overall pH. Sharma et al, 2017 reported antibacterial effects of *Lactobacillus* isolates of curd and human milk origin against food-borne and human pathogens. Davoodabadi et al, 2015 suggested that *Lactobacillus* strains have a mild inhibitory activity against the diarrheagenic *E. coli* during his investigation on antimicrobial activity of *Lactobacillus spp*. isolated from fecal flora of healthy breast-fed infants against diarrheagenic *Escherichia coli*. Kang et al, 2017 also reported that *Lactobacillus spp.* had a strong bactericidal effect against planktonic and biofilm *S. aureus*.

5) Antibiotic Susceptibility Test

The zones of inhibition observed against the two antibiotics indicated that probiotic culture isolated in the present study is sensitive to Loperamide Hydrochloride and Azithromycin (Fig 4). Sharma et al, 2017 reported LAB isolates of curd origin 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. Based on the above results, the isolated bacterial colonies were tentatively identified as belong to the genus *Lactobacillus.*

From the experiments conducted above, it was observed that, isolated lactic acid bacteria (LAB) could be used as an excellent candidate for probiotics and also for the probiotic product development.

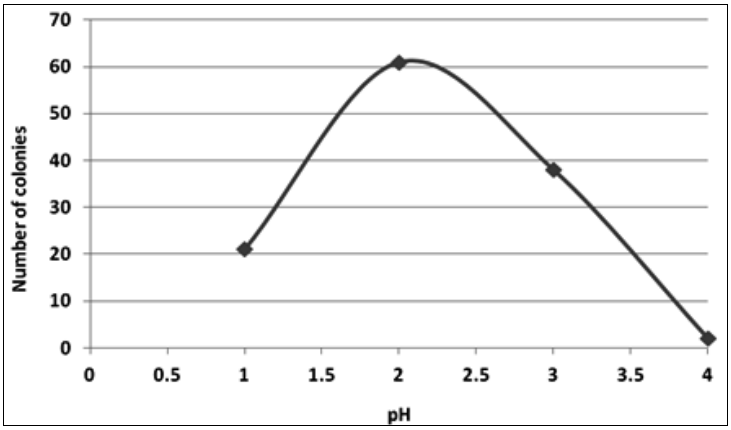


Figure 1: Acid Tolerance test

Figure 2: Milk Coagulation test

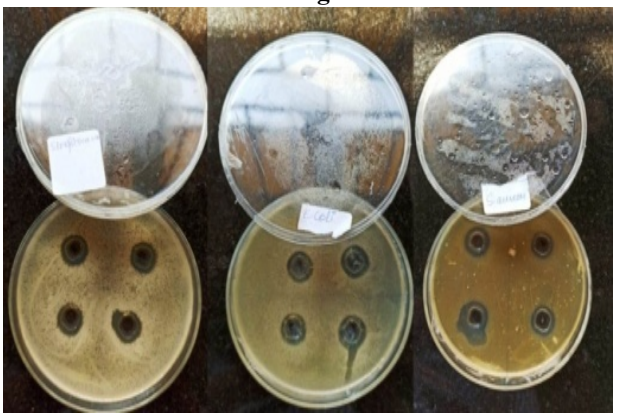


Figure 3: Antimicrobial Activity

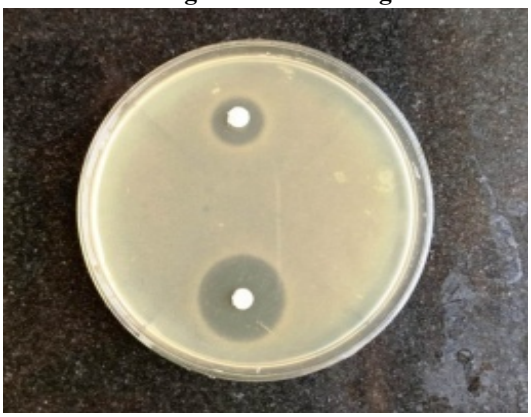
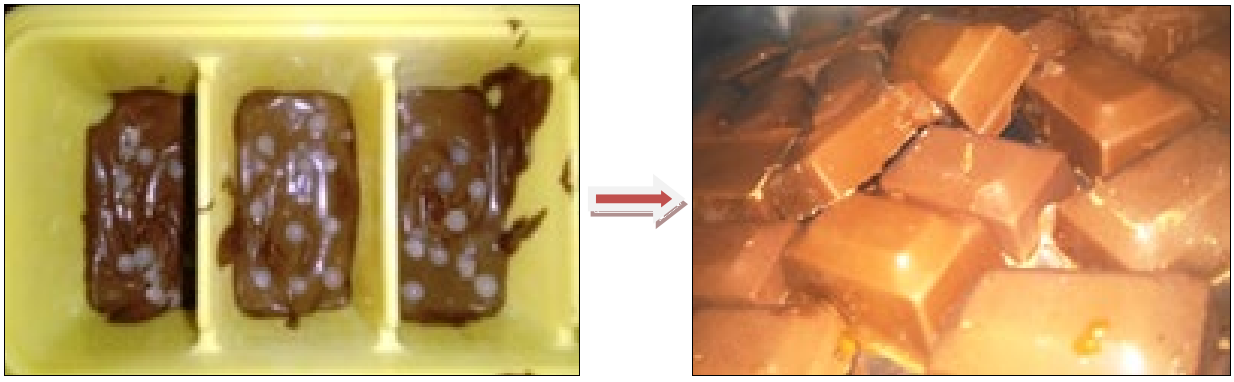


Figure 4: Antibiotic Susceptibility

B. Preparation of probiotic chocolate

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. Brown and white probiotic chocolates were prepared using two different fruits: passion fruit and strawberry that serve the purpose of pre-biotic sources (Fig 5). Strawberries are an excellent source of vitamin C, manganese, folate (Vitamin B9) and potassium and with a fibre content of 26% of the total carbohydrate and these dietary fibers are essential to feed the bacteria in the gut and improve the digestive health. On the other hand, passion fruit is a good source of nutrients, pectin and pulp extracted demonstrates properties that stimulate probiotic growth and survival in the gut. Neither the texture nor taste changed by addition of the microencapsulated *Lactobacillus* cells.



Addition of encapsulated bacteria | Probiotic chocolate

Figure 5: Preparation of Probiotic chocolate

Sensory evaluation of the probiotic chocolate

The organoleptic evaluation of the brown and white probiotic chocolates carried out for 45 participants is depicted in Fig 6 a-d. Error bar indicates mean ±SD of the three experiments. No significant differences were observed in attributes such as colour, taste, texture, flavour and sweetness among the two samples. Similar results have been reported by Nebesny et al, 2005 where sensory qualities of sucrose-free yoghurt-containing dark and milk chocolates and their yoghurt free counterparts revealed outstanding quality.

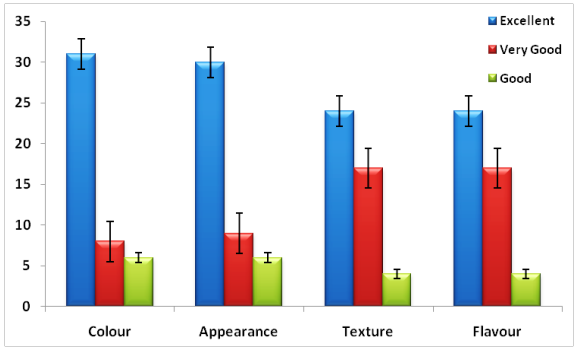


Figure 6 a: Sensory evaluation of brown chocolate

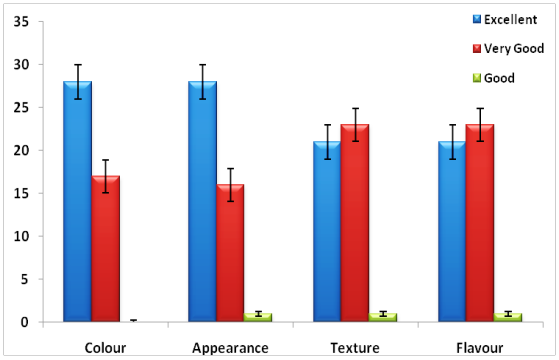


Figure 6 b: Sensory evaluation of white chocolate

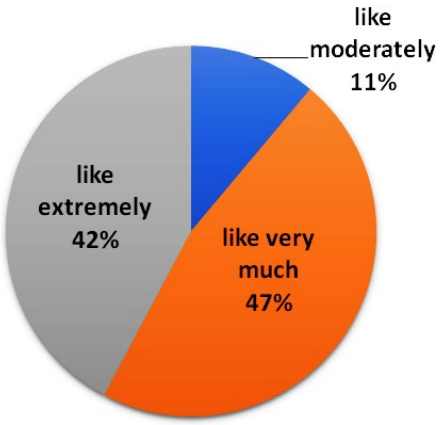


Figure 6 c: Overall acceptability of brown chocolate

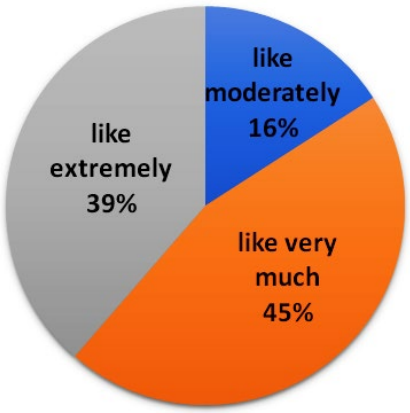


Figure 6 d: Overall acceptability of white chocolate

C. Shelf life of probiotic chocolate

Figure 7 shows the results of shelf life of the probiotic chocolate up to a period of 22 days. The bacterial colonies number remained consistent until 12 day of storage beyond which there is gradual drop in number of colonies indicating that the prepared chocolate can be stored for 12 days with the probiotic culture intact at room temperature. The reason for decline in number of probiotic bacteria over time could be due to the acidity of product, and depletion of nutrients. Taghizadeh et al, 2018 reported that the viability of the probiotic chocolate prepared by soy milk showed decline in survival from seven to twenty-one days.

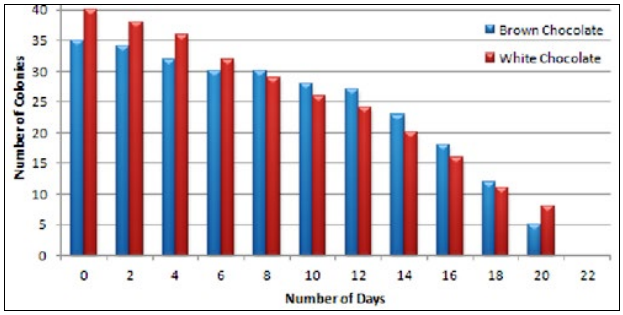


Figure 7: Shelf Life analysis of Probiotic Chocolates

4. CONCLUSION

The probiotic chocolate that was formulated using fruit extract, probiotic beads and chocolate is a functional food since it has the beneficial effects of fruit, probiotic and chocolate. The idea of combining all these ingredients and making a product with the goodness of each of the ingredients gives the combined effect in the functional food. With progression in technologies and further improvements and developments in new techniques, research in this area will continue to deliver novel bio-therapeutics as well as novel probiotic strains for the treatment and prevention of gastrointestinal disorders. For the commercialization of the product, further works are necessary.

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