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Assessment Of Probiotic Activity and Anti-Oxidant Potential Of Commercially Available Probiotic Chocolate In India

Research Article

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Abstract

Introduction: Chocolate is one of the most commonly consumed product in the world at all age groups. It is socially acceptable by most of the consumers for its taste. If its in the form of a functional food with probiotic activity without altering the taste, it becomes beneficial and can reach a wider population.
Aim: The aim of the study is to assess and compare the antimicrobial and antioxidant activity of commercially available probiotic infused green tea, green coffee and slim tea.
Materials and Methods: Synthesis of probiotic medium is done by 2 gm of each sample was taken in 1 flask of 15 ml peptone water and mixed well. Then it is inoculated in MRS agar plate and it is sealed in an anaerobic jar for 48 hours. Antimicrobial activity is tested by agar well diffusion method against *S.mutans* and the zone of inhibition is measured. Antioxidant activity is measured with DPPH and % of inhibition is measured.
Results: In the antimicrobial activity, in all the samples the activity increased with increasing concentration, but the maximum was seen in cranberry orange probiotic chocolate. In antioxidant activity, all the samples showed antioxidant activity although cranberry orange probiotic chocolate showed more antioxidant activity than peanut butter caramel chocolate
Conclusion: In our study, Bacillus coagulans incorporated probiotic chocolate bars exhibited both antimicrobial and antioxidant properties. Since there was a good antimicrobial activity against *S. mutans*, these can be incorporated as a potential antibacterial agent for oral infections and as an antioxidant carrier in the local drug delivery.

Keywords: Probiotic; Chocolate; Antimicrobial Activity; DPPH; Agar Well Diffusion Method; *S.mutans*.

Introduction

Chocolate is, in essence, composed of cocoa mass and sugar suspended in a cocoa butter matrix [1]. Major types of chocolates are dark, milk and white that differs in content of cocoa solid, milk fat and cocoa butter in the formulation. Chocolates are semisolid suspensions of fine solid particles of sugar and cocoa (and milk, depending on type); making about 70% in total, in a continuous fat phase [2]. Chocolate is consumed all over the world, by every age group and every social class. The popularity of this food appears to mainly associate with its potential to arouse sensory pleasure and positive emotions [3]. Foods with positive acceptance have been enriched with compounds that improve consumers health. For example, reducing the fat content in the diet might decrease the energy intake and therefore, contribute to the prevention of obesity. There is an opportunity using indulgent foods, such as chocolate to achieve this aim [4, 5].

Functional foods are those that provide basic nutrition and, at the same time, promote health [6]. As the market for functional foods continues to expand, research in the development of food products containing probiotic bacteria also has grown [7]. There are two dietary strategies to increase the beneficial microorganisms in the instestine. The first one is by the consumption of probiotics and the other one is by increasing the number of resident microorganisms in the gut tract, using prebiotics [8]. Probiotics are viable microorganisms that are beneficial to the host when administered in appropriate quantities [9]. They may play an important role in helping the body protect itself from infection, especially along the colonized mucosal surfaces of the gastrointestinal tract

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[10]. Ouwehand, Salminen, and Isolauri affirmed that to be considered a probiotic, the microorganism must have certain properties and functions, like adhesion in the host epithelial tissue, acid resistance and bile tolerance, pathogen elimination or reduction in pathogen adherence, production of acid, hydrogen peroxide and bacteriocin against the growth of pathogens, assurance and improvement of gut microbiota balance [11].

The attention and stress was on the survival in the gut and temporary colonization of gut mucosa surface. The species with beneficial properties belong, generally, to the genera Bifidobacterium and Lactobacillus [12]. Probiotic bacteria including bifidobacterial and lactobacilli and Bacillus coagulans are natural inhibitors of the human intestine. They beneficially affect human health by improving the intestinal microorganisms and pH balance and the defences against pathogens. Additional health benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis, anti-carcinogenic and anti-bacterial activities [13]. Other crucial criteria to determine the efficacy and the efficiency of the product containing probiotics are the acceptance of the product by the consumers and the survival of probiotic microorganisms during its production . In general, the food industry has applied the recommended level of 106 cfu g1 at the time of consumption for Lactobacillus acidophilus, bifidobacteria and other probiotic bacteria [7].

A prebiotic is a non digestible food ingredient that beneficially affects the human body by selectively stimulating the activity of certain colon bacteria. This means that some dietary compounds are resistant to digestive enzyme and are not absorbed in GIT including small intestine. These compounds when go to large intestine where most of the gut microbiota is present, these stimulate the growth of some beneficial microorganisms in the gut especially bifidobacteria [14]. Inulin is an oligosaccharide which is extracted from plants like onions, asparagus root, Jerusalem artichoke tuber, wheat, banana, Chinese chives, burdock, garlic, honey, oat, pine, rye and chicory [15]. It is officially recognized as a natural food ingredient and is classified as a dietary fibre in most European countries [14]. Studies on humans have shown significant changes in the composition of faecal microbiota, allowing the conclusion that these oligosaccharides are prebiotics [16]. The other physiological and nutritional effects associated with inulin are modulation of calcium absorption and lipid metabolism, and a possible role in reducing the risk of colon precancerous lesion.

The term synbiotic is used when a product contains both probiotic and prebiotic ingredients. The synergism is attained in vivo by the ingestion of lactobacilli and by the promotion of indigenous bifidobacteria simultaneously [17]. For example a product comprising of inulin or oligofructose and probiotic bifidobacteria or L. paracasei, for example, can be considered as a symbiotic. Synbiotic products have not been intensively studied to date. Roberfroid suggested that these ingrediants can improve the survival of bacteria when they pass into the small intestine and produce benefits in the large bowel. It is not known if the individual advantages might be additive or even synergistic [18].

Aerated dairy products have found to have great potential in the market and attracts the customers who are interested in lighter and healthier products [19]. Mousse is an aerated dessert with stabilized foamy structure that is gaining popularity in the market and produced on a large scale. The most popular mousse flavour is chocolate, followed by orange, lemon, cranberry, peanut butter, caramel and strawberry [20]. The large scale production of aerated dairy desserts is delicate, requiring knowledge about the formation and stabilization of foam, the use of functional ingredients (emulsifiers, stabilizers), and the interaction and interference of process parameters in the properties of the resulting product [19]. The aim of this study is to assess the antimicrobial activity and anti-oxidant potential of commercially available probiotic chocolate in India.

Materials and Methods

Figure 1 shows the preparation of probiotic stains from commercially available probiotic chocolate in two flavours. Peptone water was used for preparation of extract.

Figure 2 shows the picture of anaerobic jar which was used to store the culture plates after inoculation in MRS agar. The plates were stored for 5-7 days in the anaerobic jar for probiotic bacteria growth.

Figure 3 shows the subcultures of subcultures of the probiotic stains inoculated on MRS agar. Subcultures were done thrice for better isolation of the probiotic stains.

Figure 4 shows the supernatant obtained after centrifugation of the samples. The supernatant were used for antimicrobial and antioxidant properties.

Chocolate used: SIRIMIRI protein chocolate bars with probiotics.

Ingredients: Whey protein isolate, fructo-oligosaccharides, dark chocolate (sugar, cocoa solids, lecithin), almond butter, milk protein concentrate, honey, butter, , Jowar millet crisp, rock salt and Bacillus Coagulans.

Chocolate 1: Dried cranberries and orange extract

Figure 1. Preparation of the probiotic extract.

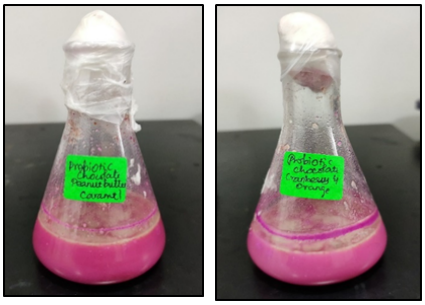


Figure 2. Anaerobic jar.

Figure 3. Subcultures of the samples.

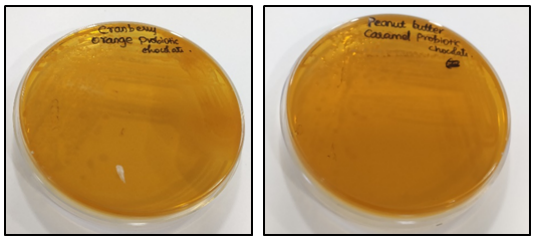
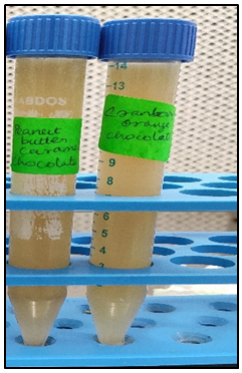


Figure 4. Collection of supernatants after centrifugation.



Chocolate 2: Peanuts and caramel extract

Synthesis of probiotic chocolate medium: 5 gm of each chocolate was taken in 2 tubes of 15 mL peptone water and mixed well. Then it is kept in a shaker at 250 rpm for 24 hours. Then it is inoculated in MRS agar plate and it is sealed in an anaerobic jar for 48 hours.

The culture is sub-cultured thrice and centrifuged and the supernatant was stored for further study.

Antimicrobial activity: Antibacterial activity of the supernatant strains against oral pathogen was determined using the agar-well diffusion method with some modifications of the protocol indicated by Chellappa et al. [30] (Streptococcus mutans).

The selected LAB isolates were inoculated from slants to fresh MRS broth containing 1% glucose and incubated overnight at 37°C overnight active culture broth of each isolate was centrifuged separately at 5000 rpm for 10 min at 4° cell-free supernatant from each separate culture was collected as a crude extract for the antagonistic study against selected oral pathogen. Pure cultures of oral pathogen were inoculated from slants to brain heart infusion broth. After 24-hour incubation at 37°C, a volume of 100 µL of inoculum of each indicator bacteria was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. plates were allowed to dry, and a sterile cork borer (diameter 5 mm) was used to cut uniform wells in the agar. Each well was filled with 100 µL culture-free filtrate obtained from each of the acid-bile-tolerant LAB isolates. After incubation at 37°C for 24 to 48 hours, the plates were observed for a zone of inhibition (ZOI) around the well.)e diameter of the inhibition zone was measured by calipers in millimeters, and a clear zone of 1 mm or more was considered positive inhibition [31,,32]. Experiment was carried out in triplicates, and the activity was reported as the diameter of ZOI ± SD.

Antioxidant activity - DPPH assay

The 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) free radical scavenging activity of the chocolate samples was determined to assess its antioxidant potential. Various concentrations (10-50µg/ml) of inoculated culture were mixed with 1ml of 0.1mM DPPH in methanol solution and 450µl of 50mM Tris-HCl buffer (pH 7.4), and incubated for 30 min. After incubation, the reduction in the number of DPPH free radicals was measured based on the absorbance at 517nm. Ascorbic acid was used as the standard controls and the percent (%) inhibition was calculated from the following equation:

% Inhibition = [Absorbance of control–Absorbance of test sample/ Absorbance of control] ×100

Results

Figure 5 shows the antimicrobial action of probiotic stains against the oral pathogen *S. mutans.* Agar well diffusion method was used

to assess the activity with 3 various concentrations of 25 µL, 50 µL, and 100 µL,.

Figure 6 shows the antioxidant activity of peanut butter caramel chocolate using DPPH assay. Various concentrations from 10 µL to 50 µL were assessed for anti-oxidant potential.

igure 7 shows the antioxidant activity of cranberry orange chocolate using DPPH assay. Various concentrations from 10 µL to 50 µL were assessed for anti-oxidant potential.

Graph 1 shows the antibiotic potential of the samples. It shows that in the peanut butter caramel chocolate, 25µL, 50 µL and 100µL showed 9 nm, 10nm and 13 nm in the zone of inhibition; in the cranberry orange chocolate, 25µL, 50 µL and 100µL showed 9 nm, 12 nm and 15 nm in the zone of inhibition although all the samples had increased activity with increasing concentration, but the maximum was seen in cranberry orange probiotic chocolate.

Graph 2 shows the antioxidant activity of the probiotic chocolates. In the peanut butter caramel sample, 13%,29%,42%,55% and 70% of inhibition was observed with increasing concentrations of 10µL, 20 µL, 30 µL, 40 µL and 50 µL respectively. In the cranberry orange sample, 26%,27%,42%, 47% and 65% of inhibition was observed with increasing concentrations of 10µL, 20 µL, 30 µL, 40 µL and 50 µL respectively. Although cranberry orange exhibited more anti oxidant activity at low concentration, at higher concentrations, peanut butter caramel had more antioxidant potent.

The growth of the probiotic stains were confirmed by subcultures (figure 3). In the antimicrobial activity, in the peanut butter caramel chocolate, 25µL , 50 µL and 100µL showed 9 nm, 10nm and 13 nm in the zone of inhibition; in the cranberry orange chocolate, 25µL , 50 µL and 100µL showed 9 nm, 12 nm and 15 nm in the zone of inhibition although all the samples had increased activity with increasing concentration, but the maximum was seen in cranberry orange probiotic chocolate (figure 5 and graph 1). In the antioxidant activity of the probiotic chocolates, the peanut butter caramel sample had 13%,29%,42%,55% and 70% of inhibition with increasing concentrations of 10µL, 20 µL, 30 µL, 40 µL and 50 µL respectively. In the cranberry orange sample2 had 6%,27%,42%,47% and 65% of inhibition with increasing concentrations of 10µL, 20 µL, 30 µL, 40 µL and 50 µL respectively. Although cranberry orange exhibited more antioxidant activity at low concentration , at higher concentrations, peanut butter caramel had more antioxidant potential (figure 6,7 and graph 2).

Discussion

Numerous methods have been explored to increase the viability and activity of probiotics in commercial products: selection of acid- and bile-resistant strains, stress adaptation, and incorporation of micronutrients . An alternative solution is represented by microencapsulation in sealed capsules of different materials, which release their content under specific environmental conditions [33]. Among these materials, lipids such as oil emulsions, milk fat and water insoluble microcapsules appear to be of con-

Figure 5. Antimicrobial activity of the samples against S.mutans.

Figure 6. Antioxidant activity of probiotics from Peanut butter caramel probiotic chocolate.

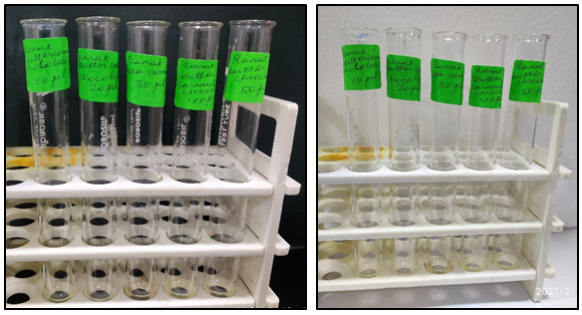
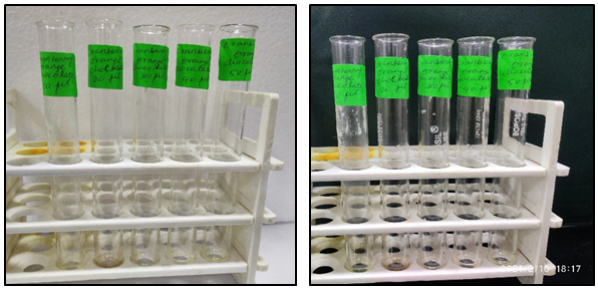
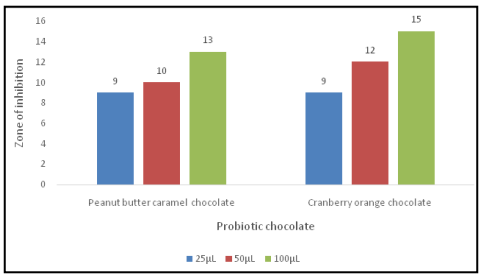


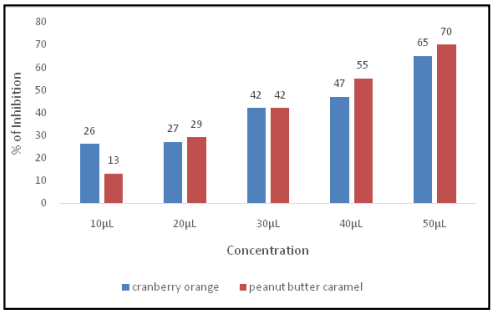
Figure 7. Antioxidant activity of probiotics from Cranberry orange probiotic chocolate.



Graph 1. Antimicrobial activity of the samples against S. mutans.



Graph 2. Antioxidant activity of both the samples.



siderable value [34-36]. Probiotics are already incorporated into a wide range of dairy products or fruit juice. Another way to increase the efficacy of a probiotic bacteria would be to use a food matrix which naturally contains a higher content of ingredients with protective properties. As the lipid fraction of cocoa butter was shown to be protective for bifidobacterial [37].

In a study conducted by S.Possemiers et al, association of a chocolate coating with microencapsulated probiotic strains have been proved to be an excellent solution to protect them from environmental stress conditions [38]. Due to this, it has been attempted to be a carrier in many ways for symbiotic activity [39, 40].

In our study, we have evaluated the anti microbial activity of probiotic chocolate against the common oral pathogen S.mutans. . Diseases such as dental caries and periodontal disease are directly linked to oral microbiota [42]. Streptococcus mutans is the often implicated initiator and plaque-resident bacterium, that begins demineralization and the metabolism of simple carbohydrates and produces a by-product which paves the way for tissue loss and further bacterial action [42, 43]. It initiates the biofilm formation by its adherence and accumulation on the tooth surface which is promoted by its synthesis of insoluble, extracellular polysaccharides. It also produces various bacteriocins that kill other bacteria, as it has a high efficiency in catabolizing carbohydrates and producing acids, and the ability to tolerate low pH [44].

Literature has shown that probiotic bacteria are not only associated with intestinal microbiota but it can also affect oral health, mucin production, competition with other flora and mucosal adherence. [45, 46].

Chocolate might be more or less responsible for tooth decay than any other carbohydrate containing foods such as bread, raisins, crackers, and fruit. However, only chocolate which is usually consumed as milk chocolate, candy bars contains large amount of sugar and complex carbohydrates and causes tooth decay and might inhibit the effect of antibacterial agents [47].

S. mutans being the arch enemy in thecaries development, more efforts have been directed towards inhibiting the known mechanisms of caries development such as; use of antimicrobial agents, fluorides, polyphenols to reduce biofilm and acid production done by *S.mutans* [46]. In our study , both the samples had antimicrobial activity. Similar findings were found in few more studies too[38, 46-48].

Chocolate has been used in antioxidant activity incorporating it with nanoparticles or probiotics. Our study also proved that probiotic chocolate had antioxidant activity. Similar findings were also found in studies conducted by Abd El-Moneim M.R. Afif et al and Dorota Najgebauer-Lejko et al [49, 50].

Conclusion

In our study , both the samples of probiotic chocolate exhibited antimicrobial and antioxidant properties. Cranberry orange exhibited more antibiotic activity and peanut butter caramel had more antioxidant potential at higher concentrations.

However , different stains of probiotic bacteria should be incorporated and tested invitro for their further potential before in vivo trials and commercial use

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