# Survival rate of *Saccharomyces boulardii* adapted to a functional freeze-dried yogurt: experimental study related to processing, storage and digestion by Wistar rats

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## ABSTRACT

**Background:** *Saccharomyces boulardii* is a probiotic clinically effective in the prevention and treatment of antibiotic induced diarrhea in both children and adults, *Clostridium difficile* infections, inflammatory bowel disease, and other gastrointestinal disorders. However, the microorganisms need to survive the gastrointestinal transit and arrive to their action site alive in order to exert their beneficial effects. Microencapsulation is an alternative to improve the viability of probiotic in foods which can also survive in the gastrointestinal conditions. Freeze-drying is a method of dehydration that does not affect nutrients and bioactive compounds, such as probiotics contained in foods. All of them will increase the survival rate of *S. boulardii*.

**Purpose of this study:** This study focused on formulae freeze-dried yogurt containing inulin, vegetable palm oil, and *S. boulardii*, both as free cells and in microencapsulated form. Also, the effect of ampicillin associated *S. boulardii*.

**Methods**. Yogurts were given to an "in vivo" digestion process, using male Wistar rats. The survival of *S. boulardii* was subsequently evaluated in colon and feces. For this study, six treatments of four of rats were used: i) control rats ii) rats fed with yogurt containing *S. boulardii* as free cells, iii) rats fed with yogurt containing *S. boulardii* in micro-encapsulated form, iv) control rats fed with penicillin, v) rats fed with ampicillin plus yogurt containing *S. boulardii* as

free cells, and vi) rats fed with penicillin plus yogurt containing *S. boulardii* in micro-encapsulated form.

**Results:** The study demonstrated it was feasible to freeze-dry the *S. boulardii* and incorporate it into a yogurt made with skim milk, inulin, and unsaturated vegetable oil. The freeze-drying process not affected the survival of the *S. boulardii* ($p < 0.05$). Microencapsulation increased the survival of *S. boulardii* on 1.77-Log CFU/g, and the presence of *S. boulardii* was only detected in colon and feces of those rats which ingested ampicillin, regardless to the formula contained the probiotic.

**Conclusion:** This study demonstrated that freeze-drying maintains the survival of *S. boulardii* in the evaluated foods and that micro-encapsulation increases the survival of this probiotic. Furthermore, *S. boulardii* was installed in the gastrointestinal tract when the microbial flora was damaged by ampicillin.

**Keywords:** Yogurt, probiotic, *Saccharomyces boulardii*, micro-encapsulation, freeze-drying.

## INTRODUCTION

Functional food provides one or more substances that contribute to human health and some of them are found naturally and can prevent ailments and chronic diseases related to diet [1, 2, 3]. These sources include the following: carrots (carotenoids), tomato (lycopene), tea (phenolic compounds), fruit and vegetables (fiber, phytochemicals) [4, 5], vegetable oils (omega-3 and omega-6), and various grains and cereals (fiber, phenol compounds, and phytochemicals). Some research has developed new functional foods based on the study of microorganism probiotics, in order to provide their biological activity and potentially beneficial effects on health — particularly as preventive measure for diarrhea, constipation, and treatment of colitis [6].

The term probiotics is derived from a Latin meaning "for life" [7]. The definition accepted by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are "Live microorganisms which, when administered in adequate doses amounts, confer benefit to the health of the host" [8]. Ideal criteria for probiotics are: i) survival of passage through the gastro-intestinal tract, ii) tolerance to acid, bile, gastric enzymes, and iii) adherence and colonization of the intestinal epithelium [9]. Lactobacilli and bifidobacterial strains are the most popular probiotics for human consumption because they improve both health and intestinal symptoms of lactose intolerance, in addition to reducing the risk of other diseases [10]. Yeasts such as *Saccharomyces boulardii* are also marketed as probiotics [11]. It has been estimated that probiotics comprise between 60% and 70% of functional foods currently available on the market [12]. The active mechanism of *S. boulardii* include: regulation of intestinal microbial homeostasis, interference with the ability of pathogens to colonize and infect the mucosa, modulation of local and systemic immune responses, stabilization of the gastro-intestinal barrier, inhibition of pro-carcinogenic enzymes, and finally induction of enzyme activity which promotes the absorption of nutrients [13]. *S. boulardii* is currently used as a probiotic in the treatment of

antibiotic induced diarrhea in both children and adults, *Clostridium difficile* infections, inflammatory bowel disease, and other gastrointestinal disorders [14].

Yogurt is one of the most popular fermented milk products worldwide, as it has many health benefits, including the reduction of lactose intolerance, lowering of the risk of certain types of cancer, anti-cholesterolemic effects, and prevention of genital and urinary tract infections, effects which are combined with other health attributes associated with probiotic bacteria such as: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* [15].

The aim of this study was to know the effect in survival rate of *S. boulardii* prepared as freeze-dried yogurt prepared with skimmed-milk. Palm vegetable oil was added to replace milk fat in order to obtain yogurt with a lower saturated fatty-acid content and to increase poly-unsaturated fatty acids, in addition to removing cholesterol from the product. Inulin was used to improve the consistency and texture of the yogurt. Another goal was to use *S. boulardii*, either as free cells or microencapsulated form. The yogurt was freeze-dried as to increase the formerly accepted shelf-life of 21 days [16], facilitate transport (stored at an ambient temperature of 25°C, 84 days) and rehydrate for consumption. We have characterized the physicochemical, microbiological, and sensory properties of re-hydrated yogurts. The survival of *S. boulardii* in yogurt was measured, not only by the effects of freeze-drying and storage at 25 °C but also by passage through the gastro-intestinal tract of Wistar rats. Finally, we tested the addition microbial flora, which had been damaged by the addition of ampicillin; yogurt containing *S. boulardii* was also given to the rodents in order to observe its capacity to restore flora to the colon.

## MATERIALS AND METHODS

Milk with 0.2% fat content. The *L. delbrueckii* subspecies *bulgaricus* and *S. thermophilus* subspecies *salvarius* (Danisco®) were used. *S. boulardii* was provided by the Laboratory of Microbiology of the Faculty of Chemical Pharmacobiology (Universidad Michoacana de San Nicolas de Hidalgo). Inulin from the *Agave tequilana* subspecies *weber* blue variety and vegetable oil were employed. Inulin (PubChem CID: 24763); Calcium carbonate (PubChem CID: 10112); Sodium alginate (PubChem CID: 5102882); Ampicillin (PubChem CID: 6249); Oleic acid (PubChem CID: 445639); Linoleic acid (PubChem CID: 5280450); α-Linolenic acid (PubChem CID: 5280934).

## Biomass production

*S. boulardii* was inoculated in a Sabouraud Dextrose Broth (BIXON, Mexico) and incubated in a bath (Lab-line 3540 brand) at 25 °C, being constantly stirred at 150 rpm over a period of 72 h, counts were conducted every 12 hours (0, 12, 24, 36, 48, 60, and 72). The survival rate of *S. boulardii* was evaluated by means of the spread-plate counting method.

Enumeration of probiotic. The samples were diluted, i.e. 1 g of sample in 9 ml sterile peptone water and then in a series of gradually less potent dilutions was obtained to $10^{10}$, an aliquot of 1 ml of appropriate decimal dilutions were spread on agar potato dextrose (Merck) acidified with 10% sterilized tartaric acid to pH $3.5 \pm 0.1$ plate, and incubated for 5 days at 25 °C [17]. *S. boulardii* colonies were quantified and expressed as the number of colony-forming units

per gram (CFU/g). The greatest concentration at 24-hour time period was registered as 9.70 ± 0.09 CFU/ml. Thus, the conditions employed for obtaining biomass of *S. boulardii* were: incubation at 25 °C, combined with constant stirring at 150 rpm for 24 h.

## Microencapsulation

The water-in-oil emulsion technique was used [18]. Microencapsulation was performed employing 1% sodium alginate, 0.05% inulin, and 0.05% mucilage extracted from cactus *Opuntia ficus-indica*, and subsequently adding the biomass obtained by centrifugation (resulting from the growth of the pure strain of *S. boulardii* in Sabouraud Dextrose Broth). Afterwards, 0.5% $CaCO_3$ was added to the aqueous mixture. Two hundred mL of canola oil was blended, separately with 2.5 mL of Tween 80 (oily phase). Constant stirring was maintained out from the aqueous to the oil phase, permitting the formation of the (water/oil) emulsion. Finally, 40 mL of the same oil was added but mixed with 1.7 ml of glacial acetic acid to initiate the microencapsulation process. The result blend was stirred for 20 minutes to complete the formation of the capsules and then left to stand for 30 min before being decanted and filtered. The capsules were washed with 200 ml of sterile distilled water and twice with 200 ml of phosphate buffer solution (pH = 7.2) to remove residual oil.

The survival of *S. boulardii* was evaluated before and after the microencapsulation process, for free cells was performed using by means of the spread-plate counting method as previously mentioned, while microencapsulated *S. boulardii* was disintegrated using the end of a sterile glass stirrer [18]. Subsequently, the quantification of *S. boulardii* was performed using the technique described above.

The microcapsules obtained were freeze-dried as follows: firstly, the microcapsules were frozen for 5 h at -20 °C and then placed in a freeze dryer with 4 kg capacity, applying pressure to 3 mmHg. —Samples were then dried at 20 °C for 24 h. Parameters have been standardized in collaboration with the SioSi Alimentos S.A.P.I. de C.V, with reference to dehydration of food susceptible to heat and moisture from 75-85%. The equipment was designed and built by SioSi Alimentos S.A.P.I. de C.V. (Morelia, Mich., Mexico). The morphology and size of the microcapsules were minutely observed with a scanning electron microscope. A sheet of carbon was placed on a brass plate, where the microcapsules were deposited. The samples were metallized with copper in a vacuum and put in a scanning electron microscope JEOL model JSM-mark SEG-7600F SEM, where the morphology and size were observed and measured.

## Yogurt-formulae

To prepare the yogurt: inulin (4.0%), vegetable oil (2.3%), mono and di glycerides (0.5%), gelatin (0.125%), and sucrose (5%) were added to skimmed milk (88.07%). The mixture was stirred and pasteurized at 63 °C for 30 min. Then, the mixture was left in an ice bath with salt for 10 min to reach approximately 4 °C. Lactic cultures (*L. bulgaricus* and *S. thermophilus*) were then added at a concentration of 0.03 g/L and incubated for 6 hours at 43 °C. The end product was stirred and cooled to 4 °C, and labelled formula F1. Two further products (using F1 as a base) were developed: F2, to which *S. boulardii* was added in the form biomass at a concentration of 0.5% cells; and F3, with added microencapsulated *S. boulardii* at a concentration of 0.5% biomass.

**Freeze-dried products and survival tests of *S. boulardii***

The F1, F2, and F3 products were freeze-dried using the technique as previously mentioned. The survival of *S. boulardii* was evaluated in fresh and freeze-dried products with the spread-plate counting method as previously mentioned. The quantification of microencapsulated *S. boulardii* was performed using the technique described above.

**Yogurt characteristics**

*Physicochemical analysis*

In order to measure the pH, the yogurt powder was re-hydrated, using a ratio of 1 part dried powder to 3 parts of water, to reach a total volume of 100 mL employing a Hanna instruments pH 213 potentiometers. Acidity was calculated and expressed in terms of lactic acid. Color, with a Hunter Lab colorimeter (Model CX0795 Colorflex). Solubility determined as per the method proposed by Shittu and Lawal [19]. The wettability of the products assessed according to the modified static method, described by Kim et al. [20].

**Chemical composition**

Moisture, ash, protein, and lipids were measured using the methods AOAC [21]; dietary fiber content determined using the method of Prosky et al. [22]. Carbohydrate content obtained by difference, i.e. subtracting the sum of the protein, lipids, ash and fiber values from 100. The fatty acid profile was measured by the Chem32 method using a gas chromatograph (Agilent Technologies 7890).

**Microbiological analysis**

A series of dilutions was prepared with sterile 0.1% buffered, peptone water. Appropriate dilutions were poured out and plated. An M17 agar (Oxoid Ltd., UK) was used to count *S. thermophilus*—and the plates were incubated aerobically at 37 °C for 48 h. *L. bulgaricus* was counted on MRS agar (Oxoid Ltd., UK) by incubating the plates anaerobically at 37 °C for 72 h. Potato dextrose agar (Merck) acidified with 10% sterilized tartaric acid to pH $3.5 \pm 0.1$ was employed to determine the total yeast and mold. The plates were incubated at 25 °C for a period of 5 days. The colonies were quantified and expressed as the number of colony-forming units per gram (CFU/g).

**Survival of *S. boulardii* during storage**

For this study, 25 g of each freeze-drying yogurt were placed in separate foil pouches, supplied by SioSi Alimentos S.A.P.I. de C.V., and the survival of *S. boulardii* was evaluated as previously described, according to established sampling time periods, day 0, 12, 24, 36, 52, 68 and 84. The freeze-dried yogurt samples were stored at 25 °C for 84 days, and the survival of *S. boulardii* monitored. The freeze-dried products were categorized as follows: F1 = Yogurt with inulin + vegetable oil (Control); F2 = Yogurt with inulin + vegetable oil + *S. boulardii* in free-cell form; F3 = Yogurt with inulin + vegetable oil + *S. boulardii* in microencapsulated form.

**Biological assay to ensure survival of *S. boulardii* in rat gastrointestinal tracts**

Twenty-four of 60-day-old Wistar rats, with an average weight of 240 g, were acclimatized for four days before being randomly assigned to the experimental groups. Animal care and management were carried out under the guidelines of Mexican Official Standard NOM-062-ZOO-1999. The animals were housed in individual cages in a room with a maintained temperature of 23±2 °C, $54 \pm 11$ % relative humidity and 12 h light/dark cycle.

During this acclimatization period, each animal was fed a standard diet (an average of 60 calories/animal/day) and water purified. Having completed this stage, the rats were randomly divided into six treatments (n = 4 rats) and fed *ad libitum* in addition to the experimental treatment and water for 14 days. Treatments were administered via an intragastrical cannula to ensure that animals ingested appropriate concentrations of saline solution 0.85%, ampicillin solution (5 mg / mL) and 4 g rehydrated yogurt (3 g of distilled water per 1 g of freeze-dried yogurt). The treatments administered were the following: Treatment I: 1 ml of saline solution was administered every 12 h for 7 days, followed by 4 ml of of saline solution every 24 h for 7 days (control). Treatment II: 1 ml of saline solution was administered every 12 h for 7 days, followed by 4 g of rehydrated formulate F2 every 24 h for 7 days. Treatment III: 1 ml of saline solution was administered every 12 h for 7 days, followed by 4 g of rehydrated formulate F3 every 24 h for 7 days. Treatment IV: The rats were administered 2 mg of ampicillin / 100 g body weight every 12 h for 7 days, followed by 4 ml of saline solution every 24 h for 7 days (control). Treatment V: The rats were administered 2 mg ampicillin / 100 g body weight every 12 h for 7 days, followed by 4 g of rehydrated formulate F2 every 24 h for 7 days. Treatment VI: The rats were given 2 mg ampicillin / 100 g body every 12 h for 7 days, followed by 4 g of rehydrated formulate F3 every 24 h for 7 days.

The F2 formulation contained $7.17 \pm 0.17$ log CFU / g of *S. boulardii* as free cells and F3 contained $6.86 \pm 0.24$ log CFU / g of *S. boulardii* in microencapsulated form.

Feces from each treatment were collected aseptically, prior to administration of the probiotic (day 1) and following its administration (days 3, 5, 7). Microbiological analysis of the feces was performed according to the methodology as previously mentioned to evaluate the presence of S. *boulardii* in the feces during yogurt administration. Colonies were reported as CFU/g.

The animals were sacrificed after the administration of treatments and a portion of the colon was resected. The specimen was weighed, washed with sterile saline solution, and placed in Petri dishes. An incision was made to the intestinal lining with a scalpel. A bacteriological loop was used to scrape the intestine to obtain a representative sample. The intestinal content was re-suspended in saline solution and subsequently plated, using the above-mentioned technique. Isolated colonies were identified by testing assimilation of sugars — employing the API 20C system AUX®.

**Data analysis**

The data obtained were statistically analyzed as per using the Tukey-Kramer test (P <0.05). Statistical analysis was performed using the program JMP6 statistical software (SAS Institute, Cary, NC, USA)

**RESULTS AND DISCUSSION**

**Microencapsulation**

The number of viable cells of *S. boulardii* after microencapsulation process was reduced by 1.59 logarithmic units, from $9.70\pm0.09$ to $8.11 \pm 0.10$ CFU/g. Microcapsules containing *S. boulardii* are shown in Figure 1. The micro-encapsulated *S. boulardii* was seen to be agglomerated or of a spherical form. When 30 microcapsules were measured individually they had an average diameter of 7.5 microns, i.e. similar to that reported average size 9.2 ∝ for Zamora-Vega et al. [18].
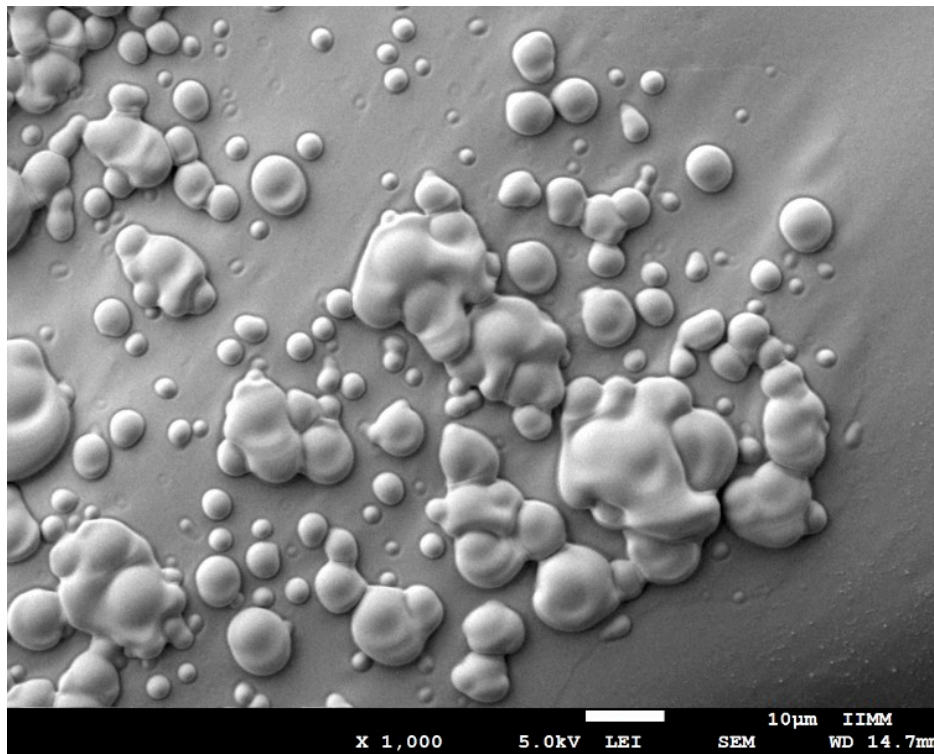
**Figure 1.** Microcapsules containing *S. boulardii*.

**Freeze-dried yogurt**

The survival of *S. boulardii* before and after freeze-drying is shown in Table 1. The variance analysis showed no significant difference (p <0.05) for F2 and F3. Therefore, the conditions used for carrying out the freeze-drying process did not affect the survival of *S. boulardii* during storage.

The concentration of yeasts in this specific type of fermented food, should be present in amounts greater than $1 \times 10^4$ CFU/g (CODEX STAN 243-2003). Consequently, the processed food evaluated in our research does meet the criteria established for this type of product and can therefore be considered a source of probiotics due to the formulations contained, 8.94-Log CFU/g for F2 and 7.41-Log CFU/g for F3.

**Table 1.** Survival of probiotics present in the fresh & freeze-dried yogurt

| Formulae | Fresh yogurt Log CFU/g | Freeze-dried yogurt Log CFU/g |
|---|---|---|
| F1 | ND | ND |
| F2 | $9.05 \pm 0.39^a$ | $8.94 \pm 0.66^a$ |
| F3 | $7.73 \pm 0.23^a$ | $7.41 \pm 0.12^a$ |

Results are expressed as mean ± standard deviations (SD) of three determinations. ND = (<1-Log CFU/g).
[a] The same letter in the same line is not significantly different (p<0.05). F1 = Yogurt + inulin + vegetable oil (Control). F2 = Yogurt + inulin + vegetable oil + 0.5% of *S. boulardii*. F3 = Yogurt + inulin + vegetable oil + 0.5% of *S. boulardii* microencapsulated.

**Yogurt—characterization**

**Physicochemical analysis**

Table 2 illustrates that the color parameter luminosity "L" where 0 represents black and 100 white — was not statistically different (p <0.05) with reference to F1, F2, and F3 products (ranging from 84 to 86). The "b" parameter, associated with color yellow for positive values, showed no significant difference (p <0.05) between them either. Consequently, the incorporation of *S. boulardii*—whether as free or microencapsulated cells—did not affect these parameters: the end products had the white color, characteristic of yogurt, but with a slightly yellow hue attributed to the addition of palm oil. The pH ranged from 4.4 to 4.5 and was thereby not notably at variance (p <0.05) when comparing the three samples (Table 2). The acid pH of yogurt helps to preserve and also prevent the growth of undesirable microorganisms—including pathogens.

We observed important variants (p <0.05) in the lactic acid values (Table 2) in the samples containing *S. boulardii*. The F2 product, of greater lactic acid and containing "free" *S. boulardii* had slightly higher lactic acid than product F1 (control sample)—probably due to the production of organic acids—such as the acetic acid—during the fermentation of free sugars by the yeast. Product F3 was less lactic acid, which may have occurred because the yeasts were microencapsulated and had no direct contact with the yogurt. It is essential that yogurt have a minimum lactic acid of 0.5 [23]. However, one must also note that all the products developed in this report meet this parameter.

Wettability and solubility tests were performed to evaluate the instantaneous hydration properties of the freeze-dried products (Table 2). Determinations were exacted taking two particle sizes into consideration. None of the three products demonstrated any significant difference (p <0.05) with reference to its solubility—which ranged from 78.53% to 79.53% in F3 for a particle 1- 2 mm in diameter. When the particle size was 0.5-1 mm, the solubility decreased significantly in F2 and F3 compared to the control (F1). Product F3 showed the lowest solubility (70.62% when compared to 76.40% in F2). Wettability tests show that in F1 and F2, particle size did have an impact on wetting time, which increased by approximately 20 min for smaller particles compared to the larger ones. Showing to the fact that the larger particles were immersed faster than the smaller. However, noteworthy differences (p <0.05) were observed in particles measuring 0.5-1 mm, which decreased the wettability time of F3 with reference to F1 and F2. Product F3 showed similar wettability for 1 to 2 mm (5 min) or 0.5 to 1.0 mm (5.29 min) particles. The microcapsules of this product favored rapid wetting, possibly because they were prepared with hydrocolloids and demonstrated greater affinity for water, and that tendency regardless of particle size. Overall, 1-2 mm particles had better wettability characteristics than those of 0.5-1 mm in all three formulae.

**Table 2.** Physicochemical analysis of yogurts.

| Product | Parameter | F1 | F2 | F3 |
|---|---|---|---|---|
| Rehydrated | pH | $4.49 \pm 0.01^a$ | $4.43 \pm 0.01^a$ | $4.50 \pm 0.06^a$ |
| Rehydrated | Lactic acid (%) | $0.81 \pm 0.009^b$ | $0.85 \pm 0.009^a$ | $0.71 \pm 0.005^c$ |
| Freeze-dried | Color L | $85.61 \pm 0.49^a$ | $85.24 \pm 0.57^a$ | $84.42 \pm 0.14^a$ |
| Freeze-dried | Color b | $15.81 \pm 0.30^a$ | $15.75 \pm 0.12^a$ | $15.85 \pm 0.14^a$ |
| Freeze-dried | Solubility (%) Particle size 1-2 mm | $79.53 \pm 0.68^a$ | $79.00 \pm 0.78^a$ | $78.35 \pm 0.86^a$ |
| Freeze-dried | Solubility (%) Particle size 0.5-1 mm | $78.55 \pm 0.86^a$ | $76.40 \pm 1.09^a$ | $70.62 \pm 0.64^b$ |
| Freeze-dried | Wettability (mins) Particle size 1-2 mm | $13.23 \pm 0.94^a$ | $13.16 \pm 0.55^a$ | $5.00 \pm 0.45^b$ |
| Freeze-dried | Wettability (mins) Particle size 0.5-1 mm | $33.9 \pm 0.30^a$ | $32.33 \pm 1.02^b$ | $5.29 \pm 0.08^c$ |

Results are expressed as mean ± standard deviations (SD) of three determinations. [a,b] The different letter in the same line is significantly different (p<0.05). F1 = Yogurt with inulin + vegetable palm-oil added (Control). F2 = Yogurt with inulin + vegetable palm-oil + 0.5% of *S. boulardii* added. F3 = Yogurt with inulin + vegetable palm-oil + 0.5% of *S. boulardii* microencapsulated added. Rehydrated product (3 parts of water: 1 part of freeze-dried product).

**Chemical composition analysis**

The chemical compositions of F1, F2, and F3 products are shown in Table 3. All three formulae demonstrated similar moisture content from 5.30% (F2) to 5.63% (F3). Protein content in F1 was not significantly modified (P<0.05) by the addition of *S. boulardii*. Nevertheless, an increase was noted in the number of lipids in F3 (15.43%) compared to F1 (11.01%) and F2 (11.53%). The rise was due to the microcapsules formula plus the water-in-oil-emulsion process resulting in higher lipid reading in the F3 product.

Milk fat contains approximately 70% saturated fatty acids, which can raise low-density lipoprotein (LDL), increasing the risk of cardiovascular ailments. Therefore, milk fat consumption should ideally be limited [24]. One objective of this study was to replace milk fat with vegetable palm oil to augment the content of unsaturated fatty acids—in particular essential polyunsaturated fatty acids (PUFAs) —such as omega-3 (fatty acid α-linolenic) or omega-6 (fatty acid linoleic), which cannot be produced by the human body. For this reason, some PUFAs, e.g. acid α-linolenic or acid linoleic and derivatives [eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA)], have to be consumed directly from food [25]. The amount of fat recommended for daily consumption is approximately 30% of total energy intake, of which 10% should be from saturated fatty acids, 6 to 11% from polyunsaturated fatty acids (2.5 to 9% of

linoleic acid and 0.5% to 2.0% of α-linolenic acid) in addition to monounsaturated fatty acid obtained by subtraction [26]. For example, with a typical diet of 2000 kcal per day, using 30% of energy from total fat and the minimum intake percentages to prevent deficiency symptoms of 2.5% linoleic acid and 0.5% α-linolenic acid, gives us 66 g/day, 5.5 g/day and 1.1 g/day respectively [27].

The formulae prepared in this study contained a low quantity of saturated fatty acids: 3.32 g/11.01 g fat (F1), 3.74 g/11.53 g fat (F2), and 3.05 g/15.43 g fat (F3). Consequently, the three products had a higher proportion of unsaturated fatty acids: 7.69 g/11.01 g fat (F1), 7.79 g/11.53 g fat (F2) and 12.38 g/15.43 g fat (F3) (Table 3). The most abundant mono-unsaturated fatty acid found in this study, was the oleic—at 4.78 g/11.01 g fat, 4.91 g/11.53 g fat and 8.29 g/15.43 g fat for F1, F2, and F3 respectively. This is significant due to the fact that reported increased intake of oleic acid raises the HDL-cholesterol fraction and thereby reduces cardiovascular risk [28]. The products demonstrated had linoleic acid values of 2.07 g/11.01 g fat, 2.08 g/11.53 g fat, and 2.70 g/15.43 g fat for F1, F2, and F3 respectively. These results are certainly worthy of note, as intake of linoleic acid is known to exert a stabilizing action on the cell membrane, producing an anti-arrhythmic effect, inhibits platelet aggregation in addition to other favorable effects on lipid profile (decreased triglycerides and VLDL cholesterol and HDL cholesterol may possibly increase) and hypotensive properties [28]. The products in our study contain 0.36 g α-linolenic acid/11.01 g fat, 0.37 g α-linolenic acid/11.53 g fat, and 0.79 g α-linolenic acid/15.43 g fat for F1, F2, and F3, respectively. Omega-3 may act as therapeutic agent with a significant role in the treatment of inflammatory illnesses —including cardiovascular and neurodegenerative ailments [29].

**Table 3.** Chemical composition analysis of freeze-dried products.

| Chemical compounds (%) | F1 | F2 | F3 |
|---|---|---|---|
| Moisture | 5.40[a] | 5.3[a] | 5.63[a] |
| Protein | 13.08[a] | 13.53[a] | 13.59[a] |
| Ashes | 3.16[a] | 3.27[a] | 3.02[b] |
| Carbohydrates | 67.35[a] | 66.37[a] | 62.33[b] |
| Fat | 11.01[b] | 11.53[b] | 15.43[a] |
| Saturated (g) | 3.32 | 3.74 | 3.05 |
| Monounsaturated (g) | 4.80 | 4.92 | 8.33 |
| Polyunsaturated (g) | 2.68 | 2.66 | 3.89 |
| Oleic acid (g) | 4.78 | 4.91 | 8.29 |
| Linoleic acid (g) | 2.07 | 2.08 | 2.70 |
| 〈-Linolenic acid (g) | 0.36 | 0.37 | 0.79 |

Results are expressed as mean ± standard deviations (SD) of three determinations. [a,b] The different letter in the same line is significantly different ($p < 0.05$). F1 = Yogurt with inulin + vegetable palm-oil added (Control). F2 = Yogurt with inulin + vegetable palm-oil + 0.5% of *S. boulardii* added. F3 = Yogurt with inulin + vegetable palm-oil + 0.5% of *S. boulardii* microencapsulated added.

## Microbiological analysis

Neither total coliforms nor molds were detected (Table 4). Moreover, these formulae showed a concentration of lactic acid bacteria of approximately 8-log CFU/g —higher than standards used as a reference for a minimum amount of 7-log CFU/g to warrant the official quantification of a "probiotic" [30]. Thus, the products obtained in this work may be denoted as probiotics. The viability of the bacteria was not affected by the adding of *S. boulardii* in F2 or F3.

**Table 4.** Microbiological analysis of freeze-dried yogurts.

| Log-CFU/g | F1 | F2 | F3 | CODEX STAN 243-2003 |
|---|---|---|---|---|
| Total coliforms | ND | ND | ND | Maximum 1 |
| Molds | ND | ND | ND | Maximum 1 |
| Lactic bacteria | $8.31 \pm 0.2^a$ | $8.47 \pm 0.29^a$ | $8.06 \pm 004^a$ | Minimum 7 |

Results are expressed as mean ± standard deviations (SD) of three determinations. [a] The different letter in the same line is significantly different ($p < 0.05$). ND= Not detected (<1 CFU/g). F1=Yogurt with inulin + vegetable palm-oil added (Control). F2=Yogurt with inulin + vegetable palm-oil + 0.5% of *S. boulardii* added. F3=Yogurt with inulin + vegetable palm-oil + 0.5% of *S. boulardii* microencapsulated added.

## Survival of *S. boulardii* during storage at 25 °C

Figure 2 shows that the viability of *S. boulardii* decreased with time. The minimum concentration of *S. boulardii* (yeast) that this kind of fermented product must have in order to be considered a probiotic dairy item is 4-log CFU / g [30]. In the case of F2, showed a concentration of 7.12-Log CFU/g on day 0 and 4.66 -Log CFU/g on day 68 and 3.93 -Log CFU/g on day 84. F3 had a concentration of 6.55-Log CFU/g on day 0 and 4.40-Log CFU/g on day 84 maintaining a yeast concentration sufficiently acceptable as a probiotic. During the whole period, the concentration of yeast present in sample F3 was sufficient to be of probiotic food level to difference of F2.

The loss of *S. boulardii* viability in F2 was of 3.92 log units, in F3, the decrease was only 2.15 log units—illustrating the protective effect of the microcapsules. A study demonstrated that when *S. boulardii* was incorporated into fresh cheese, viability was increased 1.22-log CFU/g when the probiotic was microencapsulated—compared to free cells, over 35 days of storage at 4 ° C [18]. The protective effect of the microcapsules should be specially noted, given that *S. boulardii* viability is markedly affected during the storage phase, that oxygen content, high temperatures, low pH, water activity, and elevated concentrations of solutes can affect the viability of probiotics [31, 32]. Freeze-drying did not affect the survival rate of *S. boulardii*— either as free cells or in microencapsulated form. But over time, viability significantly decreased during storage at 25 °C.
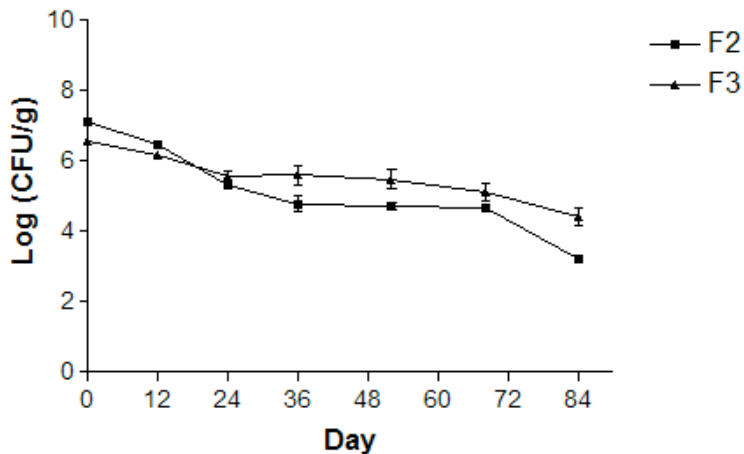
**Figure 2.** Values represent mean ± standard deviation of tree determinations. *S. boulardii* survival according to time lapse at 25 °C: F2 = Yogurt with inulin + vegetable oil + 0.5% of *S. boulardii*. F3 = Yogurt with inulin + vegetable oil + 0.5% of *S. boulardii* microencapsulated.

**Biological assay and survival of *S. boulardii* in rat gastrointestinal tract**
Table 5 shows the survival of *S. boulardii* in fecal samples of the six treatments. *S. boulardii* was not detected in treatments I, II, III, and IV, over a period of seven days. Treatments I and IV, which were the control, were not given *S. boulardii*. However, for treatment II and III, fed yogurt containing *S. boulardii* (either as free cells or in microencapsulated form, respectively) —no survival *S. boulardii* was found in the feces.

Furthermore, *S. boulardii* was also not discovered in survival form in the colon, probably because the intestinal flora in these test animals was typically balanced (i.e. normal flora), and was not affected by the administration of an ampicillin, or pathology in the treatments I, II, III, and IV (Table 5). Some authors have reported that *S. boulardii* can only be hosted in the digestive tract of axenic (totally bacteria-free) animals. Nevertheless, a study demonstrated that in holoxenic animals (complex flora), *S. boulardii* was rapidly eliminated [33]. Thus, when flora was "normal", *S. boulardii* was not deposited in the gastro-intestinal tract and, therefore, not viable in the colon—as appreciated in our investigation. Conversely, *S. boulardii* was found in the feces of treatments V and VI, which correspond to animals administered ampicillin for seven days prior to the probiotic. This antibiotic (ampicillin) damages intestinal flora—due to its 40% absorption level—and the remaining unabsorbed drug passes such as through the gastro- intestinal tract destroying all susceptible microorganisms. In this path and leaving behind free "binding sites" where *S. boulardii* can initially settle in the colon, followed by excretion after five or seven days (Table 5).

The excretion of probiotics may occur because *S. boulardii* in the early stage of administration, occupy fill the "binding sites" of the gastro-intestinal tract and consequently begin to appear in the feces [34], base these criteria on the fact that, for the proved deposit of a probiotic in the gastro-intestinal tract, its suffices that the colonizing microorganism be present in the stool culture—signifying its viability as it courses through the entire gastro-intestinal tract.

**Table 5.** Survival of *S. boulardii* in feces of Wistar rats.

| Treatment | Log CFU/g in feces | | | | Log CFU/g in colon |
|---|---|---|---|---|---|
| | Day | | | | |
| | 1 | 3 | 5 | 7 | 7 |
| I | ND | ND | ND | ND | ND |
| II | ND | ND | ND | ND | ND |
| III | ND | ND | ND | ND | ND |
| IV | ND | ND | ND | ND | ND |
| V | ND | ND | $6.02 \pm 0.37^a$ | $5.10 \pm 0.57^a$ | $2.32 \pm 0.48^a$ |
| VI | ND | ND | $4.98 \pm 0.36^b$ | $4.55 \pm 0.41^a$ | $3.10 \pm 0.72^a$ |

Results are expressed as mean ± standard deviations (SD) of four determinations. [a,b] The different letter in the same line is significantly different (p<0.05) for Log CFU/g in faeces. [a] The different letter in the same column is significantly different (p<0.05) for Log CFU/g in colon. ND= No detected (< 1-Log CFU/g).

According to our study, in the two treatments, the colonization of the gastro-intestinal tract of the animals was corroborated—in that colonies were viable in feces and colon. The adhesion mechanism during colonization was probably realized by means of mannoproteins, known as adhesins, present on the *S. boulardii* cell membrane, which can bind to specific amino-acid or sugar residues on the surface of other cells. These receptors can be for D-galactose, N-acetylgucosamine, N-acetylgalactosamine, or N-acetylneuraminic acid; associated with intestinal glycoproteins, and in carbohydrates as mucin receptors that can function as both pathogenic and probiotic bacteria [35].

Microencapsulation of the *S. boulardii* proved tendency more efficient than its administration by the *S. boulardii* as free cells. The survival of *S. boulardii* as free cells and as microencapsulated form was of 2.33 and 3.10 log CFU/g respectively.

*Saccharomyces boulardii* strains can assimilate a variety of sugars including: D-glucose, Glycerol, D-galactose, D-maltose, D-Cellobiose, D-sucrose, D-trehalose, and D-raffinose — dependent on the strain [36]. The quoted authors analyzed nine strains of *S. boulardii* and reported differences in sugar assimilation. However, it was discovered that four sugars were assimilated by all nine strains: i.e. D-glucose, D-maltose, D-sucrose, and D-raffinose—and said sugars were assimilated by the strain of *S. boulardii* that was used and isolated in this study. See Table 6.

The API AUX 20® system, specializing in assimilation of sugars, was the one used to identify *S. boulardii* in this study. Strains isolated from colon and feces in groups V and VI, showed the same chemical profile demonstrated by the *S. boulardii* strain present in the yogurt analyzed here. Nine strains of *S. boulardii* studied displayed a similar outline of assimilation of sugars [36] as *S. boulardii* strain used in this study. The biochemical profile of *S. boulardii* strains isolated from colon and feces are presented in Table 6.

**Table 6.** Identification of probiotic for test assimilation of sugars using the API 20C systemAUX®.

| Sugar | S. boulardii |
|:---:|:---:|
| D-glucose | + |
| Glycerol | - |
| Calcium 2-keto-gluconate | - |
| L- arabinose | - |
| D-Xylose | - |
| Adonitol | - |
| Xilitol | - |
| D-Galactose | + |
| Inositol | - |
| D-sorbitol | - |
| Methyl-α-D Glucopyranoside | + |
| N-Acetyl- Glucosamine | - |
| D-Celobiose | - |
| D-Lactose | - |
| D-Maltose | + |
| D-Sucrose | + |
| D-Trehalose | - |
| D-Melezitose | - |
| D-Raffinose | + |

**CONCLUSIONS**

The *Saccharomyces boulardii* was added in microencapsulated form and as free cells to yogurt, was freeze-dried, being the freeze-drying process an ideal method of preservation. Microencapsulation increased the viability of *S. boulardii* compared to yeast added as free cells, therefore extending the full benefits of the product. The prepared formulae containing *S. boulardii* can be considered probiotic food; in controlled groups of experimental rats with intestinal flora affected by ampicillin, ingested yeast—either as free cells or in micro-encapsulated form was found in feces, thereby demonstrating its resistance to gastric pH, bile salts and certain "digestive" enzymes and, finally, its ability to survive in the colon.

**Competing interests:** The authors have no conflict of interest.

**Author's contributions:** Tranquilino-Rodriguez performed the experimental research, Martinez- Flores and Rodiles-Lopez designed the study, Zamora-Vega designed the probiotic encapsulation stage, Salgado-Garciglia and Perez-Sanchez contributed to the analysis of results.

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