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Samlesh Kumari
Centre of Excellence on Soybean Processing & Utilization, ICAR-Central Institute of Agricultural Engineering, Bhopal, Madhya Pradesh, India

Corresponding Author:
Samlesh Kumari
Centre of Excellence on Soybean Processing & Utilization, ICAR-Central Institute of Agricultural Engineering, Bhopal, Madhya Pradesh, India

Development of Synbiotic functional milk chocolates with incorporation of probiotic culture (MTCC-5462), soymilk powder and prebiotic (inulin)

Samlesh Kumari

Abstract
In today's time functional foods are getting more attentions as compared to the food products present in the market from long time as they provide additional health benefits than the conventional form the products. In the present study attempt has been made for development of novel functional variants of milk chocolates. Synbiotic milk chocolates were prepared by incorporating with freeze dried or *Lactobacillus helveticus* MTC-5463, soymilk powder and inulin. *Lactobacillus* counts were remained above 8.0 log cfu/g until 30 days under refrigerated condition. Yeast and molds, and coliforms were found to be absent in the products during the storage. Sensory panelists liked the synbiotic milk chocolate. Thus, milk chocolate has been shown to be an excellent food for delivery of probiotic lactobacilli. The overall sensory scores of the developed synbiotic milk chocolates were >7 on nine-point hedonic scale. The final optimized synbiotic milk chocolate with addition of probiotic culture at the rate 1% (w/w) was subjected to storage study at the rate 08 ± 2 ^{0}C. For comparison, a branded milk chocolate was used as control. Moreover, the addition of the lactobacilli, soymilk and inulin did not affect the sensorial quality of the products.

Keywords: Probiotics, synbiotic, soymilk, inulin, chocolate, *Lactobacillus helveticus* MTC-5463, functional foods

1. Introduction
Chocolate is reportedly most craved food among female consumers although this craving is related to craving for sweets. Liking for chocolate, correlate significantly with liking for sweets or white chocolate. The liking for the sensory properties could originate in innate or acquired liking on the sweetness, texture and aroma of chocolate (Anon., 2009) [1].
Food products contains probiotic organisms are in demand (Stanton *et al*. 2001) [14] as these foods are claimed to have a health promoting properties (Lee and Salminen 1995) [7] and various probiotic functional foods have been developed all over the world. In general, the food industry has applied the recommended level of 106 cfu/g at the time of consumption for Lactobacillus acidophilus, bifidobacteria and other probiotic bacteria (Boylston *et al*. 2004) [4].
Prebiotics are digestible food ingredient that beneficially affects the human body, selectively stimulating the increase and/or activity of one or a limited group of colon bacteria. Inulin is an oligosaccharide extracted from commonly consumed plants like onions, asparagus root, Jerusalem artichoke tuber, honey, oat, chicory, etc. (Bengmark *et al*. 2001) [3], and it is a natural food ingredient and is classified as dietary fiber in most European countries (Roberfroid 2000) [11].
Plant milk-based confectionery is a fast-growing but still largely untapped opportunity, with almost one-third of the population citing milk chocolate as the product they would most like to see a plant-based version. India Soy Beverages Market is projected to record a CAGR of 3.8% during the forecast period (2020-2025). The market is primarily driven by the increasing number of consumers seeking non-dairy beverage alternatives have led to substantial growth in the demand for soymilk beverages market. In addition, new food production technology and new varieties of soybeans result in new flavors creates competition with cow's milk and dairy products. Milk is one of the most important ingredients in chocolate production but some people cannot tolerate lactose and allergic to milk proteins, thus use of soymilk as a milk substitute can be a probable solution for these problems. Soymilk is naturally free of cholesterol, low in saturated fat, good source of health proteins and carbs (Messina, 2016) [9], minerals and vitamin-E and riboflavin.

For health-conscious consumers soymilk incorporated products will be a good alternative. Replacement of dairy milk with soymilk has the potential to develop novel food products in the growing number of functional foods. Besides having excellent nutrients, milk chocolate has a higher pH and buffering capacity than the more traditional fermented probiotic foods, a more solid consistency and a higher fat content. Hence, the probiotics can be successfully incorporated in milk chocolate to derive extra health benefits from probiotic milk chocolate. Therefore, the study was undertaken to develop synbiotic milk chocolate incorporated with *Lactobacillus helveticus* MTC5463, soymilk and inulin. The resulted milk chocolate was studied for survival of lactobacilli and sensory qualities during storage at refrigerated condition.

2. Materials and Methods

2.1 Materials: The probiotic *Lactobacillus helveticus* MTCC 5463 culture was acquired from Dairy Microbiology Department, Anand Agricultural University, Anand. The lyophilized culture was propagated in sterilized reconstituted skim milk-RSM (10% T.S.) by incubation at 37 °C for 8–12 h and stored at 5±2 °C. Prior to use, three successive transfer of culture was administered into sterilized reconstituted skim milk powder following incubation at 37 °C for 8–12 h to keep it active. Inulin powder was purchased from Urban platter. Soymilk powder was taken purchased from bionutrients, Mandideep, Madhya Pradesh (India). Cocoa powder, Cocoa butter and sugar were procured from local market of Bhopal, Madhya Pradesh (India). Skimmed milk powder Amul All the chemicals were procured from Sigma-Aldrich (USA), Merck and Himedia (Mumbai, India). Soy lecithin was purchased from Urban Platter.

2.2 Preparation of freeze-dried probiotic culture

About 2% of activated culture of *L. helveticus* MTCC 5463 was inoculated in 100 ml of de Man Rogosa Sharpe (MRS) broth and incubated at 37 °C for 12 h in order to obtain sufficient cell mass. The cells were harvested by centrifugation at 4 °C, 5000 r.p.m. for 15 min, washed twice with saline water, and were stored at –20 °C in case of concentrated culture. For freeze drying, collected cells were inoculated into 10 ml sterilized skim milk containing cryoprotectant (1% glycerol), mixed thoroughly, distributed in glass vials, and frozen at –20 °C for overnight. Culture was freeze-dried using a freeze-dryer (MINI LYODEL).

2.3 Production of Synbiotic Milk Chocolate

For preparation of synbiotic milk chocolates procedure adopted by Alegro *et al*. (2007) with some modifications. Complete flow diagram for preparation of probiotic chocolate with added culture is given in Figure 1.

The formulations were individually pasteurized at 74±2 °C for 15S and then mixed simultaneously. The probiotic microorganisms including *Lactobacillus helveticus* MTCC 5463 were added with 1.0% (w w-1) at 38-40 °C. Then, all samples were stored at 4±1°C (Richter *et al*., 2011). The synbiotic milk chocolate dessert trials were produced in triplicate. The chocolate mass was made in the laboratory ball mill with a capacity of 1 Kg. Homogenization of chocolate mass was carried out at 50 °C at an agitator shaft speed of 40 rpm, recycling the mass through the balls at a medium speed of 10 Kg h-1 of the recycling pump, for 5 h. All the ingredients including inulin were added to the ball mill at the beginning of the production time. The lyophilized bacteria were added at 40 °C at least 10^7 -10^8 CFU g-1. The moulding and vibration process was conducted at 27-30°C. The chocolate samples were cooled at 5°C for 30 min, removed from the moulds, wrapped in aluminium foil and stored at 22 °C.

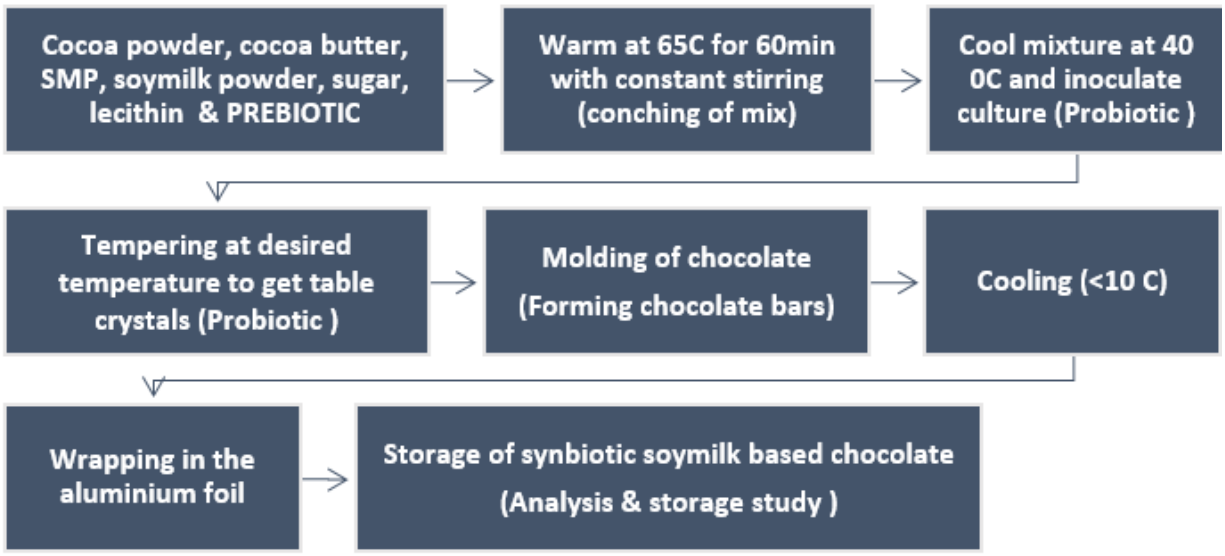

Fig 1: Flowchart for production of synbiotic milk chocolate

2.4 Physicochemical analysis of developed chocolate

The pH of samples in each day of storage was measured by a digital pH-meter. Titratable acidity was determined by titrating 10 g of sample in 100 ml of distilled water with NaOH 0.1 N to an endpoint of pH 8.3 according to AOAC, 2000. All pH and acidity measurements were carried out in triplicate. Moisture content of sample was determined according to the procedure described in SP: 18, Part XI, (1981). Fat content of chocolate was estimated as per procedure given in AOAC 963.15(31.4.02). As per procedure given in IS: 1479, Part II, (1961). Total carbohydrates of sample were determined according to the procedure described

in SP: 18, part XI, (1981). Protein content of product was estimated by Kjeldahl method as described in IS: 9617, (1980).

2.5 Texture profile analysis
Three samples of each experimental chocolate are subjected to uniaxial compression to 60 per cent of the initial sample height, using a Food Texture Analyzer.

2.6 Microbiological analysis
The first dilution was prepared by taking 11 g of the milk chocolate sample in 99 mL of phosphatbuffer (0.1 M, pH 7.2 ± 01), containing 0.1% peptone for depolymerization of alginate capsules and release of encapsulated bacteria in solution (Sheu *et al*. 1993), and serial dilutions were made in 9 mL normal saline. Viable lactobacilli were enumerated on MRS agar (37C for 48 h). Coliforms and yeast and molds were enumerated on violet red bile agar (37C for 24 h) and potato dextrose agar (25C for 3–5 days), respectively

2.8 Sensory evaluation
The product was subjected to the sensory evaluation by an expert panel of nine judges for colour and appearance, flavour, body and texture and overall acceptability criteria. Fresh product at 0 days and the stored products (15 and 30 days storage at 10 ± 2 °C) were brought to 15 °C before giving for judging. The score given by the judges on 9 point hedonic scale were taken to determine the acceptability level of product.

2.9 Statistical analysis
Quantitative data was expressed as mean ± SD values of 3 replicates. Data were analyzed by using completely randomized factorial design. Analysis of variance was conducted; when significant effect was detected, the means were separated by Fisher Least Square Analysis.

3. Results and Discussion
Lactobacillus helveticus MTCC 5463 has been used to prepare various synbiotic products *viz*, synbiotic dahi (Gawai, 2006) [5], Synbiotic Whey Drink (Madhavi, 2009) [8], Oat based functional fermented product containing probiotic culture and pineapple fruit (Jain, 2010) [6], synbiotic lassi with carrot juice (Sudheendra, 2010) [15], synbiotic lassi with honey as prebiotic (Sharma, 2010) [13]. A carbonated drink by making use of this strain has also been developed (Shah, 2009) [12]. Thus, basic characteristics, technological potential in various food forms and some of the health properties of *Lactobacillus helveticus MTCC* 5463 is well established.

3.1 Formulation Synbiotic Milk Chocolates
Chocolate manufacture involves incorporation of various ingredients, to be blended in right proportion and under right conditions. Several preliminary trials were taken to decide the formulation for making the chocolate at the laboratory scale. The final formulation which gave organoleptically acceptable best product is given in Table 1. synbiotic chocolates were prepared by incorporation of freeze dried culture of *Lactobacillus helveticus* MTCC 5463 at 3 different levels *viz*, 1, 2, 3% w/w of chocolate formulation. A control chocolate without addition of probiotic chocolate was also prepared for comparison. The quality of the chocolate was judged and compared based on sensory profile and microbiological counts.

Table 1: Formulation of synbiotic milk chocolates for per 100gm

Samples	Sucrose (gms)	Inulin (gms)	Soy milk pw. (gms)	Skim milk pw. (gms)	Cocoa butter (gms)	Cocoa pw. (gms)	Soya lecit-hin (gms)	Flavour (gms)	Probiotic culture (gms)
Control	30	0.0	0.0	26.5	33	8	0.5	0.5	0.0
Synbiotic (A)	28	1.5	6.8	20.6	33	8	0.5	0.5	1% (>10^8cfu/g)
Synbiotic (B)	28	1	5.5	22	33	8	0.5	0.5	1% (>10^8cfu/g)

3.2 Proximate analysis
The Proximate composition of the probiotic chocolate is presented in Table 2. It could be seen that the major portion of chocolate solids consist of carbohydrate and fat. In addition, developed synbiotic milk chocolates had 9. 17 ± 0.15 per cent and 9. 26 ± 0.11 protein and 1.78 ± 0.17 per cent and 1.62 ± 0.71 moisture respectively. It is easier to understand that all this conditions are adverse for microbial growth in the chocolate. However, our intention was to protect the probiotic bacteria in live condition in this matrix.

3.2 Proximate analysis of developed synbiotic Milk Chocolates

Table 2: The Proximate composition of the probiotic chocolate is presented

S.N.	Constituents	Control	Synbiotic (A) (w/w%)	Synbiotic (B) (w/w%)
1.	Carbohydrates	54.36 ± 0.11	58.32 ± 0.23	57.82 ± 0.66
2.	Fat	31.17 ±0.62	32.07 ±0.34	31.07 ±0.62
3.	Protein	8. 88 ± 0.14	9. 17 ± 0.15	9. 26 ± 0.11
4.	Moisture	1.09 ± 0.31	1.78 ± 0.17	1.62 ± 0.71
5.	Ash	0.86 ± 0.14	0.85 ± 0.19	0.89 ± 0.15
6.	Water activity (RH%)	0.51 ± 0.05	0.64 ± 0.00	0.64 ± 0.05
7.	Probiotic count Log CFU/gm	Nil	8.6 ± 0.26	9.11± 0.21

3.3 Texture analysis
The instrumental method of texture assessment aims at quantifying objectively the textural characteristics to the maximum extent possible.

Table 3: Texture analysis of probiotic chocolate.

NO.	1	2	3	4	5
Texture Attributes	Hardness (N	Cohesiveness	Chewiness (Nmm)	Adhesiveness (Nmm)	Gumminess (N)
Readings	134.23	0.03	1.99	0.01	1.58

Various instruments are in vogue for the purpose, but Food Texture Analyzer of Lloyd Instruments LRX Plus material testing machine working on parallel plate uniaxial compression principle, is one of the most widely used instruments in the food industry for study of the behaviour of food while chewing and mastication before swallowing in the mouth. Results of texture analysis are shown in table 3.

3.4 Microbiological analysis

Microbiological quality of the chocolate was evaluated based on viable lactobacillus count, yeast & mold count and coliform count (Table 4).

Table 4: Microbiological quality of synbiotic milk chocolates

Samples	Lactobacillus count (Log cfu/g)	Yeast & mold count (cfu/g)	Coliform count (cfu/g)
Control	-	< 10	< 10
Synbiotic (A)	8.03 ± 0.04	< 10	< 10
Synbiotic (B)	8.44 ± 0.03	< 10	< 10

3.5 Sensory Evaluation

Sensory score based on 9 point hedonic scale of 2 types of chocolates with different concentration of soymilk and inulin as presented in Table 3. The average score derived after statistical analysis of the data from three replications and seven judges showed non-significant differences for all the parameters of sensory evaluation *viz*, flavour, body and texture, colour and appearance and overall acceptability.

Table 5: Sensory evaluation of freshly prepared chocolates

Samples	Parameters				
	Colour & Appearance	Flavour & Taste	Mouth feel	Body & texture	Overall Acceptability
Control	8.6 ± 0.20	8.5± 0.10	8.5± 0.22	8.0±0.41	8.3 ±0.18
Synbiotic (A)	7.8±0.21	7.8±0.21	7.1± 0.31	7.9±0.33	7.5±0.12
Synbiotic (B)	7.1±0.13	7.1±0.42	7.0± 0.16	7.2±0.42	7.2±0.23

Based on the results of sensory and physical analysis of the milk chocolate formulated using different levels of soymilk powder, skim milk powder and inulin a combination of the ingredients with very good desired results was obtained. The optimized formulations are based on The Continues trials on the bases of sensory evaluation. The overall sensory scores were 7.2±0.23 and 7.5±0.12 respectively for both synbiotic milk chocolates as shown in the table 5.

4. Conclusions

In the present study, applying soy milk and inulin in synbiotic milk chocolates can produce a multi-functional confectionary which improves the viability of three probiotic bacteria (*Lactobacillus helveticus* MTCC 5463) as well as milk to the use prebiotic properties of soy milk. Although soy milk had negative effects on sensory evaluation but using milk/soy milk (combination improves organoleptic properties of synbiotic milk chocolates in an acceptable level. Overall, these are novel food products which provides functional health benefits to the consumers.

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