Original Article

Potential application of *Bacillus subtilis* SPB1 lipopeptides in toothpaste formulation

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HIGHLIGHTS

- The application of a lipopeptide biosurfactant in a toothpaste formulation.
- The investigation of the physicochemical properties and the cleaning ability of the formulated toothpaste.
- The evaluation of the antimicrobial activity of the formulated toothpaste.
- The follow-up of the formulated toothpaste stability.

GRAPHICAL ABSTRACT
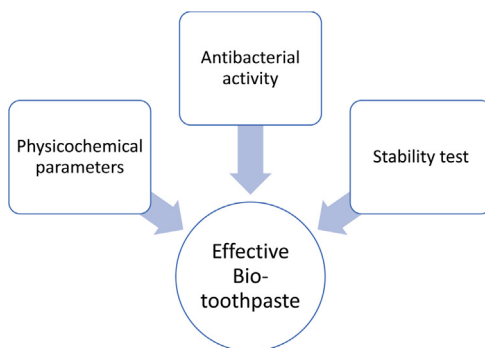
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ABSTRACT

Toothpaste is a gel dentifrice used with a toothbrush as an accessory to clean, keep and promote oral hygiene. The literature review suggests that there are many different formulations of toothpastes and that each of their individual components present specific functions. The concentration of the toothpaste ingredients must be appropriately chosen taking into account the purposes of the toothpaste. Biosurfactants are considered as suitable molecules for application in many formulations such as in toothpaste one. In the present work, two dentifrice formulations were investigated and their efficiencies were tested using chemical surfactant agent and lipopeptide biosurfactant isolated from *Bacillus subtilis* SPB1. The physicochemical properties were analyzed considering several tests mainly spreading ability, water activity, pH, foaming and cleaning tests. The obtained results indicated that the SPB1 biosurfactant was as efficient as the chemical surfactant confirming its potential utilization in toothpaste formulation compared to the commercial one. The evaluation of the antimicrobial activity of the formulated dentifrice was carried out against eight bacteria. The results demonstrated that the biosurfactant-based product exhibited an important antimicrobial activity, which was very effective against *Enterobacter sp* and *Salmonella typhinirium*.

Introduction

In daily routine, many factors can influence the success of oral hygiene procedure such as the status of the local and systemic defense mechanisms, the mechanical skills and knowledge, the

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motivation, and discipline of the consumer [1]. Therefore, this maintain of oral hygiene can prevent signs of inflammation and caries, mineralization of the inorganic portion, destruction of the organic substance, cavitation of the oral biofilm and staining of teeth [1,2]. The inconsistent eating habit of different age people and the increased consumption of sugar may continuously rise the frequency of these oral diseases. In fact, as reported by the Centre for Disease Control and Prevention (CDC), children suffer from high dental caries prevalence, with 27% of preschoolers and 42% of school-age children. Moreover, 91% of adults have dental caries experience once in lifetime [3]. This oral problem is due to significant role of microorganisms since several bacteria are present in dental plaque. It can be estimated that around 700 bacterial types exist in the human oral microbiome [2]. Therefore, to maintain ideal oral environment, it is important to control these natural processes and the most common and effective factor for cleaning, removing and preventing plaque is carried out thanks to the mechanical action of the toothbrush and not by the toothpaste [1,2,4]. However, for most people, brushing alone will be insufficient to maintain plaque control for long period [2]. Moreover, patients search to have an attractive smile, as it is considered synonymous with health [5]. This growing demand for an enhanced esthetic appearance and an improved oral health has led to a great development of dentifrices [1,6]. In fact, these products have been used since antiquity [1,4] and in 1950, the first toothpaste was invented by the dental surgeon and chemist Washington Wentworth Sheffield, [4].

As reported by Joiner [5], almost all the pastes contain the same basic functional ingredients, that have a definite function within the formulation. These include abrasives presenting an important role in removing the pigmentation and the dental plaque from the enamel surface [1,4,6,7]. Other kind of ingredients are incorporated in toothpastes, such as antimicrobial agents which reduce, control and prevent the accumulation of cariogenic and periodontopathogenic microorganisms [8]. They can also contain some other additives that present a significant part in determining the efficiency, stability, and esthetic appeal of any cosmetic formulation [9] such as sweeteners which may stop the bacteria attraction, water softeners allowing a better detergents work, thickening agent defining formulation rheological properties, preservative to maintain formulation stability, binders to provide consistency, fluoride to harden the teeth against caries and to provide health benefits and humectants for other ingredients solubilisation and for protecting the formulation from drying [4].

In addition, studies have shown that surfactants are used in dentifrice as active components [6]. As described by Iqbal et al. [4] Sodium Lauryl Sulfate (SLS), an anionic surfactant, is one of the leading toothpaste components. It is used as foaming and synthetic cleaning agent, it also imparts desirable sensorial properties during use and exhibits antimicrobial activity. In addition to providing the effervescent action of toothpastes and their distribution in the oral cavity, this molecule can improve the food particles removal [10]. Nevertheless, frequent use of this substance may cause multiple allergic and toxic reactions which include skin dermatitis, inflammation, mucosal irritation and ulcers [4,11,12]. Its uses in mouth rinses may cause desquamation of oral epithelium and a burning sensation in human volunteers as described by a study at the Stern College for Women at Yeshiva University in New York in 1997 [13]. Moreover, the addition of SLS to dentifrices raises their abilities to increase plaque fluoride concentrations and it was suggested that its ingestion may exert a carcinogenic effect [10]. It was reported by a dental association in Japan, that SLS was mutagenic when testing its effects on bacteria [9]. Owing to these adverse effects on human health, the use of SLS in commercial toothpaste should be avoided and the monitoring of environmental materials as well as the development of rapid and reliable methods

for toxicity evaluation and risk assessment should be investigated [14].

In fact, several reports indicated that biosurfactants have similar properties to the well known synthetic surfactants and can be used in the same way in detergency, emulsification, de-emulsification, wetting, foaming, dispersion, solubilization of hydrophobic substances or to modify surfaces [12]. In addition, they have some advantages including compatibility with human skin, low toxicity and irritancy [15] and higher biodegradability [12]. Moreover, researchers reported that thanks to their anti-adhesive, anti-fungal, anti-viral, and anti-bacterial activities against several pathogens, biosurfactants become very interesting for cosmetic and personal care applications [16]. For instance, Rincon-Fontan et al. [17] reported that a biosurfactant composed by 64.2% of fatty acids (linolelaidic acid, oleic and/or elaidic acid, stearic acid, and palmitic acid) and 21.9% of proteins, can be considered as an interesting and ecofriendly alternative, to other surfactants derived from petrochemicals, for cosmetic companies. Some authors have suggested the importance in the cosmetic industry of several parameters related with the composition of biosurfactants, such as the critical micelle concentration (CMC). The CMC is defined as the concentration for which the surface tension of water becomes minimal. Commonly, it is used as a measurement of biosurfactant efficiency [12]. In addition, the hydrophilic–lipophilic balance (HLB) value of biosurfactants is an important factor for their correct incorporation in cosmetic products. Depending on its HLB value, a biosurfactant can act as an emulsifier, wetting agent or antifoaming agent, among others. The ionic behavior of biosurfactants is also another crucial parameter for their application in cosmetic formulations. According to their polar head group, surfactants are divided into four groups: anionics, nonionics, cationics, and amphoteric. The anionic surfactants have the greatest wetting, foaming and emulsifying properties as compared with the cationic or non-ionic groups. However, they are more irritating to both eyes and skin than non-ionic and amphoteric ones [12].

As previously reported, the lipopeptide biosurfactants produced by the *Bacillus subtilis* SPB1 strain (HQ392822) revealed a wide spectrum of actions including antimicrobial activity towards multidrug resistant profiles microorganisms [18], antifungal activity against phytopathogenic fungi [19] and antidiabetic and antilipidemic properties in alloxan-induced diabetic rats [20]. This biosurfactant is able to reduce surface tension of the water from 70 mN/m to 34 mN/m [21] with a critical micellar concentration of 150 mg/L. Moreover, the *in vivo* potential toxicity of the SPB1 lipopeptide biosurfactant towards male mice was performed by Sahonoun et al. [15]. They proved that the daily intake of doses lower than 47.5 mg of SPB1 biosurfactant per kg of body weight had no significant adverse effect on hematological parameters and serum biochemical data.

Therefore, thanks to these great properties of the SPB1 biosurfactant, this study was carried out to evaluate its potential application in toothpaste formulation instead of using chemical surfactant.

Material and methods

Microorganism strain and biosurfactant production

Bacillus subtilis SPB1 (HQ392822) was isolated from Tunisian hydrocarbon-contaminated soil and identified by morphological, biochemical and 16S (rDNA) sequence analysis [22]. It was selected based on its high hemolytic and emulsification activities of its biosurfactant, which belongs to the class of lipopeptides [19].

One loop of cells of the wild-type strain *B. subtilis* SPB1 was dispensed into 3 mL Luria-Bertani medium (LB) then incubated and shacked 18 h at 150 rpm and 37 °C. A 0.2 mL sample of this culture was added to 50 mL of fresh LB medium and incubated on shaker until an optical density (OD600) of almost 3 was reached [22]. This culture broth was used to inoculate the production medium, composed of glucose, yeast extract, ammonium sulfate and other salts (KH_2PO_4, K_2HPO_4, $MgSO_4$), to start with an initial optical density of 0.15. After its incubation for 48 h at 37 °C and 150 rpm, the culture was centrifuged at 10,000 rpm and 4 °C for 20 min to remove bacterial cells and the supernatant-free cells served to extract biosurfactants [23].

Preparation of the crude lipopeptide powder

The supernatant-free cells was precipitated, by adding HCl solution (6 N) to achieve a final pH of 2.0, for 18 h at 4 °C. After centrifugation at 10,000 rpm and 4 °C for 20 min, the white pellet was dissolved in alkaline water (pH = 8) and followed by second centrifugation. The supernatant collected was followed by second acid precipitation (HCl 6 N) and then centrifugated. The final pellet formed was washed three times with acid water (pH = 2), suspended in alkaline water (pH = 8) and then lyophilized (Christ Alpha 1-2 LDplus, Germany) [23]. This serves as crude lipopeptide preparation to perform this study.

Formulation of toothpaste

The formulation of two different toothpastes containing different combinations of natural active ingredients, as given in Table 1, were elaborated as described by David [24] and Das et al. [9], with slight modifications, using manual mixing process. As abrasive agents, we used sodium carbonate [25] and calcium carbonate [26]. Glycerin, sodium fluoride and sodium alginate were used, respectively, as humectant, fluoride and binder agents [27]. The tested toothpastes were divided into three groups: the first one contained biosurfactant and was noted BIO, the second one contained sodium dodecyl sulfate and noted SDS and the third one, served as a control, did not contain emulsifier and was noted SS. Each formula was prepared by adding the required amounts of distilled water until the mixture reaches the same appearance of commercial toothpaste. All preparations were packed in large plastic jars with screw lid. The commercial toothpaste contains as ingredients: sodium monofluorophosphate (antimicrobial agent), sodium fluoride, dicalcium phosphate dihydrate (polishing agent), aqua, glycerin, SLS, cellulose gum (thickening agent), aroma, tetrasodium pyrophosphate (buffering, emulsifier, dispersing and thickening agents), sodium saccharin (sweetener agent), calcium glycerophosphate (mineral supplement), limonene (flavoring agent).

Physico-chemical evaluation of the toothpastes

To evaluate the prepared formulations, quality tests including physicochemical controls and visual assessment were performed. All the analyses were conducted in triplicate.

Determination of pH

The pH of 2.5% toothpaste solution was determined at room temperature (25 °C), using a previously calibrated pH meter (744 pH Meter, Metrohm (Switzerland)).

Determination of total solids

A defined quantity of toothpaste (0.1 g) was weighed on a Petri dish and heated in an oven at 105 °C until the liquid portion was evaporated (nearly 24 h). Loss by desiccation was calculated from the initial and final weights difference.

Determination of water activity

The water activity (aw) of the toothpaste formulation was measured using Novasina Aw Sprint TH-500 (Switzerland) at room temperature. Approximately, 2 g of the toothpaste was placed in a cell specific to the aw meter and the value of the aw was displayed directly.

Determination of foaming activity

In a test tube, 5 mL of distilled water was followed by 0.375 g of toothpaste. The toothpaste solution was shaken properly via Ultra-Turrax (T18 basic, Germany) for 30 s at speed 3 and then placed on the lab bench. The height of the foam above the water was measured in centimeter [9,24]. The foaming ability was determined using the following equation:

$$\text{Foaming ability } (\%) = \frac{\text{The height of the foam above the water}}{\text{The total height(foam and water)}} \times 100$$

Spreading ability test

0.5 g of toothpaste was placed at the center of a glass slide and cover with another glass slide. 1 kg weight was carefully placed on covered glass plate. After 10 min, the weight was removed and the diameter of the paste was measured in millimeter [9,24,28].

Cleaning ability test

The composition of the eggshell is very similar to that of teeth, both are made of calcium compounds [29]. For this reason, we used hard boiled and withe eggs for the cleaning test as reported in previous works [9,24,30,31], with slight modifications. In a boiling water, we put one spoon of coffee, one spoon of tea and 40 g of chocolate. After cooling, the baked eggshell was stained with this mixture for 12 h at room temperature. The stained eggshell was washed firstly with a wet tooth brush until there was no change in color of stain and secondly with known amount of toothpaste. We used 5–10 brush strokes for each toothpaste (Each stroke is a complete back and forth motion) and if necessary, we used more brush strokes. We note that the brushing procedure should be as exact as possible for each tested toothpaste.

The cleaning ability of specific toothpaste was observed and the results were interrupted as follows: '+++' very high cleaning ability, '++' high cleaning ability, '−' bad cleaning ability.

Determination of antimicrobial activity

Antimicrobial assay

The *in vitro* antibacterial activity of the tested dentifrices was evaluated against eight strains of microorganisms: *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Enterobacter sp,* *Listeria monocytogenes* (ATCC 43251), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 43972), *Salmonella typhinirium* (ATCC 19430) and *Micrococcus luteus* (ATCC 4698) using the cup plate or well diffusion method. The inocula of bacterial strains, prepared in LB medium, were adjusted, after incubation at 37 °C for 18 h, to an optical density of 0.1, corresponding to almost 1×10^8 CFU/mL. Nutrient agar plates (LB) were seeded with 1 mL of the broth cultures of each tested microorganism and were dried for 1 h.

A sterile corn borer was used to cut four wells (of 6 mm diameter); three for the formulated toothpastes (SS, BIO, SDS) and one for the commercial toothpaste. The ampicillin (100 mg/mL), served as a positive control, was tested alone. Solutions of selected toothpaste was made by mixing 0.2 g toothpaste with 1 mL of sterile distilled water and 60 μL of each dilution were poured on the designated well. The plates were then kept 2 h in the refrigerator for

Table 1
Composition of the formulated toothpastes (g).

Ingredients (g)	Emulsifier		Sodium alginate	Sodium carbonate	Calcium carbonate	Sodium chloride	Sodium fluoride	Glycerin
Formula 1	SS-1	0	1	0	4	1.5	0.5	4
	SDS-1	0.5						
	BIO-1	0.5						
Formula 2	SS-2	0	0	5	1.5	1.5	0	4
	SDS-2	0.5						
	BIO-2	0.5						

(BIO: biosurfactant-based toothpaste, SDS: SDS-based toothpaste, SS: toothpaste without emulsifier).

diffusion of samples and then incubated at 37 °C for 24 h [2]. All the experiments were conducted in duplicate.

Calculation of zone of inhibition

Zones of inhibition appeared as a clear and circular halo surrounding the wells, after the incubation. The average of vertically and horizontally measured diameter of obtained halo was taken (mm).

Stability studies

The appearance and stability of the physical-chemical properties of the formulated toothpastes were inspected for a period of 3 months at interval of one month.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 20.0). For the parametric parameters, data are presented as Means ± SD. Values were obtained from triplicate determinations and the differences were examined using one-way analysis of variance (ANOVA) followed by a Tukey *post hoc* TEST. Concerning the nonparametric parameters, data are presented as Median ± range, obtained from triplicate determinations, using kurskal-wallice the nonparametric ANOVA.

Results

In this work, two types of dentifrices were studied. In order to ensure their performance, quality and effectiveness, their characteristics were evaluated.

Evaluation of physical-chemical properties

The results of the physical-chemical characteristics of the different toothpastes are presented in Table 2.

The results showed that the desiccation loss of the formulated toothpastes was between 22 and 38%. However, the commercial product exhibited larger amount of solid residues (61.7%). Concerning the foaming ability, commercial toothpaste and formula with SDS showed almost good result (>80%), the biosurfactant-based toothpastes presented lower foaming ability (33%) and the SS-products exhibited the lowest values (<23%) as they lack any surfactant. Regarding the pH analysis, although the pH of the toothpastes was basic, we noticed a difference between Formula 1 and the commercial toothpaste, presenting a pH close to 9, on the one hand and Formula 2 showing a pH value near to 11.4, on the other hand.

The spreading ability test showed that all formulated toothpaste presented similar value of the order of 20 mm except BIO-2 indicating low value equal to 16.5 mm. The results also showed that the commercial product had a spreading ability similar to

the formula 2 and SS-1. Respecting the aw, all formulations presented comparable values between 0.2 and 0.32. However, the commercial toothpaste possessed higher activity equal to 0.87. We noticed, also, that formula 2 was heterogenic (separated into liquid and solid ingredients) whereas formula 1 and the commercial toothpaste were homogeneous and had a comparable texture.

Cleaning ability

From Fig. 1, we noted that the formula BIO-1 and the commercial toothpaste had the same ability of cleaning stains (++). In contrast, the use of formula 2 (BIO-2) showed a change in color of eggs from yellow to brown (−). In addition, we remarked that eggs previously brushed by formula BIO-1 were not pigmented by dyes which is not the case for other formulations and even for commercial toothpaste.

As a conclusion, due to the heterogeneity of formula 2, its high pH value, its low spreading ability and its low cleaning efficiency, we choose to use Formula 1 for further experiments.

Antibacterial activities

The antibacterial activity of the formulated toothpaste (Formula 1) was evaluated *in vitro* against height different strains in comparison with the commercial toothpaste. It was clear from Table 3, that BIO-1 was very effective against the tested microorganisms except *E. coli*. As remarked, the greatest inhibition diameter was observed against *Enterobacter sp* (22 mm) and *Salmonella typhinirium* (20 mm) and the lowest zone of inhibition was observed against *Listeria monocytogenes* (12.67 mm). In addition, BIO-1 was more effective than the commercial toothpaste and the SDS-1 in inhibiting *Listeria monocytogenes, Klebsiella pneumoniae* and *Salmonella typhinirium*

Stability test

As shown in Table 4, the spreading power of all formulas did not change during storage. Moreover, the foaming ability remained stable except for BIO-1. In fact, after 3 months, the values were significant different with a p-value of 0.037. We remarked also a slight variation of other parameters which cannot affect the toothpaste properties. Indeed, we noticed a decrease of the pH value of all formulas, except the biosurfactant-based toothpaste, and an increase in the aw value of all formulated toothpastes, which stays always less than the standard minimum value (0.585).

Discussion

Cosmetics are intended to be applied on the human body through rubbing, sprinkling or other methods, aiming to clean,

Table 2
Physical-chemical properties of formulated and commercial toothpastes.

Formulation	Formula 1			Formula 2			Commercial
Parameters	SS-1	SDS-1	BIO-1	SS-2	SDS-2	BIO-2	
Total solids (%)	31.78 ± 6.75	25.48 ± 0.04	30.33 ± 16.04	38.49 ± 0.61	25.25 ± 2.84	22.02 ± 0.63	61.70 ± 0
Foaming ability (%)	23.61 ± 2.78	92.85 ± 9.72	33.04 ± 8.93	16.66 ± 0.00	82.09 ± 27.82	38.64 ± 22.73	92.00 ± 0
Data are presented as median ± range values of three triplicates determinations							
pH	9.80 ± 0.21$^{a\ A}$	9.65 ± 0.12$^{ab\ AC}$	9.08 ± 0.44$^{ab\ AC}$	11.45 ± 0$^{a\ B}$	11.43 ± 0$^{a\ B}$	11.34 ± 0.03$^{a\ B}$	9.20 ± 0 ^{C}
Spreading ability (mm)	22.00 ± 2.00$^{a\ AC}$	22.50 ± 0.50$^{a\ A}$	22.50 ± 0.50$^{a\ A}$	20.50 ± 0.50$^{a\ AC}$	21.00 ± 1.00$^{a\ AC}$	16.50 ± 1.50$^{b\ BC}$	19.00 ± 0.00^{C}
Water activity	0.30 ± 0.068$^{a\ A}$	0.32 ± 0.043$^{a\ A}$	0.22 ± 0.028$^{a\ A}$	0.20 ± 0.002$^{a\ A}$	0.25 ± 0.02$^{a\ A}$	0.28 ± 0.019$^{a\ A}$	0.87 ± 0.00^{B}
Data are presented as means ± standard deviation values of three triplicates determinations							

(BIO: biosurfactant-based toothpaste, SDS: SDS-based toothpaste, SS: toothpaste without emulsifier).
Means not sharing the same letters (a–c) within a row, are significantly different ($P < 0.05$).
Means not sharing the same letters (a–c) within Formula 1 are significantly different ($P < 0.05$).
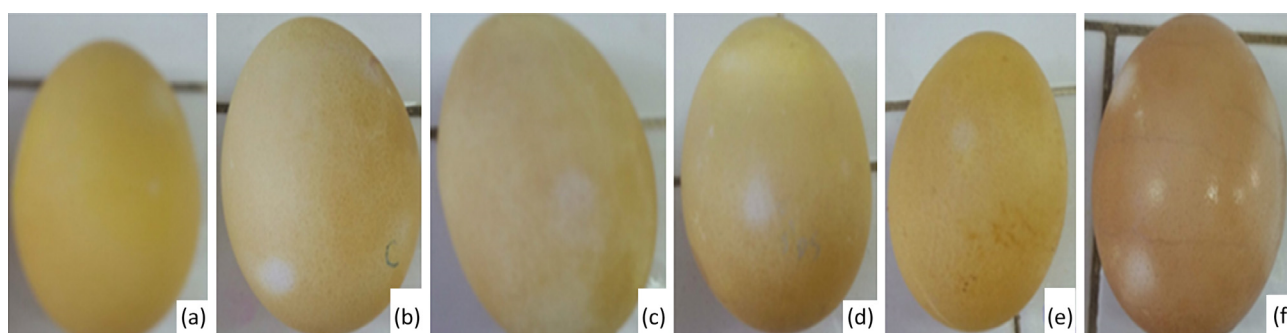Means not sharing the same letters (a–c) within Formula 2, are significantly different ($P < 0.05$).
Means not sharing the same letters (A–C) within a row are significantly different ($P < 0.05$).
Means not sharing the same letters (A–C) within (SS1-SS2-Commercial), (SDS1-SDS2-Commercial) and (BIO1-BIO2-Commercial) are significantly different ($P < 0.05$).

Fig. 1. Cleaning ability of formulated and commercial toothpastes. (a: before cleaning, b: commercial toothpaste, c: BIO-1, d: SDS-1, e: SS-1, f: BIO-2). (BIO: biosurfactant-based toothpaste, SDS: SDS-based toothpaste, SS: toothpaste without emulsifier.)

Table 3
Antimicrobial activity of formula 1 against microorganisms (mm).

Toothpaste	Formula 1			Commercial	Ampicillin
Bacteria	SS-1	SDS-1	BIO-1		
Escherichia coli	0	0	0	0	33±0.8
Enterococcus faecalis	0	15 ± 0.0	14 ± 3.0	16 ± 1.0	19.5 ± 1.1
Enterobacter sp	20	17	22 ± 0.0	23	36.5 ± 0.8
Listeria monocytogenes	0	0	12.67 ± 2.9	0	31 ± 1.6
Klebsiella pneumoniae	0	0	15	0	28
Salmonella enterica	11.5	19 ± 1.0	14.75 ± 4.6	18 ± 1.0	23 ± 3.3
Salmonella typhinirium	0	18	20	12	31 ± 1.8
Micrococcus luteus	17	18 ± 5.67	18.50 ± 2.1	20.50 ± 9.2	24.4 ± 0.1

(BIO: biosurfactant-based toothpaste, SDS: SDS-based toothpaste, SS: toothpaste without emulsifier).

beautify and enhance attractive features or to alter appearance. Therefore, various issues and aspects have to be considered such as its site and area of application, sensory and optical properties, state of matter, packaging, final product storage and stability [32]. Dentifrices are considered as a cosmetic product that have been used for many years and proven to be an important tool for improving both oral health and esthetics [1]. They are daily used products worldwide, but only little information is available about them [7], that is why it will be important to evaluate and study their properties.

Basically, toothpastes perform three main functions; they remove stain on tooth through abrasion [7,9], clean oral cavity through detergents and act as a carrier for releasing therapeutic compounds [9]. It is important to point out that increasing the dentifrice abrasiveness leads to improve the stain removal efficacy, contrariwise, it increases the tooth wear and may harm tissues and dental restoration [5,7]. As described by Das et al. [9], damage

can be even more pronounced in the dentin. The most common harms are cervical abrasion and gingival recession, which are generally combined with dentin hypersensitivity [33]. Hence, to assure their effectiveness, dentifrices must be sufficiently abrasive and the ideal one must supply maximum cleaning with minimum wear [6,7]. It was reported that higher values of solid residues were a sign of distortion in roughness, therefore, they may define the toothpaste potential to modify the surface enamel [34]. In order to understand the greater variation in abrasiveness of different formulated toothpastes, their desiccation loss was investigated. The results showed that the commercial toothpaste presented the larger amount of solid residues, which could indicate its higher abrasivity. The relation between the abrasive potential of toothpastes, alterations on enamel and restorative materials has been evaluated by many studies [35]. In fact, it was reported by Pinto et al. [7] that dentifrices abrasiveness might be affected by the quality and the quantity of abrasives, unlike other studies, where no correlation

Table 4
Stability test of formula 1.

Parameters	Water activity			pH			Spreading ability (mm)			Foaming ability (%)		
Formulation	SS-1	SDS-1	BIO-1	SS-1	SDS-1	BIO-1	SS-1	SDS-1	BIO-1	SS-1	SDS-1	BIO-1
Initial	0.30 ± 0.068[aA]	0.32 ± 0.043[aA]	0.22 ± 0.028[aA]	9.80 ± 0.21[aA]	9.65 ± 0.12[aA]	9.08 ± 0.44[aA]	22.0 ± 2.0[aA]	22.5 ± 0.50[aA]	22.5 ± 0.5[aA]	23.61 ± 2.78[A]	92.85 ± 9.72[A]	33.04 ± 8.93[A]
1 month	0.37 ± 0.057[aA]	0.41 ± 0.03[aA]	0.36 ± 0.02[aB]	9.33 ± 0.12[aAB]	9.11 ± 0.03[aA]	9.04 ± 0.02[aA]	22.0 ± 1.0[aA]	21.0 ± 2.0[aA]	22.0 ± 0.0[aA]	33.33 ± 22.22[A]	94 ± 4.00[A]	36.67 ± 6.67[AB]
2 months	0.46 ± 0.053[aB]	0.44 ± 0.068[aB]	0.42 ± 0.021[aB]	8.82 ± 0.01[aB]	8.82 ± 0[aB]	9.05 ± 0.03[aA]	21.0 ± 1.0[aA]	21.50 ± 0.5[aA]	22.0 ± 0.0[aA]	36.67 ± 6.67[A]	99 ± 1.00[A]	31.67 ± 3.33[A]
3 months	0.47 ± 0.004[aB]	0.45 ± 0.003[aB]	0.42 ± 0.008[aB]	8.82 ± 0.03[aB]	8.79 ± 0.02[aB]	8.65 ± 0.41[aA]	21.5 ± 0.50[aA]	20.50 ± 0.50[aA]	22.00 ± 0.00[aA]	32.05 ± 2.57[A]	99 ± 1.00[A]	40.00 ± 0.00[B]

Data are presented as means ± standard deviation values of three triplicates determinations

Data are presented as median ± range values of three triplicates determinations

(BIO: biosurfactant-based toothpaste, SDS: SDS-based toothpaste, SS: toothpaste without emulsifier).
Means not sharing the same letters (a–b), within a row, for each parameter alone, are significantly different ($P < 0.05$).
Means not sharing the same letters (A–B), within a column, for each formula alone, are significantly different ($P < 0.05$).

between the content of abrasives and abrasion degree has been detected [36]. In the present study, sodium carbonate and calcium carbonate were used as combined abrasive agents for formula 2, similarly to Moore and Addy [37] who formulated toothpaste with a combination of different abrasive agents (artificial silicas, tixosil, Zeodent and calcium carbonate). It is important to remind that chemically identical abrasives may have different properties and the effect of mixing these agents may differ when the components are used individually [38]. As described by Printo et al. [7] and Costa [11], calcium carbonate is the most common abrasive agents used in dentifrices thanks to its lower abrasivity comparing to other agents [9,34]. It promotes an induced raise of dental plaque pH, plaque calcium levels and supports the remineralisation process [11].

In addition to its abrasiveness, the pH value of toothpastes plays a crucial role in evaluating their properties as it gives an indication of inorganic constituents [9]. Exposing the teeth and oral tissues at low or high pH for a long time can cause adverse reactions [39]. According to the consumer voice [40], a dentifrice should have a pH between 5.5 and 10.5 which is not the case for formula 2. This higher pH value could be due to the presence of sodium carbonate. Many research aimed to study the effects of pH on enamel erosion: as described by Price et al. [39] at pH below 5.2, demineralization of enamel and root resorption were observed. Moreover, Bell [41] reported that a pH of 5.5–5 in the mouth was considered critical for demineralization. Other authors mentioned that acidic pH dentifrices increased the binding of fluoride to the teeth [33] and showed greater alteration to the dental surface [6]. Johannsen et al. [42] carried out that low pH dentifrices were more abrasive and might have harmful effects on dentin this surface, leading to the deterioration of its structure. Therefore, they recommended to use neutral or basic dentifrices. Recently, Das et al. [9], indicated that lower pH stimulated the growth of oral bacteria that caused dental carries. Thus, an alkaline pH can enhance the neutralizing of acid biofilm [7]. It was reported in a previous study, that a slightly better hardening could be measured when slurry with basic pH was used after softening [33]. In addition, it was noted that toothpaste with the alkaline baking soda (sodium bicarbonate) could neutralize the acids in the mouth, kill germs and mop up unpleasant odors [41,43]. This finding has made the manufacturers concerned about formulating dentifrices with higher pH in order to mitigate dental structure loss by abrasion caused by low pH dentifrices. Another crucial property required in a toothpaste formulation is its aspect. In fact, a toothpaste must not separate into liquid and solid ingredients [9] and should extrude from the flexible tube in the form of a homogeneous aspect with the application of normal force [40]. Das et al. [9] reported that the aw and spreading ability might reflect the consistency of toothpaste, as the large spread area showed its better consistency. Indeed, it appears that formula 1 was more consistent than formula 2 and the commercial product since it had the highest spreading area. Concerning the aw values, according to Gustavo et al. [44], a toothpaste should ideally have an aw between 0.585 and 0.984. Although the formulated toothpastes had very low aw, Formula 1 presented the nearest standard values.

Foam is a desirable characteristic of any oral care compositions as it enables the dentifrice to spread all over the oral cavity during brushing and contact tooth surfaces thoroughly. Consumers also prefer compositions with good foaming ability, usually achieved by employing surface-active agent. In fact, the foaming effect produced by the surfactants is beneficial in cleaning the teeth and contributes to remove debris and gives a feeling of cleanness. In addition, surfactants reduce the surface tension of the liquid environment in the oral cavity so that the substances in the toothpaste can contact the teeth more easily. This makes it easier to clean the teeth. Another function of the surfactant is dispersing the flavors in
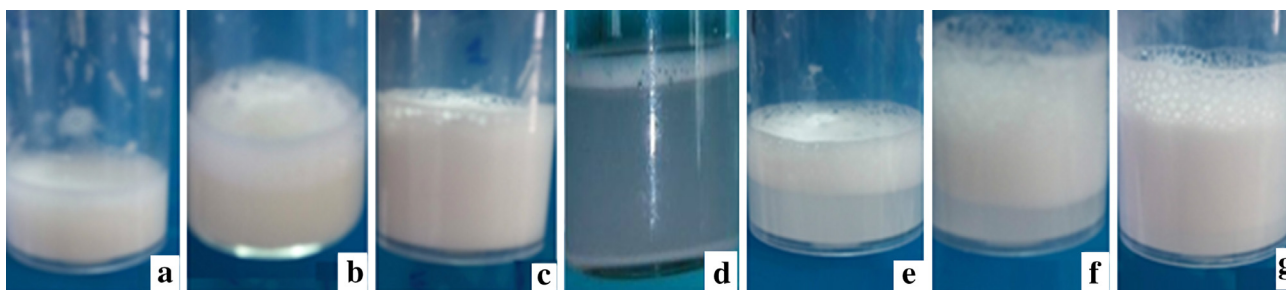
Fig. 2. Foaming ability of formulated and commercial toothpastes. (a: SS-1, b: BIO-1, c: SDS-1, d: SS-2, e: BIO-2, f: SDS-2, g: commercial toothpaste). (BIO: biosurfactant-based toothpaste, SDS: SDS-based toothpaste, SS: toothpaste without emulsifier.)

the toothpaste. The most frequently surfactant used at present is SLS [27]. As reported, surfactants are the most important class of industrial chemicals which are used widely in almost every sector of modern industry. Only within the US chemical industry the demand of surfactants has been increased by 300% during the last decade [40]. At present, the worldwide production is more than three million tonnes per annum (at an estimated value of US $4 billion) and is expected to be greater than over four million tonnes by the end of the century [45]. This global industrialization, have motivated the scientific and technological communities to seek products with high aggregate value in the world market, such as biosurfactants which are considered as suitable molecules in the industrial processes during the 21st century [46]. Moreover, biosurfactant-containing marketable products and patents have been reported for application in the healthcare and cosmetic industries [47–50]. According to Transparency Market Research™, the global biosurfactant market is estimated at USD 1735.5 million in 2011 and is expected to reach USD 2210.5 million in 2018. Europe accounts for 53.3% of the global biosurfactant market revenue share in 2018 followed by North America. Moreover, a well-known surfactants companies have already ventured into the biosurfactant market such as Ecover (Belgium) and BASF-Cognis (Germany and the USA), which is the leader with over a 20% share of the market in 2011 [51]. Despite their great potential and commercial interest, the bottleneck of biosurfactant production on the industrial scale is their high production and downstream costs. For this reason, many studies are focusing on the use of renewable agro-industrial wastes as substrates to make the biosurfactants biotechnological production competitive comparing to the chemical synthesis of their counterparts [12].

Thanks to their foaming property, the inclusion of biosurfactants in a toothpaste formulation, can reduce significantly the use of the chemical surafactants. As shown in Fig. 2, formulas contained biosurfactant were able to produce foam. This result indicates that biosurfactant acts as a good detergent in toothpaste, which is on accordance with Das et al. [9] who used biosurfactant from *Nocardiopsis* VITSISB in toothpaste instead of SLS.

In addition to its foaming property, an effective toothpaste should be able to remove stains caused by cigarette smoke, beverages, colored fruits, chocolates, etc., which adhere strongly to the teeth and resist against their suppressing. In recent years, the demand for products that promote a whitening of the teeth has increased significantly. For this reason, whitening toothpastes containing calcium carbonate and perlite, as the abrasive system, and an efficient fluoride source have been developed [11]. This new formulation appears to be effective in removing teeth extrinsic stain *in vitro*, according to Joiner [5]. In this study, the formula BIO-1, was as effective as the commercial toothpaste in cleaning stains as it contained a combination of calcium carbonate, sodium fluoride and biosurfactant. Moreover, the non-fixing of dyes in eggs previously brushed by this formula indicated its preventive

property in protecting teeth against browning due to the abusive drinking of tea and coffee. Although formula 2 containd sodium carbonate and calcium carbonate as abrasive agents, which could lead to improve stain removal efficacy clean, we observed a variation of eggs color from yellow to brown. This problem can be explained by the fact that mixing these abrasive agents may differ when each one is used individually [38].

It was reported that, when the balance, which exists in the person's oral microbial population, is lost, opportunistic microorganisms can proliferate and produce an acidic environment leading to the destruction of hard enamel tissue [2]. Hence, it is of utmost importance to control the oral hygiene by mechanical action and suitable toothpaste. As described by Bora et al. [2], the important key factor to select dentifrice is its antibacterial efficacy. The addition of antimicrobial agents to conventional toothpastes aims to reduce the microbial growth and their colonization on the tooth surface and may deteriorate their cell walls and inhibit their enzymatic activity [52]. Moreover, the complex composition of toothpastes implies that it is necessary to ensure that the active ingredients are not inactivated. For example, calcium carbonate binds to sodium fluoride rendering it ineffective as an anti-caries agent. So, the elaboration of a correct composition of a toothpaste is of crucial importance, regarding its effectiveness on oral health maintenance [11].

The methodology included agar diffusion technique has been used as standard method of checking the antibacterial sensitivity. Although this method is convenient for fluid materials like water, it has been also used for antimicrobial evaluation of semi-solid matters which are fluid in the presence of saliva or water, such as toothpaste [2,53]. As described by Fine et al. [54], several clinical studies have demonstrated the inhibitory effects of antimicrobial dentifrice on oral bacteria and gingival. Previous work showed that the SPB1 biosurfactant was effective against microorganisms with multidrug-resistant profiles [18]. Therefore, it was used in this study as antimicrobial agent. In fact, the formulated toothpaste presented a clear inhibition zone against almost all of the tested bacteria, which indicated the level of antimicrobial activity presented in the product. A larger zone of inhibition usually implies the higher antimicrobial agent efficiency. It was reported that, evaluating the antimicrobial activity of dentifrices by *in vitro* tests prior to randomized controlled trials evaluations is required in future studies to evaluate their possible *in vivo* benefits [55]. However, Inetianbor et al. [53] indicated that it cannot presumed that the efficiency of the antimicrobial results obtained by the *in vitro* test could be transferable or proportional to the oral cavity and translated into clinical effectiveness [53]. In fact, and as shown by Barry and Thornsberry [56], higher antibacterial properties may not necessarily refer to those having superior diameter of inhibition zones because a toothpaste used *in vivo* is likely to be diluted by saliva, the level to which antimicrobial properties are buffered or lost in dilution *in vitro* is of interest. In addition, it was reported

that the mean average inhibition zone of a toothpaste brand may not be directly compared with that of other toothpaste [53] since each one contains a complex mixture of active ingredients that may diffuse at different rates.

In accordance to what it was described by Das et al. [9], biosurfactant obtained either from *Bacillus subtils* or *Actinobacteria* can act as a good ingredient in place of chemical surfactant for toothpaste formulation. Both of these tow biosurfactant-based toothpastes possess a good foaming ability required by consumers, good cleaning efficiency in removing stains form eggs hells and an alkaline pH necessary for acid biofilm neutralization. However, the SPB1 biosurfactant-based toothpaste has an effective antimicrobial activity against the tested microorganisms, this property was not reported by Das et al. [9].

The stability test measures the ability of the product to retain its potency and to determine whether differences of test parameters were significant or not. Stability and acceptability of formulas properties during the storage period indicated that they are chemically and physically stable which was the case.

Conclusions

Using completely natural raw materials in order to formulate cosmetics is a difficult task. The challenge lies in choosing materials, considered as 'natural', and formulating them into cosmetics having comparable functions with their synthetic counterparts.

In order to popularize more natural toothpaste, more radical approach is to be deployed as to change the consumer expectations from a toothpaste, with emphasis on safety and efficacy.

Formulators must play an essential role in highlighting to the consumers the potential harmful effects of synthetic detergents and other chemical additives present in cosmetics. Consumers perception has to be changed concerning a good toothpaste and the onus lies with the formulators.

Conflict of Interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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