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Studies on proximate analysis and microbial analysis of probiotic chocolate

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Abstract
An increasing demand of consumers for foodstuffs supplemented with live LAB, preferentially probiotic ones, gave rise to studies on the enrichment of some other foods with these microorganisms. Despite high fat and sugar contents, chocolate consumption makes a positive contribution to human nutrition through provision of antioxidants, principally polyphenols including flavonoids such as epicatechin, catechin and notably, the procyanidins. The main objective of this work was to obtain a potentially probiotic chocolate by using microencapsulated *Lactobacillus* strains and to study the mineral composition as well as microbial characteristics of probiotic chocolate. This probiotic milk chocolate displayed the similar mineral content with the mineral content of control chocolate (without LAB). The number of live bacterial cells was maintained at the functional level of 10^7 - 10^9 Cfu/g after keeping for 4 weeks at 4^0 C. the chocolate sample was free from *Coliform* and *E. coli* when the sample was fresh and throughout the storage period of 4 weeks at refrigerator temperature (4ºC) as result of good hygienic and sanitary conditions, during the preparation of the chocolate.

Keywords: Milk chocolate, Mineral composition, Polyphenols, Microbial characteristics

1. Introduction

Chocolate is a typically sweet, usually brown, food preparation of *Theobroma cocoa* seeds, roasted and ground, often flavoured, as with vanilla. It is made in the form of a liquid, paste, or in a block, or used as a flavouring ingredient in other foods. Chocolate possesses unique taste, flavour and texture and is also a source of biologically active substances, such as polyphenols, which display significant antioxidant properties and have a beneficial impact on human health, particularly on the cardiovascular system. (Richelle M. *et al*., 2001; Schmitz H., 2001) [15, 18].

The incorporation of probiotics into chocolate could offer a good alternative to common dairy products and allow to broaden the health claims of chocolate based food products. Indeed, recent market research into functional food has shown that, in relation to chocolate, digestive health was one of the most important drivers of consumer acceptance (Callebaut 2009) [5].

Probiotic cells are defined as live microorganisms that, when administered in suitable amounts, confer a health benefit to the host. At present, probiotics are the driving force to design functional foods because they improve the properties of indigenous gut microflora and exert many positive effects on human health.

The consumption of products supplemented with live cells of lactic acid bacteria (LAB), in particular with their probiotic strains, is believed to benefit consumers' health due to their well documented positive impact on the function of gastro-intestinal tract and immune system, reduction of blood cholesterol, and apparent anticancer activity (Yoon *et al*., 2006) [21]. An increasing demand of consumers for foodstuffs supplemented with live LAB, preferentially probiotic ones, gave rise to studies on the enrichment of some other foods with these microorganisms. The development of new technologies facilitating the supplementation of confectionery with LAB can yield novel products, enriched with health-promoting ingredients that are capable of preventing civilisation disorders. Because confectionery products are consumed by children and teenagers, their supplementation with live LAB is advisable.

Cocoa is a widely consumed food ingredient. Phytochemicals' profile in cocoa beans varies for different cultivars and among cocoa beans and cocoa-containing foods. However, cocoa is a rich source of polyphenolic compounds with a high amount of flavonoids, specifically flavanols, also known as flavan-3-ols. Moreover, cocoa components are particularly rich in catechins, epicatechins and proanthocyanidin (Ellam and Williamson 2013) [7]. More importantly, flavanols may influence healing properties and offer a wide range of health benefits.

In addition to polyphenols, cocoa contains methylxanthine compounds, predominantly theobromine and caffeine, but in lower amounts than those of theobromine.

Since theobromine has been shown to possess high bioavailability and multiple biological activities, recent cocoa intervention studies have been conducted on the ability of theobromine to increase serum HDL cholesterol (Neufingerl *et al*., 2013) [13]. Furthermore, theobromine stimulates heart muscle, relaxes bronchial smooth muscles in the lungs and plays an important role in the transmission of intracellular signals (Blinks *et al*., 1972) [3]. It is noteworthy that theobromine has antioxidant activity, and several antioxidant compounds may be effective treatments for depressive disorders (Scapagnini *et al*., 2012) [17].

The cocoa powder is an extremely rich source of many essential minerals, including magnesium, copper, potassium and iron. As reviewed by (Scapagnini *et al*., 2003) most of these minerals may affect vascular health and function, improving cocoa's nutritional effects. The predominant mineral found in cocoa is magnesium, which catalyzes a multitude of biological reactions, including protein synthesis and energy production. In addition, magnesium is an antiarrhythmic and hypotensive agent, and its deficiency has been linked to the metabolic syndrome, insulin resistance and diabetes (Gums, 2004). Dark chocolate is also an important source of copper, and this mineral is required for processes, such as iron transport, glucose metabolism, infant growth and brain development (Jalil and Ismail 2008) [11].

Thus, pertaining to the above discussion, in the present investigation, the attempt was made to study mineral composition and microbial characteristics of probiotic chocolate

Materials and Methods

Raw materials

The raw materials such as Cocoa powder, skimmed milk powder, butter, sugar, lecithin etc. were procured from the local market of Parbhani.

Strains of LAB: Two LAB strains were used

- *Lactobacillus acidophilus*
- *Lactobacillus bulgaricus*

Methods

1. Preparation of starter culture

The probiotic organisms viz. *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* were individually grown in MRS broth at 37 ^{0} C for 48h. The cultivated MRS broth was then centrifuged at 4,000rpm for 10 min to harvest the cells. The harvested cells were washed twice with sterile water. The biomass was taken as starter culture.

Flow Sheet 1: Preparation of starter culture

Stock culture

↓

Activation of bacterial strains in MRS broth separately at 37 ^{0} C for 48 hours

↓

MRS broth containing desired strains

↓

Centrifugation at 4000rpm/10min

↓

Biomass (Starter culture)

2. Encapsulation of probiotics

The microencapsulation of probiotic bacteria was performed using the extrusion technique. Hydrocolloid solution was prepared by using a combination of sodium alginate and guar gum at 1 & 0.8% (w/v) respectively. For preparation of 100 gm probiotic chocolate, 10 ml of inoculum (5ml each of *L. acidophilus* and *L. bulgaricus*) was mixed in 20 ml of polymer solution. Probiotic cultures and polymer solution were mixed properly and passed through a syringe in the form of droplets into 0.3M calcium chloride solution. Interaction between the two solutions led to formation of beads (2-5mm) and the resulting beads were then stored in 0.1% peptone solution at 4 ^{0}C.

Flow Sheet 2: Microencapsulation of strains

Preparation of polymer solution

↓

Addition of probiotic cultures in the polymer solution

↓

Extrusion of the cell-polymer solution into calcium chloride solution

↓

Capsule formation by cross linking

↓

Recovery of capsules and storage in 0.1% peptone solution at 4 ^{0}C

1. Processing technology for preparation of probiotic chocolate

The major ingredient cocoa powder was mixed with skimmed milk powder. This cocoa powder and milk powder mixture was added into butter and gentle heating was carried out till it becomes smooth paste. Powdered sugar and emulsifier (lecithin) were added at this stage. Emulsifier helps to give smooth texture to chocolate. Subsequently after heating, encapsulated probiotics were added to mixture and mixed well. Moulding was carried out to get chocolates with different shapes. These moulds were kept in a refrigerator or freezer for at least 6 hours. Finally, moulded chocolates were packed in wrappers.

Flow Sheet 3: Processing technology for probiotic chocolate

Cocoa powder

↓

Mixing of Cocoa powder & Milk powder

↓

Addition of this mixture to melted butter

↓

Gentle heating

↓

Addition of sugar and emulsifier (lecithin) to mixture

↓

Smooth chocolate paste

↓

Addition of encapsulated probiotics to this chocolate paste

↓

Moulding & Freezing

↓

Packaging & storage at refrigerated temperature (4 ^{0} C)

Proximate analysis

All samples were analyzed for moisture, crude protein, crude fat, total ash and total carbohydrate contents according to their respective standard methods as described in (A.O.A.C., 2000) [1].

Mineral analysis

Minerals content like calcium, magnesium and zinc, iron,copper, mangnese were determined by using titration and spectrophotometric method respectively.

Microbial analysis of probiotic chocolate

Microbial examination is the perfect quality assessment protocol performed in quality analysis of food products. However, in a probiotic product it is a mandatory one. Total Plate count, Yeast and mold count and *coliform* count were analysed.

Results and Discussion

Chemical analysis of Raw Material

The results pertaining to chemical analysis of raw materials are presented in Table 1. It was revealed from (Table 1) that, the moisture for cocoa powder (6.72%), protein (9.52%), crude fat (12.60%), ash (6.35%), crude fiber (2.60%) and Carbohydrate (62.21%). The results of the chemical analysis of the skimmed milk powder are moisture for skimmed milk powder (3.2%), protein (35.5%), crude fat (1.0%), ash (8.4%) and Carbohydrate (51.9%).These values of chemical properties recorded in the present study are similar to the values reported earlier by Ndife Joel *et al.*, (2013) [12] and Reference Manual for U.S. Milk Powders (2005) [14].

Table 1: Chemical Composition of Raw Material

Raw material	Moisture %	Crude Protein %	Crude Fat %	Ash %	Crude Fiber %	Total carbohydrates %
Cocoa powder	6.72	9.52	12.60	6.35	2.60	62.21
Skimmed Milk powder	3.2	35.5	1.0	8.4	-	51.78

*Each value is average of three determinations.

Chemical Analysis of probiotic chocolate

The data pertaining to various chemical properties of probiotic chocolate is depicted in Table 2. Data showed in table 2 revealed that the chocolate sample contained 5.64% moisture, 6.81% crude protein, 31.73% crude fat, 51.27% carbohydrates and 2.43% crude fiber. It was observed that the protein content of chocolate (6.81%) was comparatively less than the protein content of cocoa powder (8.50%) and skimmed milk powder (35.5%). This may be as a result of the heating process which could have denatured some protein in the chocolate. (Ndife Joel *et al*., 2013) [12]

It was observed that the fat content of chocolate was much higher (31.73%). The significant increase in the fat content of chocolate was as a result of the contribution of ingredients added in the production of chocolate such as butter, milk powder. Fats, especially the unsaturated fat are prone to oxidation and shorten shelf-life of food products (Borchers *et al*., 2000; Afoakwa *et al*., 2007) [4, 2].

The ash content of probiotic chocolate was 2.12%. Ash is an indication of mineral contents of foods and has been shown by Ieggli *et al*., (2011) [10] to be high in cocoa products. Afoakwa *et al*., (2007) [2] reported that chocolates are good sources of minerals, specifically calcium, magnesium, copper and iron.

Table 2: Chemical Analysis of probiotic chocolate

Parameter (%)	**Values**
Moisture	5.64
Crude fat	31.73
Crude Protein	6.81
Crude fiber	2.43
Ash	2.12
Carbohydrate	51.27

* Each value is average of three determinations.

Mineral analysis of Raw Material

Table 3: Mineral composition of Cocoa powder and milk powder

Samples	Mineral composition of raw materials (mg/100gm)				
	Calcium	Magnesium	Iron	Zinc	Mangnese
Cocoa powder	128	89.1	10.2	104	1.90
Skimmed Milk powder	1242	112	0.44	4.05	ND

*Each value is a mean of three determinations

The observations from table 3 revealed that the calcium content of cocoa powder and skimmed milk powder was 128 and 1242 mg/100g respectively. The magnesium content of cocoa powder and skimmed milk powder was 89.1 and 112 mg/100g respectively. Iron content of cocoa powder and skimmed milk powder viz. 10.2 and 0.44 mg/100g respectively and zinc content of cocoa powder and skimmed milk powder was 104 and 4.05 mg/100g respectively. The manganese content of cocoa powder was 1.90 mg/100g. Similar results were found by Emmanuel (2016) [8] and Reference Manual for U.S. Milk Powders (2005) [14].

Mineral analysis of probiotic chocolate

Table 4: Mineral analysis of probiotic chocolate

	Mineral composition of Probiotic chocolate (mg/100gm)				
Sample	Calcium	Magnesium	Iron	Zinc	Mangnese
Control	1451	490	21.2	9.16	2.84
Probiotic chocolate	1443	487	22.5	10.1	2.80

The observations from table 4 revealed the mineral content of probiotic chocolate. Calcium content of probiotic chocolate was (1443mg/100gm), the magnesium content of probiotic chocolate was (487mg/100gm), the iron content of probiotic chocolate was (22.5mg/100gm), and the zinc content of probiotic chocolate was (2.80mg/100gm). These values were found similar with the mineral content of control chocolate (without LAB) reported by Sager M (2012) [16].

Microbial analysis of probiotic chocolate during storage

The prepared probiotic chocolate sample was further analyzed for microbial properties during storage up to 4 weeks. The accepted chocolate sample was subjected to microbial studies for total plate count, yeast and mould count and coliform growth during the storage period as per method adopted by Cappuccino and Sherman, (1996) [6]. The results recorded during the present investigation are presented in Table 5.

Table 5: Microbial analysis of probiotic chocolate during storage

Time in Weeks	Total Plate Count (cfu/gm)x10^8	Yeast & Mould Count (cfu/gm)x10^3	Coliform Count (cfu/gm)x10^3
1	2.3x10^8	ND	ND
2	3.7x10^8	1.6x10^3	ND
3	5.5x10^8	1.2x10^3	ND
4	4.6x10^8	1.0x10^3	ND

The accepted sample was subjected to microbial studies for total plate count, yeast and mold count and *Coliform* growth during the storage period as per method adopted by Cappuccino and Sherman, (1996) [6]. The results recorded during the present investigation are presented in Table 12. Steinkraus, (1996) [20] reported that yeast counts were strongly correlated with LAB count. Co-metabolism between yeast and LAB may exist, where the bacteria provide the acid environment, which selects the growth of yeast, that in turn; provide vitamins and other growth factors to the bacteria. The progressive decrease in yeast and mold count might be due to resultant increase in acidity during storage.

The results from table also shows that, the chocolate sample was free from *Coliform* and *E. coli* when the sample was fresh and throughout the storage period of 4 weeks at refrigerator temperature (4 ºC) as result of good hygienic and sanitary conditions, during the preparation of the chocolate.

Conclusion

The trend to enrich new foodstuffs with live Lactobacillus cells is a novel and promising approach to the application of LAB in the food production. The supplementation of chocolate with encapsulated live LAB cells is one of these new applications. Thus in the light of the scientific data of the present investigation, it can be concluded that milk chocolate was a good carrier for *Lactobacillus acidophilus* and *L. bulgaricus* cells. Cocoa and cocoa products are important sources of phytocompounds with nutritional and therapeutic value. A growing body of scientific evidence is becoming available to support that cocoa components with antioxidants and anti-inflammatory activities contribute to endogenous photoprotection and are crucial for the maintenance of skin health. However, several studies have shown that the beneficial effects of cocoa vary among the wide range of cocoa and chocolate products. The process of preparation of probiotic chocolate can be techno-economically feasible, justifies the suitability of chocolate as a carrier for in microencapsulated mixture of probiotic *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. Chocolate is willingly consumed by children and teenagers. The supplementation of this product with encapsulated live probiotic cells can enrich their snacks.

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