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Development of a potential probiotic lozenge containing *Enterococcus faecium* CRL 183

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ABSTRACT

In this work, a diet lozenge with the microencapsulated probiotic strain *Enterococcus faecium* CRL 183 was developed. The microbiological, physicochemical and sensorial characteristics were analyzed and the anticariogenic potential of the strain was also evaluated. The results showed that the complex coacervation microencapsulation technique enables higher viability during storage at room temperature. Lozenges were produced through three treatments: PC—control formulation; PPP1—probiotic formulation; PPP2—probiotic formulation with inulin. The probiotic strain had the viability decreased after lozenges production and during the storage from 5.53 to 4.83 log CFU/g at the beginning to 1.05–1.86 log CFU/g after 28 days, for both PPP1 and PPP2. Formulations were different concerning water activity, moisture and color ($p < 0.05$). All formulations remained microbiologically safe during the storage period. Association of probiotic bacteria and inulin improves the lozenges flavor acceptance and the purchase intent. The anticariogenic evaluation showed that the probiotic bacteria were able to survive in saliva and inhibit the growth of *Streptococcus mutans* ATCC 25175 in this study. The lozenge with *E. faecium* CRL183 and inulin represents an alternative to the diversification of the probiotic segment which aggregates anticariogenic potential. However, a process refinement and use of gas resistant packaging to preserve the probiotic viability, is necessary.

1. Introduction

Probiotics and oral health have evoked new researches in the last years. Results indicate that probiotics provide important benefits to the oral health, preventing caries, helping periodontal and halitosis disease treatment and reducing the amount of bacteria related to oral pathology (Cagetti et al., 2013; Haukioja, 2010).

The action mechanism of probiotics in the oral cavity involves the formation of biofilms, immune system modulation and competition with bacteria that cause caries and periodontal diseases, such as *Streptococcus mutans* (Çaglar, Kargul, & Tanboga, 2005).

The probiotic strain *Enterococcus faecium* CRL 183, isolated from Tafí cheese (typical of Tucuman, Argentina), has several proven health effects, as it modulates the lipidic profile, intestinal microbiota and immune system, reduces colon and breast cancer risks, and improves symptoms related to ulcerative colite (Cavallini et al., 2009, 2011; Celiberto, 2014; Kinouchi, 2006; Rossi, Vendramini, Carlos, Oliveira, & Valdez, 2003, Rossi et al., 2008; Sivieri et al., 2008). It is known that such strain is safe to human consumption and presents gastrointestinal resistance, although, its oral effects have not been investigated so far (Saavedra, Taranto, Sesma, & Valdez, 2003).

The viability of probiotic bacteria is important in order to provide health benefits to the consumer. Therefore, the microorganism should survive adverse conditions during processing and storage (pH and temperature variations and oxygen exposure), besides resisting the gastrointestinal transit (Folign, é, Daniel, & Pot, 2013). Probiotic microorganisms used to maintain the oral health must as

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well stay longer in the mouth and resist the oral environment.

Lozenges are candies manufactured in a simple way, which can deliver a variety of compounds as they disintegrate slowly in the mouth (Edwards, 2001). Considering the time in which lozenges are kept in the mouth, they could start a local effect such as reduction of caries and other oral pathologies risks, representing a potential vehicle for probiotic microorganisms.

Microencapsulation, a process that surrounds microorganisms in a polymeric membrane, confers greater protection to probiotic cells, allowing their inclusion in products stored at room temperature and their release under specific conditions (Tripathi & Giri, 2014).

Thus, this study aimed at developing a probiotic diet lozenge with the addition of microencapsulated and lyophilized *E. faecium* CRL 183, evaluating its anticariogenic potential, *in vitro*.

2. Material and methods

2.1. Material

Diet lozenges with probiotic strain *E. faecium* CRL 183 addition (CERELA - Centro de Referência para Lactobacilos, Argentina).

2.2. Methods

2.2.1. Probiotic bacteria

2.2.1.1. Probiotic culture preparation. An aliquot (1.0 mL) from a probiotic stock culture *(E.faecium* CRL 183) maintained at −80.0 °C in sterilized milk with yeast extract, glucose and glycerol addition, was inoculated into 10.0 mL of Man Rogosa and Sharp Media (MRS) (Acumedia®) and incubated at 37.0 °C, for 16 h. After this, 0.5 mL was again inoculated into 5.0 mL of MRS and incubated at 37.0 °C, for 16 h.

2.2.1.2. Probiotic culture microencapsulation and freeze drying. Two methods were used to microencapsulate *E. faecium* CRL 183 cells.

In method 1 (M1), the extrusion technique was carried out according to Lee, Cha, and Park (2004), with modifications. The probiotic suspension (200.0 g/L, 8.99 log CFU/g) was added to a sterile solution composed by sodium alginate (Synth®, Brazil, 20.0 g/L), glycerol (Synth®, Brazil, 50.0 g/L), MRS broth (Acumedia®, USA, 5.5 g/L), xanthan gum (Synth®, Brazil, 2.6 g/L), tween 20 (Synth®, Brazil, 1.0 g/L) and inulin GR (Beneo® Orafti, Belgium, 10.0 g/L). After complete homogenization, this solution was dripped in a sterile calcium chloride (Vetec®, Brazil, 74.0 g/L) solution. After 15 min, the beads were rinsed with sterile distilled water.

Method 2 (M2) used the complex coacervation technique, carried out as described by Baracat, Nakagawa, Freitas, and De Freitas (2004) and Oliveira (2006), with changes. In order to promote coacervation, pectin (Vetec®, Brazil, 20.0 g/L) and casein (Vetec®, Brazil, 20.0 g/L) were dispersed (8% of total solids) in distilled water with constant mechanical stirring. The pH was adjusted to 8.0 (±0.1) with NaOH solution (4.0 M), and then, this dispersion was sterilized. The *E. faecium* CRL 183 suspension (200.0 g/L, 8.99 log CFU/g) was added to the cooled dispersion, under aseptic conditions, and the pH fall monitored (pH = 4.8).

The microcapsules obtained through M1 and M2 were frozen at −20.0 °C. Then, the samples were freeze-dried (Modulyod Freeze Dryer, Thermo Electron Corporation), and stored in sterile glass flasks at room temperature (25.0 ± 2.0 °C).

2.2.1.3. Viable cells count. The *E. faecium* count was performed in free cells and freeze-dried materials (M1 and M2). The analyses were performed immediately after obtaining the freeze-drying microcapsules (T = 0) and weekly, during the storage or by the time the count reached values below 6 log colony forming unity (CFU)/g.

Cell viability was determined by the pour plating technique in M17 agar (Difco®, USA), in aerobic conditions at 37.0 °C, for 48 h. The results were expressed as log CFU/g (Rossi et al., 2008).

2.2.2. Production of diet lozenge

The diet lozenges were prepared with xylitol (Labonathus®, Brazil), gelatin (Gelita®, Brazil), inulin GR (Beneo® Orafti, Belgium), mint flavor (Firmenich®, Brazil), microencapsulated and freeze-dried *Enterococcus faecium* CRL 183, and water.

Three formulations were defined: control formulation (PC) with xylitol (89.60 g/100 g), water (8.40 g/100 g), gelatin (1.50 g/100 g) and mint flavor (0.50 g/100 g); potentially probiotic formulation 1 (PPP1) with xylitol (88.41 g/100 g), water (8.25 g/100 g), gelatin (1.47 g/100 g), microencapsulated and freeze-dried *E. faecium* CRL 183 (1.38 g/100 g) and mint flavor (0.50 g/100 g); and potentially probiotic formulation 2 (PPP2) with xylitol (85.66 g/100 g), water (8.25 g/100 g), gelatin (1.47 g/100 g), microencapsulated and freeze-dried *E. faecium* CRL 183 (1.38 g/100 g), inulin (2.75 g/100 g) and mint flavor (0.50 g/100 g).

In order to reduce the amount of *E. faecium* added to the probiotic formulations, the microorganism suspension was previously concentrated by centrifugation.

The lozenges were produced in three batches, through the agglutination of ingredients by the gelatin solution, followed by molding and drying at 35.0 °C for 20 h (Edwards, 2001), stored in plastic bags (low density polyethylene) and kept in a temperature-controlled chamber (Fanem®, Biochemical oxygen demand), at 23.0 ± 0.5 °C.

2.2.3. Characterization of the diet lozenge

2.2.3.1. Microbiologic evaluation. Samples containing 25.0 g of each formulation were collected and diluted in 225.0 mL of peptone water. Serial dilutions were subsequently prepared with the same diluent and used for the viability and safety analyses of the lozenges.

Countings of *E. faecium* in the product were weekly enumerated by the pour plating technique, using 1.0 mL of each dilution in M17 agar followed by aerobic incubation at 37.0 °C, for 48 h. The results were expressed in log CFU/g (Rossi et al., 2008).

Microbiologic safety assays were determined in triplicate, through yeasts and molds, thermotolerant coliforms and total mesophiles counts. The thermotolerant coliforms analysis were carried out in Petrifilm® (3 M®, USA), while the total mesophiles one was performed in PCA (Plate Count Agar, Himedia®, India) and incubated at 37.0 °C, for 48 h. The yeasts and molds analysis was performed in PDA (Potato Dextrose Agar, Himedia®, India) and incubated at 30.0 °C, for 120 h.

2.2.3.2. Physicochemical assessments. All formulations were analyzed for their physicochemical characteristics. The pH, water activity, moisture and color were determined fortnightly, in triplicate.

The pH was determined using a digital potentiometer (Qualxtron®, Model 8010), through the dissolution of a 3.0 g-sample in 20.0 mL of distilled water. The water activity (Aw) was measured in a 3.0 g-sample, using a specific device (Aqualab® CX2). The moisture content was measured in an infrared analyzer (Sartorius®, MA35). A colorimeter (Hunterlab® Color Quest XE) was used for the assessment of color parameters.

2.2.3.3. Sensory evaluation. The sensory panel was composed of 112 untrained individuals. The acceptance test of attributes

(appearance, color, flavor, taste, texture and overall acceptance) using a 9-point hedonic scale (1 = disliked very much and 9 = liked very much) and purchase intent test using a 5-point scale (1 = would not certainly buy and 5 = would certainly buy) (Meilgard, Civille, & Carr, 1988; Stone & Sidel, 1993) were performed immediately after lozenges processing. The consumers evaluated 3 formulations in a session. Each sample was coded by using a 3-digit random number and presented in a monadic form (approved by the Research Ethics Committee of the UNESP School of Pharmaceutical Sciences, Araraquara-Brazil, protocol number 661487).

2.2.4. Anticariogenic potential evaluation

The probiotic strain survival capacity in saliva was evaluated in PPP1 and PPP2 samples and in free cells. Such analyses was carried out, as described by Haukioja (2009), with modifications. The saliva donated by a healthy volunteer was sterilized at 121.0 °C for 15 min and 1.0 g-samples of PPP1 and PPP2 formulations and 1.0 mL of free cells were inoculated into 10.0 mL of saliva and incubated at 37.0 °C, for 24 h. *E. faecium* counts were enumerated in triplicate, at the beginning (T0 = before incubation) and after 24 h of incubation, by pour plating technique, using 1.0 mL of each dilution in M17 agar, followed by aerobic incubation at 37.0 °C, for 48 h. The results were expressed in log CFU/mL (Rossi et al., 2008).

The *E. faecium* CRL 183 ability to inhibit the multiplication of *S. mutans* was measured with an agar well diffusion assay (Lewus & Montville, 1991). A 1.0 mL aliquot of *Streptococcus mutans* ATCC 25175 suspension cells (8.26 log CFU/mL) was inoculated into 10.0 mL of BHI broth (Brain heart infusion, Difco®, USA), and 1.0 mL of *E. faecium* cells (8.13 log CFU/mL) was inoculated into 10.0 mL of MRS. Both were incubated at 37.0 °C, for 16 h, followed by centrifugation at 7000 rpm, for 10 min. The supernatant was discarded and the biomass re-suspended in 10.0 mL of peptone sterile water. To proceed with the well diffusion test, a layer of LAPTg medium (10.0 g yeast, 15.0 g peptone, 10.0 g glucose, 10.0 g tripton, 1.0 mL tween 80, 1.5 g agar and 70.0 g sucrose) was placed in triplicates of petri plates. After its complete solidification, 10.0 mL of a softer overlay of LAPTg medium (0.75 g agar) containing 10.0 μL of *S. mutans* was deposited in the plates. Following its solidification, 30.0 μL of *E. faecium* was deposited in a 5 mm diameter well and the plates were incubated in aerobiose at 37.0 °C, for 24 h, the inhibition halo being measured in millimeters.

2.2.5. Statistical analysis

The results were expressed as mean ± standard deviation and statistical analyses of the averages were calculated by Analysis of Variance, Tukey and T-student test ($p < 0.05$), using the Biostat software.

3. Results and discussion

3.1. Viable cells counts

It was established that the probiotic microorganism viability would be observed until reaching values below 6.0 log CFU/g and also, that the amount of probiotic cells added to the final product could be inferior to the range (8.0–9.0 log CFU in the daily portion) preconized in international surveys and local guides (Anvisa, 2008). The following aspects were considered: 1) the amount of inoculum added to the final product should be minimum so as to avoid unpleasant sensory characteristics, and 2) the probiotic effect would be local, without the exposure to the conditions found in the gastrointestinal tract, therefore, an amount lower than the one suggested would be enough to observe the effects.

The viability of *E. faecium* free cells, microencapsulated by complex coacervation (M1) and extrusion technique (M2) followed by freeze-drying is shown in Table 1.

At first (T = 0), the population of free cells was 8.84 log CFU/g. After the microencapsulation by complex coacervation (M2), followed by freeze-drying, the count was 8.73 log CFU/g, showing that the technique did not affect the *E. faecim* survival ($p < 0.05$). Only after 210 days of storage, 1 log cycle reduction was observed in the probiotic cells population (M2). The results obtained show that the complex coacervation technique was able to protect the strain, for 273 days, since only at the 280th day of storage the viability reached values below 6.0 log CFU/g. This long storage period was superior to that of Oliveira (2006), who also used complex coacervation to microencapsulate probiotic strains.

The viability of *E. faecium* microencapsulated by the extrusion technique (M1) followed by freeze-drying, suffered substantial reductions of 3.1 log at the beginning (T = 0) and 5.49 log after 14 days of storage. Therefore, the extrusion technique is not suitable to protect *E. faecium* CRL 183 cells.

The use of alginate as a wall material results in porous beads, which can allow the passage of internal material to the external environment (Mandal, Hati, Puniya, Khamrui, & Singh, 2014; Rokka & Rantamaki, 2010). Thus, we hypothesize that *E. faecium* passed to the external environment reducing the microcapsules' viability.

This result differs from that of Haghshenas et al. (2015) who observed higher *Lactobacillus plantarum* viability in capsules obtained through the extrusion technique, using at least 1.5% of alginate as a wall material. The present study used 2.0% of alginate in the wall material composition, however, since the effect and performance of a bacterium are strain dependent, it is not possible to observe the same results for strains in the same species (Sha, 2007).

Table 1
Viability of the *E. faecium* CRL 183 during the storage period.

Time (days)	M1	M2	Inoculum
0	5.74 ± 0.03 $^{a\ A}$	8.73 ± 0.06 $^{a\ B}$	8.84 ± 0.04 $^{a\ B}$
14	3.35 ± 0.05 $^{b\ A}$	8.59 ± 0.02 $^{b\ B}$	6.97 ± 0.05 $^{b\ C}$
28	–	8.65 ± 0.03 $^{abc\ A}$	5.06 ± 0.04 $^{c\ B}$
42	–	8.59 ± 0.06 $^{bcd\ A}$	2.50 ± 0.07 $^{d\ B}$
56	–	8.13 ± 0.05 ^{e}	–
70	–	8.05 ± 0.03 ^{ef}	–
84	–	8.38 ± 0.02 ^{g}	–
98	–	8.49 ± 0.05 ^{bdgh}	–
112	–	8.39 ± 0.02 ^{ghi}	–
126	–	8.31 ± 0.02 ^{gij}	–
140	–	8.31 ± 0.02 ^{gijk}	–
154	–	8.02 ± 0.01 ^{efl}	–
168	–	8.30 ± 0.01 ^{gijkm}	–
182	–	8.03 ± 0.05 ^{efl}	–
196	–	8.31 ± 0.02 ^{gijkm}	–
210	–	7.43 ± 0.03 ^{n}	–
224	–	7.57 ± 0.06°	–
238	–	7.13 ± 0.05 ^{p}	–
252	–	7.16 ± 0.02 ^{p}	–
266	–	6.93 ± 0.01 ^{q}	–
280	–	5.93 ± 0.02 ^{r}	–

Results are presented as means ± standard deviation, in log CFU/g.
Means in the same column followed by different lowercase letters are significantly different by the Tukey's test ($p < 0.05$) – M2 method and inoculums.
Means in the same column followed by different lowercase letters are significantly different by the T-student test ($p < 0.05$) – M1 method.
Means in the same line followed by different capital letters are significantly different by the T-student test ($p < 0.05$).
M1 - E.faecium microencapsulated by the extrusion technique followed by freeze-drying.
M2 - E.faecium microencapsulated by the complex coacervation technique followed by freeze-drying.
Inoculum – free cells of the E.faecium.

E. faecium free cells had their viability reduced below 6.0 log CFU/g after 28 days of storage at room temperature. Comparing such a result to the one achieved with complex coacervation, it is possible to say that the technique associated with freeze-drying raised the probiotic viability in 200 days. Thus, the coacervation technique (M2) was selected for the next step of the study.

3.2. Lozenges preparation

The viable counts of the concentrated and microencapsulated *E. faecium* (M2) before being added to the probiotic lozenge formulations, were 10.62 log CFU/g. It was added in enough quantity to reach 7.0 log CFU/g of lozenges in formulations PPP1 and PPP2.

Considering that the average weight of each lozenge is 1.5 g, the ingestion of one unit would not be enough to reach the viable cell counts of 10^8–10^9 CFU/g recommended in the daily portion of the final product. It is important to emphasize that probiotic lozenges focus would be a local effect in the oral cavity. In such cases, lower viable counts would be accepted if the benefits were proved.

3.3. Diet probiotic lozenges characterization

3.3.1. Microbiological evaluations

3.3.1.1. Viable counts. The survival of *E. faecium* in the lozenges formulations is shown in Table 2. The viable counts in both PPP1 and PPP2 formulations were reduced to values below 6.0 log CFU/g at the initial time (T = 0). The viable counts in PPP1 formulation suffered a reduction superior to 2.0 log after 7 days of storage, whereas a reduction of 1.53 log was observed after 21 days in formulation PPP2, which might indicate a protective inulin effect. However, this trend was not maintained until the end of the storage period. After 28 days, the bacteria viability was severely reduced in both probiotic formulations, thus, the viability follow up ended.

The rapid loss of probiotic viability was not expected, since the microencapsulated *E. faecium* presented high viability for nine months, at room temperature. Several factors can influence the survival of probiotic bacteria including the processing temperature, oxygen incorporation, pH, mechanic stress, raw material and ingredients (Shah, 2000). Lozenges preparation stages do not involve high temperature, oxygen incorporation or mechanic stress and the pH was close to neutrality (6.23–6.28), thereby, some ingredients were investigated.

Possibly, the flavoring used could have played a role in probiotic survival, since alcohol is a common ingredient in flavoring compositions. Thus, lozenges formulations PPP1 and PPP2 were processed again without it. However, the viability loss was very similar to that observed in formulations processed with flavoring.

Xylitol is a polyol which is widely used as a sweetener by the food industry, and like other polyols and disaccharides it plays a role in cells protection during the drying process and storage period (Carvalho et al., 2004). Studies relating the use of polyols to probiotic viability loss were not found.

Further studies are necessary to evaluate the *E. faecium* viability in relation to each ingredient present in lozenge formulations, so as to identify the ingredient involved in viability loss.

The material used for wrapping the lozenges is another important factor that may be related to the decrease of probiotic cells viability. Polyethylene presents an excellent barrier to water vapor, however, it is permeable to gases (Jorge, 2013). The passage of oxygen from the external environment to the interior of the package may have caused *E. faecium* viability loss in the lozenges. This interaction of oxygen with the probiotic strain probably was minimized during the storage of the microcapsules of *E. faecium* obtained by coacervation, considering that glass has excellent barrier properties and is impermeable to vapors and gases (Jorge, 2013).

The results accomplished in the present study were different from those of Toiviainen et al. (2014), who used xylitol and sorbitol tabletes as *Lactobacillus rhamnosus* and *Bifidobacterium lactis* vehicle, focusing on the oral microbiota of adults. The viability of such probiotic tablets was maintained at 8.0 log CFU/g, during the 4 weeks of treatment. Çaglar, Kuscu, Cildir, Kuvvetli, & Sandali, (2008) used isomalt lozenges as *Lactobacillus reuteri* vehicles, focusing on the oral microbiota of young women. According to the study, the lozenges viability was 8.0 log CFU/g during the 10 days of treatment.

3.3.1.2. Microbiological safety. Microbiological counts during the storage of lozenges remained below 5.0 log CFU/g for total mesophiles; 4.0 log CFU/g for yeasts and molds; and 3NMP/ml for total and thermotolerant coliforms. These results confirm the suitability of processing operations and conformity to the local microbiological patterns of the category (Anvisa, 2001).

3.3.2. Physicochemical evaluations

The results of physicochemical analyses of *diet* lozenges are shown in Table 3. The water activity, moisture and color values presented differences ($p < 0.05$) among formulations PC, PPP1 and PPP2. The lowest levels of water activity and moisture observed in formulations PPP1 and PPP2 are probably related to a lower water concentration (8.25 g/100 g), owing to the addition of the probiotic culture and inulin, which can reduce the water availability.

The water activity range suggested for lozenges is 0.40–0.75 (Bussiere & Serpelloni, 1985). It is known that water activity values around 0.80 present susceptibility to yeast and mold growth. Since food components are concentrated, values below 0.60 are recommended in order to avoid microbial development (Bobbio & Bobbio, 2001).

The addition of the probiotic strain and inulin made the lozenges darker. Color analysis results proved such a fact, since the control formulation (PC) exhibited higher averages for parameter L^* and lower ones for parameters a^* and b^* ($p < 0.05$).

3.3.3. Sensory evaluation

The sensory assessment of lozenges was conducted at the initial time of storage. As an unexpected rapid loss of viability was observed in formulations PPP1 and PPP2, it was not possible to conduct another sensory assessment. The acceptability and purchase intent are shown in Table 4 and Fig. 1, respectively.

All formulations had the attributes scores above 6.0, ranging from “liked slightly” and “liked moderately”, however, formulation PPP2 achieved 5.75 and 5.68 for the appearance and color. Consumers have declared a positive purchase intent (would certainly or probably buy the product), especially for PC and PPP2 formulations

Table 2
Viability of the *E. faecium* CRL 183 added to lozenges during the storage period.

Time (days)	PPP1	PPP2
0	$5.53 \pm 0.05^{a\ A}$	$4.83 \pm 0.01^{a\ B}$
7	$3.35 \pm 0.02^{b\ A}$	$4.15 \pm 0.01^{b\ B}$
14	$2.71 \pm 0.07^{c\ A}$	$4.01 \pm 0.01^{c\ B}$
21	$2.52 \pm 0.07^{d\ A}$	$3.30 \pm 0.07^{d\ B}$
28	$1.86 \pm 0.07^{e\ A}$	$1.05 \pm 0.08^{e\ B}$

Results are presented as means ± standard deviation, in log CFU/g.
Means in the same column followed by different lowercase letters are significantly different by the Tukey's test ($p < 0.05$).
Means in the same line followed by different capital letters are significantly different by the T-student test ($p < 0.05$).
PPP1 - Potentially probiotic formulation 1, with the E.faecium CRL 183.
PPP2 - Potentially probiotic formulation 2, with the E.faecium CRL 183 + inulin.

Table 3
Physicochemical characterization for the three lozenges formulations (PC, PPP1 and PPP2).

	PC	PPP1	PPP2
Water activity	0.79 ± 0.01^{A}	0.77 ± 0.02 ^{B}	0.75 ± 0.02 ^{C}
Moisture (%)	2.31 ± 0.07^{A}	2.12 ± 0.07 ^{B}	1.99 ± 0.06 ^{C}
pH	6.28 ± 0.08^{A}	6.25 ± 0.05 ^{A}	6.23 ± 0.07 ^{A}
Color	L* 92.77 ± 0.90 ^{A} a* −0.20 ± 0.07 ^{A} b* 1.30 ± 0.08 ^{A}	L* 85.84 ± 0.92 ^{B} a* 0.38 ± 0.09 ^{B} b* 7.00 ± 0.29 ^{B}	L* 88.64 ± 0.78 ^{C} a* 0.51 ± 0.09 ^{C} b* 8.13 ± 0.39 ^{C}

Results are presented as means ± standard deviation.
Means in the same line followed by different capital letters are significantly different by the Tukey's test ($p < 0.05$).
PC – Control formulation, without the probiotic strain.
PPP1 - Potentially probiotic formulation 1, with the *E.faecium* CRL 183.
PPP2 - Potentially probiotic formulation 2, with the *E.faecium* CRL 183 + inulin.
L* = luminosity, from black to white.
a* = from green to red.
b* = from blue to yellow.

Table 4
Sensory evaluation of lozenges formulations PC, PPP1 and PPP2.

	PC	PPP1	PPP2
Appearance	7.26 ± 1.43 ^{A}	6.11 ± 1.53 ^{B}	5.75 ± 1.51 ^{B}
Color	7.59 ± 1.2 ^{A}	6.13 ± 1.49 ^{B}	5.68 ± 1.48 ^{B}
Flavor	6.40 ± 1.25 ^{A}	6.32 ± 1.42 ^{A}	6.47 ± 1.56 ^{A}
Taste	6.18 ± 1.71 ^{A}	6.43 ± 1.67 ^{A}	6.97 ± 1.40 ^{B}
Texture	6.09 ± 1.79 ^{A}	6.23 ± 1.63 ^{A}	6.61 ± 1.44 ^{A}
Overall Acceptance	6.58 ± 1.49 ^{A}	6.21 ± 1.39 ^{A}	6.58 ± 1.21 ^{A}

Results are presented as means ± standard deviation.
Means in the same line followed by different capital letters are significantly different by the Tukey's test ($p < 0.05$), $n = 112$.
PC – Control formulation, without the probiotic strain.
PPP1 - Potentially probiotic formulation 1, with the *E.faecium* CRL 183.
PPP2 - Potentially probiotic formulation 2, with the *E.faecium* CRL 183 + inulin.

(48.6% and 62.2%, respectively).

Formulations PPP1 and PPP2 had inferior averages on appearance and color ($p < 0.05$) as compared to PC. Such result reflects the addition of microencapsulated and freeze-dried *E. faecium* which changes the lozenges color and could have been perceived as a defect. Flavor, texture and global impression had no differences among the formulations ($p < 0.05$). The addition of probiotic associated with inulin improved taste perception, indicating that the changes in appearance and color had no negative effect on this attribute. This was confirmed by the purchase intent, where formulation PPP2 had the lowest negative results, that is, 12.7% of consumers would not probably or certainly buy the product.

Satisfactory results have been found in recent studies with different categories of food products and the addition of probiotic microorganisms (Lalicic-Petronijevic et al., 2015; Pimentel, Madrona, & Prudencio, 2015). However, no studies relating the effect of probiotic bacteria addition on the sensory characteristics of lozenges or similar products, were found.

3.4. Anticariogenic potential evaluation

The *E. faecium* CRL 183 survival in saliva is shown in Table 5. The probiotic strain was able to propagate in saliva in the three forms evaluated: free cells, PPP1 and PPP2 lozenges formulations. The viable counts observed after 24 h increased 0.82 log CFU/ml in the free cells, whereas in formulations PPP1 and PPP2 they increased 3.64 and 3.31 log CFU/ml, respectively, showing that *E. faecium* CRL 183 resists human saliva. Haukioja (2009) evaluated the survival of

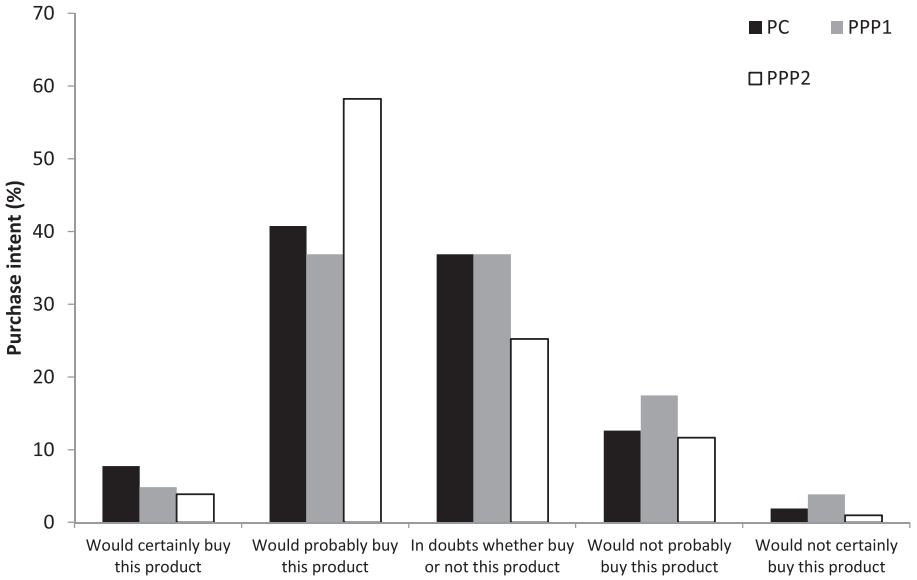

Fig. 1. Lozenges Purchase intent.
PC – Control formulation, without probiotic strain.
PPP1 - Potentially probiotic formulation 1, with *E. faecium* CRL 183.
PPP2 - Potentially probiotic formulation 2, with *E. faecium* CRL 183 + inulin.

Table 5
Viability of *E. faecium* CRL 183 inoculated in saliva.

	t = 0	t = 24 h
PPP1	5.45 ± 0.03 $^{a\ A}$	9.09 ± 0.02 $^{a\ B}$
PPP2	5.66 ± 0.06 $^{b\ A}$	8.97 ± 0.03 $^{b\ B}$
Inoculum	8.04 ± 0.06 $^{c\ A}$	8.86 ± 0.03 $^{c\ B}$

Results are presented as means ± standard deviation, in log CFU/g.
Means in the same column followed by different lowercase letters are significantly different by the Tukey's test ($p < 0.05$).
Means in the same line followed by different capital letters are significantly different by the T-student test ($p < 0.05$).
PPP1 - Potentially probiotic formulation 1, with the E.faecium CRL 183.
PPP2 - Potentially probiotic formulation 2, with the E.faecium CRL 183 + inulin.

various probiotic strains in saliva and all were viable after the inoculation, but none presented increases.

No studies were found in the literature reporting that the presence of xylitol could stimulate the probiotic multiplication, although, it is known that such ingredient can reduce the cell multiplication rate and acid production, leading to a reduction in the *S. mutans* population (Marsh & Martin, 2005).

The inhibition potential of *E. faecium* CRL 183 in relation to *S. mutans* was proved through the formation of a 1.7 ± 0.3 mm diameter halo (Fig. 2), showing that the probiotic strain inhibited *S. mutans* growth under these conditions, thus, reducing the dental caries risk.

Local and systemic effects of probiotic microorganism are related to the inhibition of some pathogenic strains, by the competition for binding sites, adhesion reductions, co-aggregation, production of organic acids, hydrogen peroxide and/or bacteriocin-like compounds and immune-modulation (Cagetti et al., 2013; Hassanzadazar, Ehsani, & Mardani, 2014). Redondo (2008) showed that *E. faecium* CRL 183 and *Lactobacillus helveticus* 416 have the ability of auto-aggregation (80.0%), co-aggregation (25.4%), besides inhibiting the *E. coli* 0157:H7 adhesion to intestinal cells Caco 2. The same strains did not produce antimicrobial substances in relation to different pathogenic strains (*E. coli* 0157:H7, *L. monocytogenes* V2 and *S. enteritidis*).

In the present study, it is not possible to state the exact mechanism involved in the inhibition of *S. mutans*, since specific tests have not been conducted. The anticariogenic effect should be better investigated through analyses involving the oral microbiota and biofilm formation.

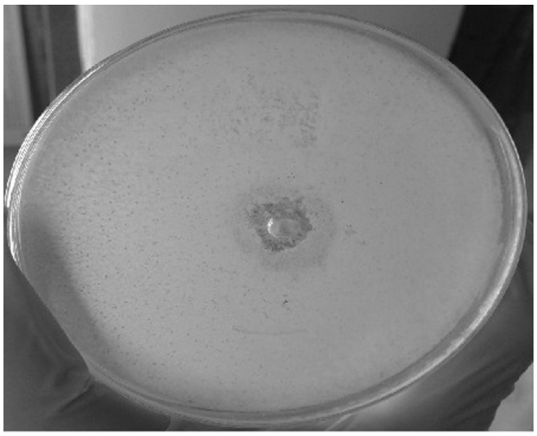

Fig. 2. Agar well plates diffusion assay, with the inhibition halo.
Indicator strain: (inoculated in the culture medium): *Streptococcus mutans* ATCC 25175.
Probiotic strain (inoculated in the well): *Enterococcus faecium* CRL 183.

4. Conclusions

The present study showed that the complex coacervation technique was efficient in preserving the *E. faecium* CRL 183 viability, at room temperature. However, the food matrix was not suitable for the probiotic veiculation, due to the severe loss of viability in formulations PPP1 and PPP2.

The probiotic strain investigated was able to survive and multiply in human saliva and also inhibit the multiplication of *Streptococcus mutans* ATCC 25175, in the study conditions.

The proposal of a diet probiotic lozenge with *E. faecium* CRL 183 and inulin is interesting, since it poses an alternative to the diversification of the segment and for aggregating anticariogenic potential, nevertheless, a process refinement and the use of gas resistant packaging to preserve the probiotic viability, is necessary.

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