MICROBIOLOGICAL-PHYSICOCHEMICAL ASSESSMENT AND GASTROINTESTINAL SIMULATION OF FUNCTIONAL (PROBIOTIC AND SYMBIOTIC) GOUDA-TYPE CHEESES DURING RIPENING

EVALUACIÓN MICROBIOLÓGICA, FISICOQUÍMICA Y SIMULACIÓN GASTROINTESTINAL DE QUESOS FUNCIONALES (PROBIÓTICO Y SIMBIÓTICO) TIPO GOUDA DURANTE LA MADURACIÓN

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Abstract
The objective of this research was to obtain a ripened gouda-type cheese added with probiotic bacteria (Bifidobacterium lactis) and a prebiotic ingredient (Nutriose®FB), evaluating its potential as a functional food. The probiotic bacteria were encapsulated by spray drying using reconstituted skim milk (RSM) and a mixture of β-cyclodextrin-arab gum (BC-AG) like encapsulant agents. Latterly, these ingredients were added during the production of gouda-type cheeses and microbiological as well as physicochemical evolution were assessed during ripening. At the end of this process, cheeses were subjected to gastrointestinal simulated conditions to evaluate probiotic survival. Results showed that the survival of microencapsulated cells was above \(10^{10}\) CFU·g\(^{-1}\) in the probiotic powder and it is maintained above \(10^{8}\) CFU·g\(^{-1}\) in the cheese, during all the ripening process (40 days). Furthermore, tests carried out on the final product indicate that the probiotic bacteria resist the gastrointestinal conditions, remaining viable at a high concentration (> \(10^7\) CFU·g\(^{-1}\)). An important aspect is that probiotic bacteria (Bifidobacterium lactis) and soluble fiber (Nutriose®FB) do not affected the physicochemical parameters of the gouda-type cheese. In conclusion, symbiotic gouda-type cheese obtained guarantees a beneficial effect to consumer, while keeps intrinsic physicochemical properties of the original cheese.

Keywords: ripened cheese, symbiotic food, microencapsulation, spray drying.

Resumen
Esta investigación tuvo como objetivo obtener un queso madurado tipo gouda adicionado con bacterias probióticas (Bifidobacterium lactis) y un prebiótico (Nutriose®FB) evaluando su potencial como alimento funcional. Las bacterias probióticas se encapsularon mediante secado por aspersión, empleando leche descremada reconstituida y una mezcla de β-ciclodextrina-goma arábica como agentes encapsulantes. Posteriormente, se adicionaron a un queso tipo gouda y se evaluó el comportamiento microbiológico y fisicoquímico durante la maduración. Al final de este proceso, se realizó una simulación gastrointestinal para evaluar la supervivencia de los probióticos. Los resultados mostraron que la supervivencia de las células microencapsuladas es superior a \(10^{10}\) UFC·g\(^{-1}\) en el polvo probiótico y se mantiene superior a \(10^8\) UFC·g\(^{-1}\) en el queso, durante los 40 días del proceso de maduración. Además, pruebas realizadas en el producto final, indican que el probiótico resiste las condiciones gastrointestinales permaneciendo viable en una alta concentración (> \(10^7\) UFC·g\(^{-1}\)). Un aspecto relevante es que las bacterias probióticas (Bifidobacterium lactis) y la fibra soluble (Nutriose®FB) no modificaron los parámetros fisicoquímicos del queso tipo gouda. En conclusión, se obtuvo un queso madurado simbiótico que garantiza un efecto benéfico al consumidor sin modificar las propiedades fisicoquímicas del queso original.

Palabras clave: queso madurado, alimento simbiótico, microencapsulado, secado por aspersión.

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1 Introduction

Several authors have shown the importance of achieving a high probiotic microorganism count at the beginning of the shelf life of the functional food. These counts must remain above $10^7 \, \text{CFU/g}$ throughout all its shelf life to exert the expected probiotic effect, once this has been ingested (Stanton et al., 2001; Kailasapathy, 2006; Masuda et al., 2005; Mendoza-Madrígal et al., 2017).

The use of dairy products, like cheese, as a functional food, enriched with probiotic microorganisms, is considered an attractive option to diversify functional foodstuff, with a great potential to improve consumers health (Lollo et al., 2015; Dantas et al., 2016). Cheese is a good alternative for probiotics delivery into the intestine and, as a result, has been the subject of marketing and research studies in recent years (Gomes da Cruz et al., 2009; Karimi et al., 2012b). Indeed, the addition of probiotic bacteria to cheese represents an added value to a product that provides health benefits, such as improvements in the immune system, oral and intestinal health in the elderly and reinforcement of intestinal immunity (Lollo et al., 2012; Albenzio et al., 2013).

Cheese has certain advantages as a carrier of probiotics compared to more acidic fermented dairy products such as yogurt. It creates a buffer against the high acidic environment in the gastrointestinal tract (GIT) and thus generates a more available environment for probiotic survival throughout gastric transit (Karimi et al., 2012a; Ortakci et al., 2012). As already reported by several authors, cheese is a promising food matrix for probiotics. Nevertheless, strain selection and possible process modifications should be carefully evaluated to maximize probiotic cell survival during cheese manufacture and ripening (Takanashi et al., 2014; Karimi et al., 2015).

In addition to the probiotic bacteria survival in cheese, it is important that the incorporation of these complementary bacteria does not affect the sensorial (flavor, texture, and appearance) or physicochemical characteristics of the conventional non-probiotic cheeses. This is particularly important for foodstuffs registered under a scheme of protected designation of origin (PDO), like several ripened cheeses all over the world. Therefore, it is necessary to compare the final characteristics of the functional products, as well as their evolution over time, when a new product is elaborated (Souza et al., 2008).

Despite the advantages of dairy products as vehicles of probiotics, usually their survival in this type of food has shown high variability. Even with numerous favorable results, it has been observed some limitations in the viability of probiotic bacteria due to the microenvironmental conditions. Indeed, the degree of survival of the microorganisms is mainly due to its sensitivity to stress caused by the characteristics of the food and environmental conditions during storage as well as preservation (Boylston et al., 2004).

In this context, the immobilization of probiotics using microencapsulation may improve the survival of these microorganisms in products, both during processing and storage, as well as during digestion process. The availability of spray-dried powders, protecting high numbers of viable probiotic microorganisms, is desirable for commercial applications. This process provides stability during shelf life storage and transportation of these cultures and their subsequent application in functional food development (Dianawati et al., 2016; Martín et al., 2015).

Ortakci et al. (2012) evaluated microencapsulation by spray drying as a method to supply probiotics to Mozzarella cheese and noted that probiotic survival improved, when the processing and storage conditions of the cheese are extremely stressful. However, this approach did not show an increase in the survival of probiotic bacteria in traditional feta cheese (Kailasapathy & Masondole, 2005). According to authors, this is probably due to open texture of cheese and dissolution of microcapsules. So, it is important to test another kind of encapsulant agents and to integrate microcapsules in suitable-texture ripened cheeses.

Besides, prebiotics incorporation into cheese could maintain or even increase the cell survival into the intestine. Indeed, prebiotics composed by non-digestible dietary components pass through the colon and selectively stimulate the proliferation and/or activity of desirable probiotic bacteria in-situ (Mattila-Sandholm et al., 2002). The addition of prebiotics, generally dietary fibers as inulin, resistant starch, pectin and beta-glucans, has induced the growth of probiotic bacteria. In this sense, Nutriose®FB, which is a dextrin obtained from wheat starch, represents a good alternative. In contrast to maltodextrin, Nutriose®FB is partially hydrolyzed and is not absorbed due to many $\alpha-1-6$ linkages as well as the presence of non-digestible glucoside linkages (e.g. $\alpha-1.2$ and $\alpha-1.3$) and is a predilect substrate to Bifidobacterium strains (LeFranc-Millot et al., 2009).
Unexpectedly, only few studies relate the potential of ripening cheese as a functional food, especially as a food matrix carrier for probiotic bacteria and prebiotic ingredients, characterizing them as a symbiotic food. In this context, this research has aimed to obtain a ripened gouda-type cheese as a functional food added with encapsulated probiotic bacteria (*Bifidobacterium lactis*) and a prebiotic ingredient (Nutriose® FB), evaluating its potential as probiotic and symbiotic food.

2 Materials and methods

2.1 Encapsulation of *Bifidobacterium lactis* by spray drying

2.1.1 Probiotic strain and culture preparations

Fresh cultures of *Bifidobacterium lactis* BLC1 (Sacco System Company, Italy) were obtained after activation by two successive transfers in Man-Rogosa-Sharpe broth (MRS)(DIFCO, Becton Dickinson and Co., USA) supplemented with 0.05% w/v of L-cysteine (Sigma-Aldrich) to generate anaerobic conditions. Cellular cultures in late log phase were centrifugated (Sigma 3-18K ASPELAB) at 3900 rpm, for 10 min and at 4 °C and washed (buffer phosphate water, pH 7.2). This process was repeated three times and the cell biomasses obtained were mixed with the carrier material in sterile conditions.

2.1.2 Preparation of solutions with carrier material

According to Table 1, a pre-established quantity of encapsulant agent was mixed and balanced to 100 g with sterile distilled water. The reconstituted skim milk (RSM) (DIFCO®, Becton Dickinson and Co., USA) was heat treated at 90 °C for 10 min to destroy pathogens. Prior to dissolution in water, β-cyclodextrin (BC) (Sigma®, Sigma-Aldrich Inc., USA) was mixed with arabic gum (AG) (EMPROVE®, Merck KGaA, Germany) in a ratio of 9:1 (w/w). Stirring was performed using a homogenizer (PRO260, Laboratory Homogenizer with Digital Speed Control, America Pro Scientific Company, USA) at 10000 rpm for 5 min. For each experiment, 93.8 mL of solution was mixed with 6 mL of biomass and 0.2 mL of Tween 60 was added, followed by a second stirring cycle at 5000 rpm for 10 min.

2.1.3 Encapsulation of *Bifidobacterium* by spray drying

Encapsulant solutions containing *Bifidobacteria* were dried using a laboratory spray dryer (BUCHI B-290, Switzerland) operated at set conditions (Table 1). The outlet temperature was 76±2 °C and the carrier solution was constantly agitated using a magnetic stirrer. The dry probiotic microcapsules were collected, placed in sealed glass vials, and stored at 25 °C.

2.1.4 Probiotic powder yield

The Probiotic Powder Yield (PPY) was determined considering the solids introduced into the fed solutions before spray drying treatment and the total powder recovered in the cyclone dryer. PPY was calculated using the next formula:

\[ PPY = \frac{P}{P_i} \times 100 \]  

where, \( P_i \) is the weight of dry solids fed in the spray dryer and \( P \) is the weight of final spray-dried powder obtained.

2.1.5 Morphology and particles size

The microcapsules were analyzed in a Jeol® scanning electron microscope SEM model JSM 6390 LV (Tokyo, Japan) at an accelerating voltage of 10 and 15 kV. Before introducing in SEM, the samples were placed on a piece of adhesive paper and were coated with gold with a vacuum sputtering coater Leica® model EM SCD 500 (Wetlar®, Germany) as described by Lian *et al.* (2002).

2.2 Pilot-scale functional cheese processing

To produce cheeses, a single batch of cow milk was pasteurized in a pilot plant at 72.5 °C during 30 min, cooled to 37 °C and transferred in vats for the cheese-making. Five batches of cheese-making were produced and were inoculated with a commercial starter culture mix (Lyofast MS 064 CP; Sacco System Company, Italy). All batches were inoculated with the commercial starter mix culture composed of *Streptococcus thermophilus*, *Lactococcus lactis* biovar diacetylactis and *Lactococcus lactis* subsp. *lactis* (counter ratio 100:1) incubated after 30 minutes and rennet (Chr Hansen, Cuamex®) were added into the milk at a strength of 1:10000 (75% chymosin, 25% pepsin). After 30 minutes, the curd was cut, stirred for 10 minutes, drained from the whey and placed
into molds. The obtained cheeses (250 g) were drained under pressure during 24 h (25 °C, 90% relative humidity RH) and then brine salted (21% w/w NaCl aqueous solution at 9 °C during 24 h). Cheese ripening was carried out through 40 days at 9 °C and 75% RH into a cooling chamber. Cheese samples were collected at 1, 10, 20, 30, 40 days for further analysis and sampling was monitored by duplicate.

To investigate the effect of encapsulate agent as well as the presence of a prebiotic over the probiotic bacteria in the gouda-type cheese, different combinations were prepared. In the case of probiotic (batches 2 and 4) and symbiotic cheeses (batches 3 and 5), additional ingredients were incorporated before molding, as follows: probiotic cheeses were enriched with 1% w/w of BC-GA and RSM probiotic powders, respectively; in the case of symbiotic cheeses, 1% w/w of probiotic powder and 1% w/w of prebiotic ingredient (Nutriose® FB) were added. Finally, for the control cheese (batch 1) none additional ingredient was incorporated.

### 2.3 Cell survival extraction and counting

Cheese samples (10 g) were suspended in phosphate buffer solution (90 mL, pH 7.2)) and were shaken at room temperature to ensure a complete dispersion. The use of a disperser-homogenizer (Polytron® PT1200 Carl Roth, Germany) at 15000 rpm for 2 min ensures total release of immobilized probiotic bacteria from microcapsules, as reported in literature (Doherty et al., 2010). Control cheese samples were subject to the same conditions to keep consistent treatment conditions. Serial decimal dilutions were prepared from the initial suspension and following the Miles-Misra technique 20 µL drops were inoculated by triplicate on the surface of MRS agar, added with 0.05% L-cysteine Sigma-Aldrich®, (Picot & Lacroix, 2004). Plates were incubated at 37 °C for 48 h under anaerobic conditions and subsequently the number of colonies was counted. Results are expressed as a logarithm of colony-forming unit per gram (log<sub>10</sub>CFU·g<sup>−1</sup>).

### 2.4 Survival of encapsulated and free bacteria during in-vitro digestion

The ability for in-vitro survival of encapsulated and free cells was compared under digestion conditions, simulating the human gastric and intestinal environments. The method was based on a previous published procedure (Picot & Lacroix, 2004). The transit tolerance of the probiotic cultures was determined by exposing the microorganisms at 37 °C to simulated gastric juice for 30 min (pH 1.9) and simulated small intestinal juice for 6 h (pH 7.5), successively. Then, changes in total viable counts in the digestion mixture were periodically monitored.

Simulated gastric juice was prepared by dispersing pepsin 1.60000 (porcine gastric mucosa P7012, Sigma Aldrich®) in HCl 0.1N (Hycl®) and adjusting the pH to 1.9 with NaOH 1N (Hycl®), in order to achieve a final pepsin concentration in the digestion mixture of 0.26 g·L<sup>−1</sup>. Simulated pancreatic juice was prepared by dispersing pancreatin (Hycl®) in sterile sodium phosphate buffer (0.02M, pH 7.5) and adjusting the pH to 7.5 with NaOH 1N in order to achieve a final pancreatin concentration in the digestion mixture of 1.95 g·L<sup>−1</sup>. Concentrated bile salt solution (150 g·L<sup>−1</sup>) was prepared by dissolving a bile extract powder (bile bovine B3883, Sigma Aldrich®) in distilled water. The resulting suspension was filtered and sterilized. The simulated gastric juice, bile salt solution and pancreatic juice were prepared just before use to avoid eventual degradation.

A cheese sample of 1.0 g was mixed with a phosphate aqueous solution (9 mL) and transferred aseptically to a 125 mL Erlenmeyer flask; temperature was maintained at 37 °C using a stirred water bath. Then, 5 mL of pepsin solution was added and after 30 min the reaction was stopped by increasing the pH to 7.5 using NaOH 1N. A sample of 1 mL was withdrawn and kept in ice before quantification of viable cells. Next, 5 mL of concentrated sodium phosphate buffer (0.5 M, pH 7.5) and 2.0 mL of bile salt solution were added. After adjusting the pH to 7.5 and the volume to 20 mL with sterile distilled water, 10 mL of the pancreatic juice was added for a final volume of 25 mL. At different times (1, 2, 3, and 6 h), 1 mL aliquots were removed, and the reaction was stopped by placing the samples in ice for 5 min. Formerly to bacteria enumeration, the sample was submitted to a process of releasing cells from microcapsules, as described in section 2.3. Survival at each stage is expressed on log<sub>10</sub>CFU·g<sup>−1</sup> of cheese. During all experiments the pH was monitored, and no changes were observed.

### 2.5 Compositional analysis

Milk samples were analyzed for lactose, protein and fat contents by using a Milko Scan™ FT 120 (Foss®, Padova, Italy). As explained before, cheese samples were collected at 1, 10, 20, 30 and 40 days for both
microbiological and chemical analysis. Furthermore, one entire cheese was used for each sample. Cheese samples during manufacturing and ripening were analyzed for moisture using the oven drying method at 102 °C (IDF, 1982), salt content using a titration method with AgNO₃ (IDF, 1988), total protein and pH 4.6-soluble nitrogen using the Kjeldhal method (IDF, 1993), and fat content using the FIL-IDF Standard 5A method (IDF, 1997). The pH was determined with a Portamess pHmeter (Knick®, Berlin, Germany), placing the measurement electrode into the sample bulk. All analyses were performed by triplicate.

The Nutriose®FB concentration in the final product was obtained using the AOAC method 991.43 “Total, soluble, and insoluble dietary fiber in foods” and the AOAC method 32-07.01 “Determination of soluble, insoluble, and total dietary fiber in foods and food products” (AOAC, 2000).

### 2.6 Sanitary quality of functional cheeses

The sanitary quality of cheeses was evaluated. Molds and yeasts were determined according to the methodology proposed in Mexican Official Norms (NOM-111-SSA-1-1994), total coliform organisms and E. coli were carried out using a Petrifilm E.C. method (3M, approved AOAC official Method No. 2003.07), Salmonella spp. was determined by 1-2 test (Biocontrol Systems, approved AOAC official method No. 989.13) and S. aureus was determined by the Staph Express Count Plate (3M, approved AOAC official method No. 989.13).

### 2.7 Statistical analysis

Cell counts were converted to log₁₀ CFU·g⁻¹ or log₁₀ CFU·mL⁻¹ for statistical analysis. Moreover, to compare the survival of Bifidobacterium population after the simulated gastrointestinal digestion, microbiological and chemical analyses results were expressed as mean values of two independent experiments and standard deviations were calculated. Statistical analyses were performed with Statgraphics 7.0 (StatSoft Inc., Tulsa, OK, USA), using the Tukey’s honest significant difference test with a significance level of p = 0.05 in all assays.

## 3 Results and discussion

Table 1 shows the results obtained for the probiotic microencapsulation. Bifidobacterium lactis was encapsulated using BC-AG and RSM, achieving viabilities of 1.89±0.25×10¹¹ CFU·g⁻¹ and 1.98±0.38×10¹⁰ CFU·g⁻¹ as well as powder percentage yield of 77.53±1.2 and 63.26±1.8, respectively. Additionally, the microcapsules exhibited water activity (aₜ) values below 0.25 in both cases (0.215±0.004 and 0.190±0.007, respectively). Moreover, low aₜ values and low residual moisture contents (< 4 – 5% w/w) are prerequisites for the commercial production of spray-dried powders with good handling characteristics such as high flow ability, low stickiness and agglomeration as well as maximum probiotics survival (Barbosa-Canovas, 2005; Borges et al., 2017). At conditions of low aₜ, the matrix moves from the rubbery state toward the glassy state and water mobility is reduced consequently. This phenomenon inhibits cell metabolic activity of the bacterial cells, leading to extended shelf-life.

Figure 1 shows the SEM micrographs of the Bifidobacterium microcapsules obtained using RSM and BC-AG as encapsulant agent.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>AIT (°C)</th>
<th>AIF (mL·min⁻¹)</th>
<th>CM (%)</th>
<th>CMC</th>
<th>LAB</th>
<th>CFU·g⁻¹dm before spray drying</th>
<th>CFU·g⁻¹dm after spray drying</th>
<th>aₜ</th>
<th>PPY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>160</td>
<td>5</td>
<td>BC-AG</td>
<td>15</td>
<td>BL</td>
<td>3.79±0.15×10¹¹</td>
<td>1.89±0.25×10¹¹</td>
<td>0.215±0.004</td>
<td>77.53±1.2</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>5</td>
<td>RSM</td>
<td>20</td>
<td>BL</td>
<td>5.33±0.43×10¹²</td>
<td>1.98±0.38×10¹⁰</td>
<td>0.190±0.007</td>
<td>63.26±1.8</td>
</tr>
</tbody>
</table>

AIT=air inlet temperature; AIF=air inlet flow rate; CM=core material; CMC=core material concentration; (LAB=lactic acid bacteria, BL=B. lactis); PPY=powder percentage yield; dm=dry material.
Fig. 1A reveals the absence of free bacteria, confirming the formation of probiotic microcapsules. The particles showed a pseudo-spherical shape and dispersed sizes, with typical concavities of microcapsules produced by spray drying. Sáenz et al. (2009) reported that the formation of concavities in the surface of atomized particles can be attributed to the rapid evaporation of liquid drops. The size of the microcapsules obtained is in a range from 7 to 15 µm. Such values are accepted for microcapsules obtained through spray drying, which vary from 10 to 100 µm, according to Fang and Bhandari (2010). Similar results were obtained for microcapsules obtained using BC-AG mixture (Figure 1C).

Figure 1B shows the Bifidobacterium lactis bacteria trapped into the capsules, released after microcapsule rupture by sonication. As expected, Bifidobacterium lactis exhibits a size between 1.7 and 2.2 µm.

The average composition characteristics of raw cow’s milk used in making cheese were: density 1.029 g·mL⁻¹, fat content 4.3%, lactose content 4.5% and total protein content 3.8%. These compositional results are typical for cow milk (Muehlhoff et al., 2013).

The initial survival and evolution during ripening of B. lactis obtained for cheese batches are shown in Figure 2. The results of this study, corresponding to the counts of B. lactis, showed in all cases a decrease (~1 log-cycle), during the first 15 days. Later, probiotics cells survival remains almost constant until the end of cheese ripening process. Besides considering standard deviations, no significant differences were observed for different treatments at the end of the ripening process.
According to several reports (Stanton et al., 2001; Kailasapathy, 2006; Masuda et al., 2005), the count of probiotic cells in a foodstuff should be approximately $10^9$ CFU·g$^{-1}$, to provide a health beneficial effect. So, a daily ingestion of $10^8$-$10^9$ CFU for each portion is recommended. In this study cheese exhibit an initial survival nearby $10^9$ CFU·g$^{-1}$ and no significant differences ($p < 0.05$) were found for samples with or without Nutriose®FB, during the ripening process.

Özer et al. (2008) observed the positive effects of microencapsulation, on viable counts of *B. bifidum* Bb-12 in Kasar-type cheese, these results show

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Table 2. Physicochemical properties of gouda-type cheese during ripening. (BC-AG=β-cyclodextrin arabic gum mixture; RSM=reconstituted skim milk; NUT=Nutriose®FB).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Treatment</th>
<th>Initial</th>
<th>10 days</th>
<th>20 days</th>
<th>30 days</th>
<th>40 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>Control</td>
<td>5.85±0.33$^{aA}$</td>
<td>5.78±0.01$^A$</td>
<td>5.90±0.04$^A$</td>
<td>5.80±0.01$^A$</td>
<td>5.89±0.04$^A$</td>
</tr>
<tr>
<td></td>
<td>BC-AG</td>
<td>5.79±0.06$^{aA}$</td>
<td>5.75±0.04$^A$</td>
<td>5.79±0.07$^A$</td>
<td>5.79±0.07$^A$</td>
<td>5.76±0.07$^A$</td>
</tr>
<tr>
<td></td>
<td>BC-AG+NUT</td>
<td>5.12±0.07$^{aA}$</td>
<td>5.14±0.03$^A$</td>
<td>5.44±0.05$^A$</td>
<td>5.21±0.10$^A$</td>
<td>5.37±0.07$^A$</td>
</tr>
<tr>
<td></td>
<td>RSM</td>
<td>5.49±0.12$^{aA}$</td>
<td>5.47±0.07$^A$</td>
<td>5.75±0.04$^A$</td>
<td>5.59±0.05$^A$</td>
<td>5.52±0.40$^A$</td>
</tr>
<tr>
<td></td>
<td>RSM+NUT</td>
<td>5.53±0.02$^{aA}$</td>
<td>5.46±0.02$^A$</td>
<td>5.71±0.02$^A$</td>
<td>5.48±0.02$^A$</td>
<td>5.69±0.03$^A$</td>
</tr>
</tbody>
</table>

| Moisture (%) | Control           | 4.45±0.93$^{aA}$ | 5.80±0.13$^A$ | 6.36±0.04$^A$ | 6.63±0.09$^A$ | 6.25±0.12$^A$ |
| Moisture (%) | BC-AG             | 4.45±0.93$^{aA}$ | 5.80±0.13$^A$ | 6.36±0.04$^A$ | 6.63±0.09$^A$ | 6.25±0.12$^A$ |
| Moisture (%) | BC-AG+NUT         | 57.78±0.90$^{aA}$ | 61.65±0.42$^A$ | 62.81±0.32$^A$ | 62.59±0.10$^A$ | 66.01±0.74$^A$ |
| Moisture (%) | RSM               | 56.32±1.31$^{aA}$ | 58.11±1.39$^A$ | 59.41±0.67$^A$ | 59.14±2.81$^A$ | 64.81±1.71$^A$ |
| Moisture (%) | RSM+NUT           | 57.97±2.17$^{aA}$ | 59.82±0.38$^A$ | 62.16±0.75$^A$ | 62.98±0.72$^A$ | 66.72±0.38$^A$ |

| Ash (%)    | Control           | 4.45±0.93$^{aA}$ | 5.80±0.13$^A$ | 6.36±0.04$^A$ | 6.63±0.09$^A$ | 6.25±0.12$^A$ |
| Ash (%)    | BC-AG             | 4.25±0.21$^{aA}$ | 5.60±0.02$^A$ | 5.34±0.06$^A$ | 5.49±0.12$^A$ | 5.76±0.05$^A$ |
| Ash (%)    | BC-AG+NUT         | 3.82±0.02$^{aA}$ | 4.31±0.04$^A$ | 4.04±0.15$^A$ | 4.09±0.10$^A$ | 4.47±0.08$^A$ |
| Ash (%)    | RSM               | 3.60±0.08$^{aA}$ | 5.37±0.07$^A$ | 5.17±0.07$^A$ | 5.62±0.18$^A$ | 5.57±0.17$^A$ |
| Ash (%)    | RSM+NUT           | 4.85±0.15$^{aA}$ | 5.03±0.06$^A$ | 5.84±0.06$^A$ | 5.12±0.06$^A$ | 5.83±0.17$^A$ |

| Fat (%)    | Control           | 22.55±0.71$^{aA}$ | 23.00±0.50$^A$ | 22.50±0.71$^A$ | 23.00±1.41$^A$ | 23.00±0.75$^A$ |
| Fat (%)    | BC-AG             | 21.25±1.60$^{aA}$ | 23.25±0.35$^A$ | 23.50±0.70$^A$ | 24.00±0.50$^A$ | 24.37±1.19$^A$ |
| Fat (%)    | BC-AG+NUT         | 21.75±0.35$^{aA}$ | 22.00±0.50$^A$ | 22.00±1.41$^A$ | 24.50±1.71$^A$ | 23.75±0.45$^A$ |
| Fat (%)    | RSM               | 22.25±0.35$^{aA}$ | 22.25±1.06$^A$ | 23.00±0.70$^A$ | 22.00±1.41$^A$ | 22.50±1.10$^A$ |
| Fat (%)    | RSM+NUT           | 22.00±1.41$^{aA}$ | 21.50±1.12$^A$ | 22.50±0.71$^A$ | 22.50±0.05$^A$ | 23.00±0.80$^A$ |

| Proteins (%) | Control          | 21.05±1.20$^{aA}$ | 19.08±1.00$^A$ | 20.05±1.40$^A$ | 19.55±1.00$^A$ | 21.50±1.30$^A$ |
| Proteins (%) | BC-AG            | 20.15±1.50$^{aA}$ | 21.15±1.50$^A$ | 20.81±1.30$^A$ | 21.50±1.60$^A$ | 22.40±1.20$^A$ |
| Proteins (%) | BC-AG+NUT        | 18.27±1.30$^{aA}$ | 19.72±1.30$^A$ | 20.81±1.50$^A$ | 19.97±1.30$^A$ | 21.00±1.40$^A$ |
| Proteins (%) | RSM              | 20.83±1.60$^{aA}$ | 19.81±1.80$^A$ | 21.38±1.90$^A$ | 22.10±1.40$^A$ | 21.95±1.60$^A$ |
| Proteins (%) | RSM+NUT          | 22.05±1.50$^{aA}$ | 23.50±1.40$^A$ | 24.50±1.50$^A$ | 24.00±1.40$^A$ | 24.50±1.20$^A$ |

Results are averages of three replicates.

At initial time, the mean values denoted by the same lowercase letters in each column (within investigated formulation) are not significantly different ($p < 0.05$; $n = 4$).

In each row, the mean values denoted by the same capital letters (within investigated ripening time evolution) are not significantly different ($p < 0.05$; $n = 4$).

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that addition of encapsulated probiotics, into the cheese, enhances probiotic survival during ripening and storage. Similar results are shown by Hernández-Rodríguez et al. (2017), who demonstrated that entrapped L. plantarum cells within coacervates, incorporated in yogurt, did not suffer loss of survival during 21 days of refrigeration storage time, contrasting with high survival losses of free cells.

In addition to probiotic survival in the product until its consumption, survival after exposure to gastrointestinal tract (GIT) conditions is also crucial. Food matrices possess significant effects in successful delivery of probiotics into the intestine compared to delivery of these microorganisms via a liquid carrier or suspension (Abadía-García et al., 2013). Figure 3 shows results obtained under GIT simulated conditions for all treatments. Results for the control represent the counting for starter mix culture, while for the probiotic and symbiotic cheeses represent only the counting for Bifidobacterium lactis added in microcapsules. For it, colonies were discriminated during the counting on behalf of their appearance. As it can be seen, free starter mix culture do not resist under GIT conditions, obtaining concentration below 10^6 CFU·g^-1 at the first stage of GIT simulation and even below 10^5 CFU·g^-1 at the end of all GIT phases. In contrast, the cheeses enriched with encapsulated B. lactis using different encapsulant agents (BC-GA and MRS) and Nutriose®FB presented an increase of resistance under GIT conditions, with a final concentration from 10^7 to 10^9 CFU·g^-1. These results confirm, on the one hand, the effectiveness of the microencapsulation and, on the other hand, the almost complete release of the encapsulated bacteria.

Fig. 3. Survival of free bacteria (control) and probiotic bacteria in formulated cheeses under gastrointestinal simulated conditions (gj=gastric juice at pH 1.9; pj=pancreatic juice at pH 7.5; RSM=reconstituted skim milk; BC-AG= β-cyclodextrin arabic gum mixture).

In fact, the orders of magnitude obtained in the systems enriched with probiotic powder correspond to the quantities added at the beginning of the process (∼10^8-10^9 UFC·g^-1). It is important to note that the final concentration of viable free cells in the control cheese, at the end of the GIT simulation, is negligible compared to that of encapsulated cells (10^5 vs. 10^7-9 UFC·g^-1). Furthermore, RSM microcapsules showed more stability until the end of GIT conditions, while BC-GA showed a reduction of 2 logarithmic-cycles. Nevertheless, all enriched cheeses keep their probiotic character throughout the process. As probiotic and symbiotic cheeses showed similar results for each encapsulant agent, it can be established that prebiotic Nutriose®FB only acts as an ingredient and do not promote probiotic proliferation during the ripening process.

Table 3. Fiber analysis obtained at the end of ripening period for different formulated cheeses (BC-AG=β-cyclodextrin arabic gum mixture; RSM=reconstituted skim milk).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insoluble fiber</th>
<th>Soluble fiber</th>
<th>Total fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g·100 g^-1 cheese</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.05±0.01a</td>
<td>2.08±0.16a</td>
<td>2.13±0.17a</td>
</tr>
<tr>
<td>BC-GA</td>
<td>0.48±0.08b</td>
<td>3.42±0.04a</td>
<td>3.91±0.05a</td>
</tr>
<tr>
<td>RSM</td>
<td>0.20±0.11a</td>
<td>3.63±0.05a</td>
<td>3.83±0.17a</td>
</tr>
<tr>
<td>BC-GA+Nutriose®FB</td>
<td>0.30±0.26b</td>
<td>8.81±0.82b</td>
<td>9.11±1.08b</td>
</tr>
<tr>
<td>RSM+Nutriose®FB</td>
<td>0.05±0.04a</td>
<td>8.31±0.41b</td>
<td>8.36±0.37b</td>
</tr>
</tbody>
</table>

The mean values denoted by the same letters in each column (within investigated formulation) are not significantly different (p < 0.05; n = 4).
Table 4. Initial and final microbiological analysis for different formulated gouda-type cheeses during ripening (BC-AG=β-cyclodextrin arabic gum mixture; RSM=reconstituted skim milk).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E. coli (CFU/g)</th>
<th>Molds (CFU/g)</th>
<th>Yeasts (CFU/g)</th>
<th>Coagulase-Positive Staphylococcus (CFU/g)</th>
<th>Salmonella spp. (In 25 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial (day 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;10</td>
<td>48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>BC-AG</td>
<td>&lt;10</td>
<td>47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>BC-GA+Nutriose@FB</td>
<td>&lt;10</td>
<td>51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>RSM</td>
<td>&lt;10</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>RSM+Nutriose@FB</td>
<td>&lt;10</td>
<td>47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>Final (40 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;10</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>BC-AG</td>
<td>&lt;10</td>
<td>17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>BC-GA+Nutriose@FB</td>
<td>&lt;10</td>
<td>29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>RSM</td>
<td>&lt;10</td>
<td>21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
<tr>
<td>RSM+Nutriose@FB</td>
<td>&lt;10</td>
<td>19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10 Absent</td>
<td></td>
</tr>
</tbody>
</table>

The mean values denoted by the same letters in each column (within investigated formulation) are not significantly different (p < 0.05; n = 4).

Similar results were obtained by Nejati et al. (2011) for a symbiotic ultra-filtrated (UF) cheese, enriched with encapsulated Bifidobacterium lactis Bb-12 and inulin as prebiotic. Their results showed that encapsulation can significantly increase the survival rate of probiotic bacteria during the cheese shelf life as well as in-vitro GIT simulated conditions, while the addition of inulin had no effect on these parameters. In Table 2, the comparison of the results of the statistical analysis applied to the initial time of the different treatments shows a significant difference in some parameters (pH, acidity and proteins). However, they can be attributed to the inherent variability of the manufacturing process since lots of cheeses were produced for each treatment.

In addition, the small differences found are within the ranges of variability found in commercial Gouda cheeses (Bazaes, 2004; Ameerally, 2015). Based on this, to analyze the evolution during the ripening, the results for each physicochemical property were contrasted as a function of time in each treatment. In this way, no significant differences were observed at the different times analyzed.

3.1 Determination of fiber content (Nutriose®FB)

The addition of Nutriose®FB did not show significant effect (p < 0.05) on the physicochemical properties of symbiotic cheeses as shown in Table 2. However, comparing the results of the total fiber content, as expected, the addition of prebiotic Nutriose®FB had a significant effect (p < 0.05) (Table 3). These symbiotic cheeses showed the highest content of soluble and total fiber. So, it can be hypothesized that prebiotic was not metabolized during the ripening process. Hence, it will be available for probiotic bacteria during the gastrointestinal process, fulfilling in this way its prebiotic character.

3.2 Sanitary quality of functional cheese

The microbiological analysis results for Salmonella spp., Escherichia coli, Staphylococcus aureus as well as molds and yeast are shown in table 4. The functional cheeses (probiotic and symbiotic) were analyzed at the beginning and end of the ripening process. The analysis showed a low microbiological contamination.
In the industrial dairy products, the pathogens are generally contained in raw milk. Then, they may colonize the environment as well as equipment or final products (Kousta et al., 2010). The concentration of all pathogen microorganisms in the different cheeses (Table 5), was below the maximum allowable limits considering the official Mexican regulations for dairy products (NOM-243-SSA1-2010) for each microorganism evaluated, which means that the milk was pasteurized properly and the good manufacturing practices (GMP) were respected. Similar results were obtained by Zamora-Vega et al. (2012), they developed and characterized a functional food (fresh cheese) incorporating a probiotic microorganism (Sacharomyces boulardii) and a prebiotic agent (inulin), both encapsulated with sodium alginate and cactus mucilage. The results obtained showed that sanitary quality of symbiotic cheese is not modified by the addition of probiotic bacteria or symbiotic compounds, if the GMP are respected.

Conclusions

Functional matured cheeses with physicochemical and sanitary characteristics similar to a conventional gouda-type cheese were obtained. Spray drying by microencapsulation using different encapsulant agents (RSM, BC-AG) allowed to obtain a high probiotic survival \((>10^8 \text{ CFU g}^{-1}\text{cheese})\) after the ripening period as well as all the stages of gastrointestinal simulated conditions \((>10^7 \text{ CFU g}^{-1}\text{cheese})\). In addition, enriched cheeses with Nutriose®FB (symbiotic cheeses) exhibited the highest concentration of soluble fiber, characteristic greatly desirable in functional foods. Therefore, this research proved the stability of the elaborated microcapsules, which effectively protected the probiotic microorganism but also do not affect the main characteristics of this food, which establishes the possibility of producing functional cheeses without changes that affect the acceptance by the consumer.

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References


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