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FOOD MICROBIOLOGY - RESEARCH PAPER

Enhancing cell resistance for production of mixed microbiological reference materials with *Salmonella* and coliforms by freeze-drying

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Abstract

The reference material (RM) is a technical requirement for the quality assurance of analytical results and proficiency tests or interlaboratory comparisons. Microbiological RMs are most available in the dehydrated form, mainly by freeze-drying, and maintaining bacterial survival after preparation is a challenge. Thus, obtaining the most resistant cells is essential. Considering that bacteria present cross-response to dehydration after being submitted to an array of stress conditions, this study aimed to evaluate the influence of growth conditions on enterobacteria for the production of mixed microbiological RMs by freeze-drying in skim milk powder. *Salmonella enterica* serovar Enteritidis, *Cronobacter sakazakii*, *Escherichia coli,* and *Citrobacter freundii* were grown in a minimal medium with 0.5 M of NaCl and 0 to 5.0 mM of manganese sulfate ($MnSO_4$) until stationary phase. *Salmonella* Enteritidis presented an increased resistance to dehydration in the presence of Mn, while *C. sakazakii* was the most resistant to freeze-drying and further storage for 90 days. Mixed microbiological RMs were produced by freeze-drying containing *Salmonella* Enteritidis and coliforms in skim milk powder with 100 mM of trehalose and the *Salmonella* survival rate was 91.2 to 93.6%. The mixed RM was stable after 30 days at -20 °C, and *Salmonella* and coliforms were detected by different methods being, the Rambach Agar the best for the bacterial differentiation. The results showed that the culture conditions applied in this study resulted in bacterial cells being more resistant to dehydration, freeze-drying, and stabilization for the production of mixed microbiological RMs more stable and homogeneous.

Keywords *Citrobacter freundii* · *Cronobacter sakazakii* · Enterobacteria · *Escherichia coli* · Homogeneous · Resistance

Introduction

Reference material (RM) is an indispensable, reliable, and widely accepted tool for comparing the results of the analyses, representing one of the main bases for methodological quality control [1–5]. In the quality assurance context, the use of RM is mentioned in ISO/IEC 17025 [6] as a technical requirement for the quality assurance of analytical results, through regular use as the internal control of analysis and in the calibration, as well as for proficiency tests or interlaboratory comparisons [1, 5–9].

Microbiological reference materials (microbiological RMs) can be used in food microbiology laboratories as part of the quality assurance program, internal control of trials, validation of methods, training of workers, evaluation of laboratory accuracy, and scientific research [1, 5, 8, 10, 11]. Thus, microbiological RMs have to meet minimum requirements, such as showing homogeneity and stability within limits established over a period of time.

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Also, microbiological RMs should be representative of the intended use, that is, they should resemble routine samples [12–14] to guarantee to assess of participants performance and characterize measurement accuracy [15]. Therefore, mixed microbiological RMs containing the target microorganism plus interfering and, or competitors microorganisms may aid in the performance evaluation of laboratory analysis methods. The target microorganism's detection and, or quantification should be possible even in the presence of interfering microorganisms [1, 16, 17].

Most microbiological RMs are dehydrated by freeze-drying or spray drying of reconstituted skim milk powder containing bacteria and posteriorly packaged in vials or presented in capsule or lenticular forms [1, 4, 5]. After production, these materials must present desirable homogeneity and stability characteristics, and microbial cells must remain viable throughout storage and analysis [1, 4, 18]. For this, the cells need to preserve their biological integrity under dehydration [19]. However, the freeze-drying method combines freezing and drying stresses harmful to bacterial cells [20]. The use of the most resistant cells is recommended to reduce these cellular damages.

Bacteria present cross-response to dehydration after being submitted to nutritional, osmotic, oxidative, or thermal stresses [21–23]. Nutritional stress, that is, growth in a medium with deprivation of carbon, nitrogen, phosphate, or other nutrients, independent of the growth phase of the bacteria, also increases the resistance to dehydration and other stresses by cross-response [24–26]. Also, bacteria in the stationary growth phase are more adapted to multiple stresses when compared to bacteria in the logarithmic phase [27–31]. Thus, bacteria at the end of the logarithmic phase and the beginning of the stationary phase of growth should be used in freeze-drying since they have greater resistance to adverse conditions [19, 29, 32–35].

The response to osmotic stress induces intracellular accumulation of ions such as potassium (K^+) and compatible solutes such as proline, glycine betaine, and trehalose, as well as activation of other osmoregulatory responses that also increase the survival of bacterial cells to dehydration [26, 36–43]. Trehalose is an osmoprotectant and cryoprotectant that can replace water molecules around macromolecules, preventing cell damage [44]. This disaccharide plays a central role in the protection of bacteria against dehydration through stabilizing membrane proteins and phospholipids [19, 35, 45–49]. Besides, trehalose may also exert a protective effect against cold stress in *Salmonella* and *Escherichia coli* [44, 50].

Adding manganese (Mn) to the culture medium may reduce the effects of oxidative stress from dehydration [51, 52]. This element can act as a cofactor of Mn-dependent superoxide dismutase (SOD) enzymes and replace iron (Fe), exerting a possible protective effect against oxidative damage in certain proteins [26, 53, 54]. In *E. coli*, when iron from the ribulose-5-phosphate-3-epimerase enzyme was replaced by Mn, the protein became less sensitive to hydrogen peroxide [55]. Fredrickson et al. [51] reported that bacteria with a high Mn/Fe ratio were less susceptible to oxidation of protein induced by dehydration. *Salmonella enterica* serovar Typhimurium cells showed higher activity of the enzyme SOD when grown in the presence of 5 mM of Mn [52].

Considering the interest in enhancing the resistance of cells subjected to stresses during the preparation of microbiological RMs, this study aimed to evaluate the influence of Mn on *Salmonella* and other enterobacteria under nutritional and osmotic stress. Besides, it was produced and analyzed mixed microbiological RMs with cells in the stationary phase of growth by freeze-drying in skim milk powder containing trehalose.

Materials and methods

Bacterial species

The enterobacteria species *Salmonella enterica* serovar Enteritidis phage type 4 (PT4) 578 (GenBank: 16S ribosomal RNA gene - MF066708.1), *Cronobacter sakazakii* ATCC 29004, *Escherichia coli* ATCC 29214, and *Citrobacter freundii* ATCC 8090 were used in this study. Bacterial cultures were stored at -20 °C in Brain Heart Infusion broth, pH 7.4 (BHI broth; Himedia, India) supplemented with 20% (v/v) of sterilized glycerol.

Evaluation of the growth of enterobacteria in a minimal medium containing NaCl

The bacterial species were cultivated in 3 mL of BHI broth for 12 h at 37 °C. Then, an aliquot of 200 µL of each specie was transferred to 20 mL of minimal medium (MM; 0.7% of K_2HPO_4, 0.2% of KH_2PO_4, 0.1% of $(NH_4)_2SO_4$, 0.02% of $MgSO_4.7H_2O$, 0.4% of glycerol, 1 mM of $CaCl_2$), pH 7.1 and incubated at 37 °C. After 24 h of incubation, 2 mL of each species was transferred to 200 mL of MM containing 0.5 M of NaCl (MMS), pH 7.1 and incubated at 37 °C. The optical density at 600 nm (OD 600 nm) was determined by spectrophotometry (Thermo Scientific, USA). Besides, the water activity (*aw*) of MM and MMS was measured in an automatic analyzer (Decagon Aqualab CX-2, USA).

Evaluation of the resistance of enterobacteria to dehydration in the presence of Mn

The resistance to dehydration was performed according to Breeuwer et al. [27], with modifications. The bacteria were

cultivated at 37 °C in 200 mL of MMS containing 0.0, 0.1, 2.5, or 5.0 mM of manganese sulfate ($MnSO_4$), pH 6.6, separately. After 38 h of incubation, an aliquot of 20 µL of each species in different concentrations of $MnSO_4$ was transferred to 96-well microplates, incubated, and kept at 25 °C. Every seven days up to 49 days of dehydration, 20 µL of 0.85% (w/v) of saline solution was added to each well to resuspend the cells, diluted in 0.1% (w/v) of peptone saline. Then, an aliquot was plated by drop plate method in Plate Count Agar (PCA) for enumeration of viable and culturable cells [56].

Evaluation of enterobacteria survival after freeze-drying and stabilization

The treatment described in item "Evaluation of the resistance of enterobacteria to dehydration in the presence of Mn" that provided the cells most resistant to dehydration was selected and used to produce the inoculum. An aliquot of 20 mL of each species was harvested by centrifugation at 2,500 × *g* at 4 °C for 30 min (Sorvall, USA), washed with 0.1% of peptone saline, and the pellet resuspended in 12 mL of 10% (w/v) of reconstituted skim milk powder containing 100 mM of trehalose. Then, volumes of 1.5 mL were distributed in glass vials with a capacity of 10 mL, frozen immediately at -196 °C in liquid nitrogen (N_2), stored at -80 °C for 18 h in an ultra-freezer (Thermo Scientific, USA) and freeze-dried for 24 h (Liotop, Brazil). These vials were kept at 4 °C for 90 days for stabilizing cells. Before and after freeze-drying, as well as after stabilization, the contents of a vial were resuspended in 1.5 mL of 0.1% of peptone saline, diluted in 0.1% of peptone saline and an aliquot was plated by drop plate method in PCA [56].

Selection of differential medium for enterobacteria

This analysis was performed to select at least one medium to differentiate colonies from *Salmonella* Enteritidis, *C. sakazakii*, *E. coli,* and *C. freundii*. These four enterobacteria species were cultivated in BHI broth for 12 h at 37 °C. Then, an aliquot of 0.1 and 1.0 mL of each one and their mixture were plated by spread plate and pour plate methods, respectively, in the differential medium, such as Brilliant Green Agar (BGA; Oxoid, England), Xylose-Lysine Deoxycholate Agar (XLD; Oxoid, England), Hektoen Enteric Agar (HE; Becton Dickinson, Germany), Violet Red Bile Glucose Agar (VRBG; Himedia, India), Eosin Methylene Blue Agar (EMB; Himedia, India), MacConkey Agar (Merck, Germany) and Rambach Agar (Merck, Germany). The inoculation was also carried in Petrifilm *E. coli*/Coliform Count Plates (Petrifilm EC; 3 M, USA) according to fabricant instructions.

Production of mixed microbiological RMs

Mixed microbiological RMs were produced according to Schulten et al. [57], with modifications. Mixed microbiological RMs were produced in three independent repetitions (RM1, RM2, and RM3) containing 3.0 to 4.0 log CFU.g^{-1} of *Salmonella* Enteritidis and 4.0 to 5.0 log CFU.g^{-1} of a mix of *C. sakazakii, E. coli,* and *C. freundii*. Initially, 1.5 mL of each species was freeze-dried and stabilized at 4 °C for 90 days as described in item "Evaluation of enterobacteria survival after freeze-drying and stabilization." Posteriorly, an aliquot of 0.6 g of *Salmonella* Enteritidis freeze-dried in 10% (w/v) of reconstituted skim milk powder containing 100 mM of trehalose was mixed aseptically with a mortar and pestle in a laminar flow cabinet with 59.4 g of skim milk powder sterilized by radiation. The same procedure was performed for 0.6 g of each previously mentioned coliform culture. Then, 0.6 g of skim milk powder contaminated with *Salmonella* Enteritidis was mixed with 59.4 g of skim milk powder contaminated with coliforms to obtain 60 g of mixed microbiological RMs. The mixed microbiological RMs were stored at -20 °C for 30 days. After storage, 0.3 g of each RM was weighed, and 2.7 mL of 0.1% of peptone saline was added. Then, dilutions were performed in 0.1% of peptone saline, and 1 mL of dilution was plated on PCA and incubated for 4 h at 37 °C. An overlay of Rambach Agar was added and incubated for another 24 h at 37 °C. It is noteworthy that Rambach Agar was used based on the results of the experiment described in item "Selection of differential medium for enterobacteria."

Assessment of homogeneity of mixed microbiological RMs

The homogeneity of mixed microbiological RMs was assessed according to Schulten et al. [57], with modifications. After 30 days of storage of the microbiological RMs at -20 °C, 10 replicates of 0.3 g of each microbiological RM were weighed into disposable Petri dishes, added 2.7 mL of 0.1% of peptone saline, performed dilutions in 0.1% of peptone saline and 1 mL of dilution plated on PCA with an overlay of Rambach Agar as described in item "Production of mixed microbiological RMs."

The statistical methods and models used to assess homogeneity depending on the microbiological contamination level of microbiological RM [58]. Homogeneity evaluation of microbiological RMs was performed in two stages [58, 59]. First, homogeneity was determined by calculating the dispersion index T1 to check for a variation between the duplicates of the analytical portions followed by a Poisson distribution. T1 was calculated as:

$$T_1 = \sum_{i=1}^{I} \sum_{j=1}^{J} \frac{\left(Z_{ij} - \frac{Z_{i+}}{J}\right)^2}{\frac{Z_{i+}}{J}},$$

- J represents the subsamples number. In this work, J was equal to 2;
- I represents the replica number. In this case, I was equal to 10;
- Zij is a colony-forming units (CFU) count of the subsample j of fraction i;
- Zi + is a CFU sum of the number in all subsamples of a fraction i.

T1 is the Chi-square distribution with I (J-1) degrees of freedom. When 10 replicates of the microbiological RMs were used for duplicate enumeration, the value found should not exceed 18.3 ($T1 \leq 18.3$, significance level $\alpha = 0.05$) for the dispersion of the cells in the microbiological RM to follow the Poisson distribution. The homogeneous distribution of microorganisms in a liquid or powder is reported in the Poisson distribution. A value of T1 greater than the critical value of $\chi 2$ indicates low repeatability of the counts in the subsamples or analytical portions of the microbiological RMs [56].

The second assessment consisted of the application of test T2, which determines the variation between the counts of different replicates. T2 was calculated as:

$$T_2 = \sum_{i=1}^{I} \frac{\left(Z_{i+} - \frac{Z_{++}}{I}\right)^2}{\frac{Z_{++}}{I}},$$

- Zi + represents the sum of counts in each replica;
- Z + + is the total counts of all subsamples of all replicates;
- I is the total number of replicas.

T2 test provides a valid result only when the T1 test result is not significant. The homogeneity of microbiological RM is determined by calculating the value of T2/(I-1) [58–60]. Microbiological RM showing T2/(I-1) value of at most 3.0 was accepted as homogeneous ($T2/(I-1) \leq 3.0$) [61]. However, a more rigorous criterion of homogeneity determines a value of $T2/(I-1) \leq 2$ for homogeneous microbiological RM [60].

Detection of *Salmonella* in mixed microbiological RMs

The detection of *Salmonella* in mixed microbiological RMs was performed by a conventional method, immunomagnetic separation and polymerase chain reaction (PCR).

Conventional method

The conventional method for research of *Salmonella* in mixed microbiological RMs was performed according to ISO 6579 [62], which involved first, the pre-enrichment of 25 g in buffered peptone water (Himedia, India) with incubation at 37 °C for 18 h. Subsequently, the selective enrichment step was performed in Rappaport Vassiliadis broth (Himedia, India) and tetrathionate broth (Himedia, India) with incubation at 41 and 37 ºC for 24 h, respectively. The selective isolation was carried out in XLD, HE, and Rambach Agar and incubated at 37 °C for 24 h. The typical colonies of *Salmonella* in each differential medium were grown in BHI broth at 37 °C for 12 h and subsequently striated in PCA. Then, selected colonies were characterized biochemically by the API 20E kit (Biomeriéux, USA) and by the oxidase test (Probac, Brazil), as well as performed the serological test using the *Salmonella* O Antiserum Poly A-I & VI (BD Difco, USA).

Immunomagnetic separation

The immunomagnetic separation for research of *Salmonella* in mixed microbiological RMs using the Dynabeads anti-*Salmonella* kit (Invitrogen, USA) was performed according to the manufacturer's recommendations. Initially, an aliquot of 1 mL of the pre-enrichment sample of the conventional method described in item "Conventional method" was added in a microtube containing 20 µL of Dynabeads anti-*Salmonella* and mixed for 30 min in Dynabeads MX4 Mixer (Invitrogen, USA). Then, the microtube was kept in the magnetic separator for 10 min; the liquid was removed and was added 1 mL of 1% of peptone saline solution buffered with 0.02% (v/v) of Tween 20. The mixture was homogenized by inversions without the magnetic separator, and then this separator was replaced and mixed for 5 min. This step was repeated two more times. Subsequently, the microtube was kept in the magnetic separator for 10 min; the liquid was removed and was added 1 mL of phosphate-buffered saline. This buffer was removed, and an additional 20 µL was added. Posteriorly, this volume plus Dynabeads anti-*Salmonella* was transferred to 10 mL of Rappaport Vassiliadis broth and followed the analysis of the conventional method described in item "Conventional method."

Polymerase chain reaction (PCR)

The PCR for research of *Salmonella* in mixed microbiological RMs was performed for the *invA* gene. Initially, an aliquot of 1 mL of the Rappaport Vassiliadis broth of the conventional method described in item "Conventional method" was used to extract the genomic DNA using the Wizard Genomic DNA Purification kit (Promega; USA), according to the recommendations of the manufacturer. The DNA was amplified using the oligonucleotide specific for the *invA* gene of *Salmonella,*

5′-GCATGAAATGGCAGAACAGC-3′ (forward) and 5′-ATGAGTGAAGGATCGCAACC-3′ (reverse). The PCR program was as follows: an initial denaturing step at 95 °C for one min, followed by 40 cycles of 95 °C for one min, 51 °C for one min and 72 °C for 2.5 min and, posteriorly, a final extension at 72 °C for 5 min. The amplified samples and molecular weight marker (1 kb DNA ladder; Promega, USA) with *GelRed* (Biotium, USA) were subjected to an electrophoretic run on 0.8% (w/v) of agarose gel at 80 V, and then the gel was exposed to ultra-violet light. The band with approximately 2 kb corresponds to the *invA* gene of *Salmonella*.

Enumeration of coliforms and *E. coli* in mixed microbiological RMs

The enumeration of coliforms and *E. coli* in mixed microbiological RMs was performed on Petrifilm EC and on PCA with an overlay of Rambach Agar and Violet Red Bile Agar (VRBA), separately. Initially, an aliquot of 1.0 g of each mixed microbiological RM was weighed into disposable Petri dishes and added 9 mL of 0.1% of peptone saline. This mixture was kept at room temperature for 45 min, further diluted with 0.1% of peptone saline and plated to enumerate coliforms as described below.

The enumeration of coliforms and *E. coli* in mixed microbiological RMs using the Petrifilm EC was performed according to AOAC [63]. An aliquot of 100 µL of dilution was plated on Petrifilm EC and incubated at 37 °C for 24 h. The red and blue colonies with and without gas were counted as coliforms, and the blue colonies with gas as *E. coli*.

The enumeration of coliforms in mixed microbiological RMs was performed using PCA with an overlay of differential media [64]. Aliquots of 1 mL of dilution were inoculated in duplicate in PCA by the pour-plate method. After incubation of 2 h at room temperature or 4 h at 37 °C, an overlay of VRBA (Merck, Germany) or Rambach Agar was added, respectively. The colony counts were performed after 24 h incubation at 37 °C.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) followed by Tukey's test using the Statistical Analysis System and Genetics Software [65]. A p-value of < 0.05 ($p < 0.05$) was considered to be statistically significant.

Results

Enterobacteria survive and grow in minimal medium containing NaCl

Salmonella Enteritidis, *C. sakazakii*, *E. coli,* and *C. freundii* showed similar growth in MMS ($p > 0.05$) and entered in stationary phase after 18 h of cultivation (Fig. S1). These enterobacteria showed to be halotolerant due to their ability to survive and grow in the MM containing 0.5 M of NaCl (MMS), which corresponds to approximately 3% (w/v). It is noteworthy that the addition of this concentration of NaCl in the MM to produce MMS changes the *aw* from 0.993 to 0.977 at 25 °C.

Mn increases the resistance to dehydration of *Salmonella* Enteritidis

Growth in MMS containing 2.5 and 5.0 mM of $MnSO_4$ promoted an increase in survival of *Salmonella* Enteritidis to dehydration for up to 42 days when compared to the control, i.e., cells growing in the absence of Mn (Fig. 1A). The high survival of *C. sakazakii* and *E. coli* to dehydration occurs independently of the presence of Mn (Fig. 1B and C, respectively). Among the coliforms evaluated, *C. freundii* was more sensitive to dehydration, and Mn did not increase its resistance (Fig. 1D).

C. sakazakii is the most resistant to freeze-drying and stabilization

Considering that MMS containing 2.5 and 5.0 mM of $MnSO_4$ increased resistance to dehydration of *Salmonella* Enteritidis, the lowest concentration was chosen for the preparation of cells for dehydration before freeze-dried. On the other hand, as Mn did not alter the survival of *C. sakazakii*, *E. coli*, and *C. freundii* for 49 days of dehydration, the cells were grown in the absence of $MnSO_4$ before freeze-dried. After freeze-drying in reconstituted skim milk powder containing 100 mM of trehalose and stabilization at 4 °C for 90 days, the cultivable cells were quantified and the log CFU.mL^{-1} is presented in Table 1.

C. sakazakii was the most resistant to freeze-drying in reconstituted skim milk powder containing 100 mM of trehalose and after stabilization at 4 °C for 90 days ($p < 0.05$) (Table 1). This result can be better evidenced by the 0.72 cycle log CFU.mL^{-1} reduced after the whole process, including freeze-drying and stabilization ($p < 0.05$) (Table 1). The other evaluated enterobacteria presented up to 2 log CFU.mL^{-1} reduced after freeze-drying and stabilization (Table 1).

Rambach Agar allows differentiating *Salmonella* and coliforms colonies

The definition of a differential medium for counting the four enterobacteria used in the mixed microbiological RM was necessary to evaluate the survival of each bacterial species. The plating by the spread plate method in the Rambach

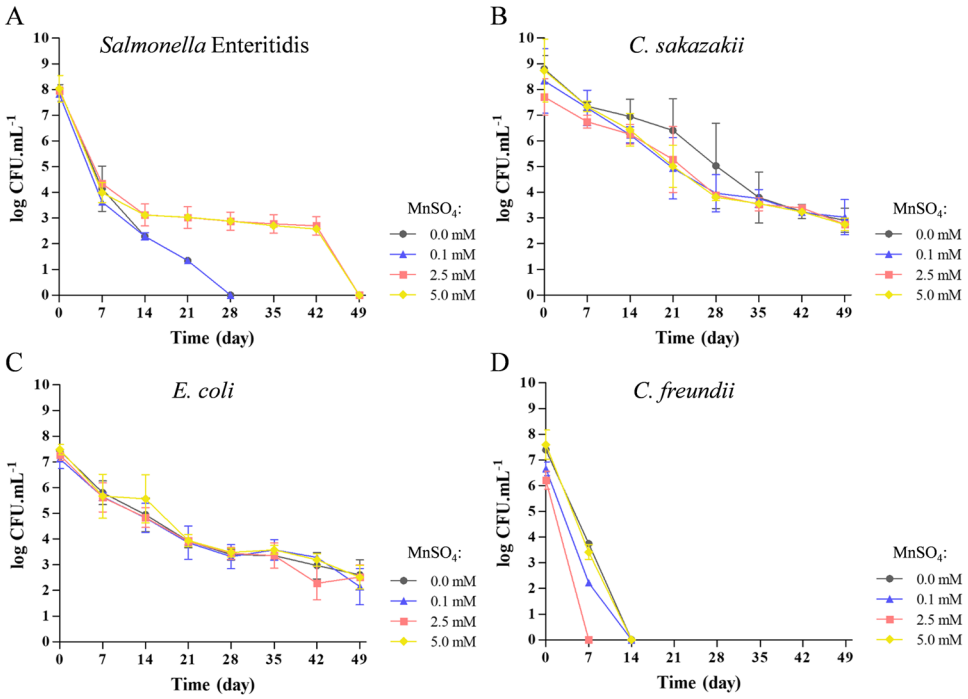

Fig. 1 Evaluation of resistance to dehydration at 25 °C up to 49 days of *Salmonella* Enteritidis (**A**), *C. sakazakii* (**B**), *E. coli* (**C**), and *C. freundii* (**D**), previously cultivated for 38 h in MMS containing 0.0, 0.1, 2.5, or 5.0 mM of $MnSO_4$, separately

Table 1 The means of the logarithm of colony-forming units per milliliter (means log $CFU.mL^{-1}$) of *Salmonella* Enteritidis, *C. sakazakii*, *E. coli,* and *C. freundii* before and after freeze-drying in reconstituted skim milk powder containing 100 mM of trehalose and after stabilizing at 4 °C for 90 days

Bacterial species	Means log $CFU.mL^{-1}$			Means log $CFU.mL^{-1}$ reduced (% reduced)		
	Before freeze-drying	After freeze-drying	After Stabilization	After freeze-drying	After stabilization	After freeze-drying and stabilization
Salmonella Enteritidis	8.48^{a}	7.59^{b}	6.24^{c}	0.89^{A} (10.5)	1.35^{AB} (17.8)	2.24^{A} (26.4)
C. sakazakii	8.64^{a}	8.51^{a}	7.92^{a}	0.13^{B} (1.5)	0.59^{B} (6.9)	0.72^{B} (8.3)
E. coli	8.06^{a}	7.83^{a}	5.27^{b}	0.23^{AB} (2.7)	2.56^{A} (32.7)	2.79^{A} (34.6)
C. freundii	7.31^{a}	6.70^{a}	4.66^{b}	0.61^{AB} (8.3)	2.04^{A} (30.4)	2.65^{A} (36.3)

log $CFU.mL^{-1}$ = logarithm of colony-forming units per milliliter;

The comparisons can be drawn among processes or bacteria. Mean followed by different superscript lowercase letters in the same line (among processes for the same bacteria) and followed by different superscript uppercase letters in the same column for log $CFU.mL^{-1}$ reduced (among bacteria for each process, separately) differs at 5% probability ($p < 0.05$) by Tukey's test

Agar allowed differentiating the colonies of *Salmonella* Enteritidis from the coliforms (Fig. 2A). In this medium, *Salmonella* Enteritidis showed pink colonies, while coliforms showed blue or purple colonies (Fig. 2A). On the other hand, in HE the colonies of four species were different (Fig. 2B). *Salmonella* Enteritidis and *C. freundii* showed black-bluish colonies, whereas *C. freundii* had a yellow halo around the colonies (Fig. 2B). *E. coli* and *C. sakazakii* showed colonies with yellow to orange coloration, but *C. sakazakii* may be differentiated by the mucoid appearance on the surface (Fig. 2B). Petrifilm EC allowed differentiation of *E. coli* and *C. sakazakii* from other species. In this medium, *E. coli* showed blue colonies with gas production, while *C. sakazakii* showed red coloration with gas production (Fig. 2C). *Salmonella* Enteritidis and *C. freundii* colonies were small with red coloration without gas production, which did not distinguish them (Fig. 2C).

The plating by pour plate method in Rambach Agar allowed differentiation of *Salmonella* colonies from coliforms, while in HE it was impossible to differentiate them with the same precision as the other plating method. It was impossible to differentiate the enterobacteria colonies when the BGA, XLD, VRBG, EMB, and MacConkey Agar were used.

Enterobacteria survive in mixed microbiological RMs

Salmonella counts in the mixed microbiological RMs ranged from 3.0 to 4.0 log $CFU.g^{-1}$, and coliforms from

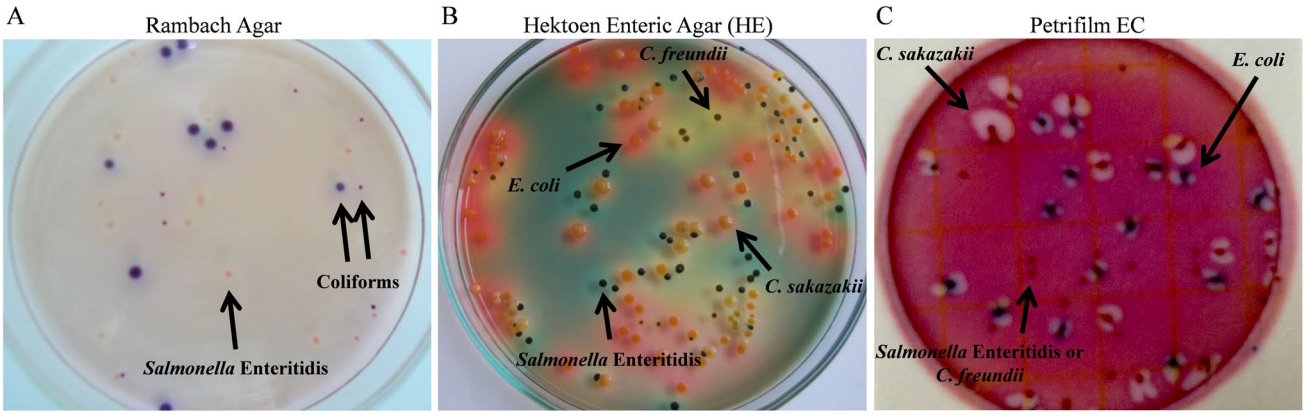

Fig. 2 Characteristics of colonies of *Salmonella* Enteritidis, *C. sakazakii*, *E. coli,* and *C. freundii* plated by spread plate method in Rambach Agar **(A)**, Hektoen Enteric Agar (HE) **(B)** and Petrifilm EC **(C)**

4.0 to 5.0 log CFU.g^{-1} after production and these values were statistically different between microbiological RMs repetitions ($p < 0.05$) (Table 2). Similar reductions of the cycles log CFU.g^{-1} of *Salmonella* were detected in all microbiological RMs after 30 days of storage at -20 °C with a *Salmonella* survival rate of 91.2 to 93.6% ($p > 0.05$). However, the same variation was not observed for coliforms ($p < 0.05$) (Table 2).

The homogeneity of the mixed microbiological RMs was evaluated by two parameters: T1 and T2/(I-1) for *Salmonella* and coliforms, separately (Table 2). The numbers of viable *Salmonella* showed T1 ≤ 18.3 in mixed microbiological RMs (Table 2), indicating repeatability of the counts in the analytical portions or fractions of the mixed microbiological RMs. The counts of coliforms showed values of T1 within the limit for microbiological RM1 and RM2 (Table 2). On the other hand, only microbiological RM1 presented values of T2/(I-1) ≤ 3.0 for counts of *Salmonella* and coliforms (Table 2). However, considering a more rigorous value of T2/(I-1) ≤ 2.0, only the counts of coliforms of microbiological RM1 met this criterion (Table 2). Thus, only microbiological RM1 was homogeneous considering the two stages of homogeneity evaluated (Table 2).

Table 2 Microbiological evaluation of the homogeneity of the microbiological RMs after production and after storage for 30 days at -20 °C

Bacteria	Microbiological RMs	Means log CFU.g^{-1}			Homogeneity		
		After production	After storage	Reduced (% reduced)	Mean [1] (log CFU.g^{-1})	T_1 [2]	T_2/(I-1) [3]
Salmonella	**RM1**	**3.06^{aC}**	**2.79^{bC}**	**0.27 (8.8)**	**2.70**	**10.3**	**3.0**
	RM2	3.93^{B}	3.66^{B}	0.27 (6.9)	3.43	9.3	5.1*
	RM3	4.56^{aA}	4.27^{bA}	0.29 (6.4)	4.18	6.9	4.4*
Coliforms	**RM1**	**4.69^{aB}**	**4.35^{bB}**	**0.33^{B} (7.0)**	**4.26**	**13.9**	**1.9**
	RM2	5.39^{aA}	4.81^{bA}	0.58^{A} (10.8)	4.72	4.0	5.4*
	RM3	4.39^{C}	4.07^{C}	0.32^{B} (7.3)	3.96	20.9*	3.5**

log CFU.g^{-1} = logarithm of colony-forming units per gram of microbiological RM;

The comparisons can be drawn among time or microbiological RM. Mean followed by different superscript lowercase letters in the same line (among time of production and storage for the same microbiological RM) and followed by different superscript uppercase letters in the same column (among microbiological RMs for the same microorganism and time or among log CFU.g^{-1} reduced of microbiological RMs for the same microorganism, separately) differs at 5% probability ($p < 0.05$) by Tukey's test. Where a letter is not shown, no statistical difference among samples was observed;

[1] Mean of log CFU.g^{-1} of 10 analytical portions or fraction of 0.3 g of each microbiological RM;

[2] The T_1 value of homogeneous microbiological RM is less than or equal to 18.3 ($T_1 \leq 18.3$);

[3] The T_2/(I-1) value of homogeneous microbiological RM is less than or equal to 3.0 (T_2/(I-1) ≤ 3.0);

[*] The T_1 or T_2/(I-1) values exceed the limits for a mixed microbiological RM;

[**] The T_2/(I-1) value is invalid;

Homogeneous mixed microbiological RM is shown in bold

Different methods detect *Salmonella* in mixed microbiological RMs

The three mixed microbiological RMs (RM1, RM2, and RM3) were analyzed by three methods for research of *Salmonella* after storage at -20 °C for 30 days. This pathogen was detected in these mixed microbiological RMs through the conventional method, immunomagnetic separation, and PCR (Fig. S2). In addition, all colonies suspected isolated in the conventional method and immunomagnetic separation were characterized biochemically by the API 20E kit and oxidase test and confirmed serologically by *Salmonella* O Antiserum Poly A-I & VI as *Salmonella*.

PCA with an overlay of Rambach Agar recovers more coliforms from mixed microbiological RMs

The enumeration of coliforms in mixed microbiological RMs after storage at -20 °C for 30 days showed a similarity to results obtained in Petrifilm EC and VRBA ($p < 0.05$) and evidenced the superiority of the Rambach Agar in recovering coliforms from RMs (Table 3). In addition, it was possible to enumerate *E. coli* in these RMs by Petrifilm EC (Table 3).

Discussion

The ability of *Salmonella* Enteritidis, *C. sakazakii*, *E. coli,* and *C. freundii* to survive and grow in the MMS that contains a high concentration of NaCl, that is, to be halotolerant, can be explained, at least in part, by some mechanisms, such as regulation and accumulation of osmoprotectants, filamentation, capsule production, and biofilm formation including the production of various extracellular polysaccharides [37, 66–69].

The varied resistance to dehydration, freeze-drying, and stabilization found in this study for the evaluated enterobacteria is recognized. According to Barron and Forsythe [70], enterobacteria may be divided into three groups considering the survival over time of storage of the dehydrated cells. The first group consists of *C. freundii*, *Citrobacter koseri,* and *Enterobacter cloacae*, which were not recovered after six months of storage under dehydration. The second group is composed of *Salmonella* Enteritidis, *E. coli*, and *Klebsiella pneumoniae* that were not recovered after 15 months of storage under dehydration. The third group is composed of *Pantoea* sp., *Klebsiella oxytoca*, and *Escherichia vulneris* that persisted for more than two years of storage under dehydration, and some strains of *C. sakazakii* that were recovered after 2.5 years.

C. sakazakii was the most resistant to dehydration, freeze-drying, and stabilization compared to *Salmonella* Enteritidis, *E. coli*, and *C. freundii* in this study. Breeuwer et al. [27] also showed that *C. sakazakii* was more resistant to osmotic stress and dehydration conditions than *Salmonella*, *E. coli,* and *C. freundii* and suggested that the resistance may be related to the accumulation of trehalose in the cells. The desiccation tolerance of *C. sakazakii* was also significantly higher than *E. coli* O157:H7, *Salmonella enterica,* and *Listeria monocytogenes* in powdered infant formula [71]. Besides, the freeze-drying of *C. sakazakii* ATCC 29544 in whole milk had the highest protective effect, followed by skim milk and tryptic soy broth [72]. Milk contains proteins that provide an additional protective layer for cells during drying by stabilizing membrane [45, 48, 73–77] and, when added with trehalose or sucrose,

Table 3 Enumeration of coliforms and *E. coli* by different methods in the mixed microbiological RMs after 30 days at -20 °C

Microorganisms	Methods	Means log CFU.g^{-1}		
		Microbiological RMs		
		RM1	RM2	RM3
Coliforms	PCA + Rambach Agar overlay	4.35^{bA}	4.81^{aA}	4.07^{cA}
	Petrifilm EC for coliform count plates	3.65^{cB}	4.45^{aB}	3.78^{bB}
	PCA + VRBA overlay	3.56^{bB}	4.24^{aB}	3.78^{bB}
E. coli	Petrifilm EC for *E. coli*	2.65^{b}	3.72^{a}	2.39^{c}

Microbiological RM = Microbiological reference material;

PCA = Plate Count Agar;

Petrifilm EC = Petrifilm *E. coli*/Coliform Count Plates;

VRBA = Violet Red Bile Agar;

log CFU.g^{-1} = logarithm of colony-forming units per gram of microbiological RM;

Mean followed by different superscript lowercase letters in the same line (among mixed microbiological RMs for the same method) and followed by different superscript uppercase letters in the same columns (among methods as Rambach Agar, Petrifilm EC for coliform count plates and VRBA for the same mixed microbiological RM) for log CFU.g^{-1} differs at 5% probability ($p < 0.05$) by Tukey's test

provide a higher survival rate during subsequent storage [73, 74, 78].

The high resistance of *Cronobacter* to desiccation may also be related to several genes as those of the Cpx system that encode an envelope stress response regulator, *dnaK* and *dnaJ* genes that encode two molecular chaperones, and the sigma factors RpoN and RpoS seem to be the main signals regulating the bacterial response to hyperosmotic conditions [79]. Furthermore, all *Cronobacter* contain genes for the production of β-carotene, which is believed to protect bacteria from harmful oxygen radicals and add tolerance to physical desiccation [80, 81].

The high survival of *E. coli* to dehydration shown in our study can be attributed to the intracellular accumulation of trehalose. *E. coli* MC4100 grown in an M9 medium containing 1% (w/v) of glucose, trace elements (0.015 mM of $FeSO_4$, 0.015 mM of $ZnSO_4$, and 0.015 mM of $MnSO_4$) and 0.6 M of NaCl until the stationary phase presented high levels of intracellular trehalose and was more tolerant to desiccation [82]. Although the accumulation of trehalose increases the tolerance of *E. coli* to desiccation, this is not the only factor related to the resistance of this bacterium [36, 82]. The *rpoS* also was required by *E. coli* O157:H7 to tolerate osmotic and desiccation stress [83]. Furthermore, Chen and Goulian [32] showed that the general stress response regulator RpoS and the transcriptional regulators DksA, LexA, RecA, and ArcA play a critical role in the survival of *E. coli* to dehydration. These authors also identified two additional regulators, Crl and ArcZ that promote dehydration tolerance through modulation of RpoS.

Considering the effect of Mn, only *Salmonella* Enteritidis increased resistance to dehydration in the presence of this element, and this may be related to the observation by Maserati et al. [84]. These authors showed that the Mn transport transcriptional regulator (*mntR*) of *Salmonella* Typhimurium ATCC 14028 was induced by low *aw*, and consequently, the increase in intracellular Mn can reduce the effects of oxidative stress from dehydration, promoting cell survival. Interestingly, the RNA polymerase sigma factor RpoS (σ^S), which is required for stress resistance, such as starvation, hyper-osmolarity, and oxidative stress, induces transcription of the Mn transporter genes *mntH* and *sitABCD* and prevents their repression by the Mn-responsive regulator MntR and the ferric uptake regulator Fur in *Salmonella* Typhimurium ATCC 14028 [85].

Thus, we have applied the results found in this study to the development of mixed microbiological RMs of *Salmonella* Enteritidis and the three coliforms. The conditions adopted, such as growth in MMS containing 2.5 mM of $MnSO_4$ until the stationary phase followed by freeze-drying in skim milk powder containing trehalose, may have contributed to the cell stability of *Salmonella* Enteritidis in the mixed microbiological RMs. It is noteworthy that minor variations of cell viability result from changes that may occur during dehydration and storage [35].

Of the mixed microbiological RMs produced, only one was homogeneous, and the lack of homogeneity of the others may be due to the difficulty in dispersing the bacterial cells in the milk powder during the various mixing steps. In microbiological RMs containing approximately 2.0 and 3.0 log CFU of *Salmonella* Enteritidis per capsule in the presence of competing microorganisms from chicken feces, the values of T2/(I-1) found were 1.7 and 3.1, respectively [86]. These authors also produced microbiological RMs containing, on average, 2.44 log CFU of *Salmonella* Typhimurium per capsule in the presence of a competing microbiota and observed a T2/(I-1) value of 5.57. In a second study, microbiological RMs containing about 2.0 and 2.7 log CFU of *Salmonella* Enteritidis per capsule and the same competing microorganisms presented T2/(I-1) values of 1.91 and 1.58, respectively [87]. Microbiological RMs produced with 2.0 log $CFU.mL^{-1}$ of *Salmonella* Enteritidis ATCC 13076 by freeze-drying in 14% of fish protein hydrolysate (FPH) from catfish (*Clarias batrachus*) [3] and tuna (*Euthynnus affinis*) [7] were homogeneous and stable at a storage temperature of -20 ºC for 28 days, and survival rates were between 1.2 and 1.4 log $CFU.mL^{-1}$, respectively. On the other hand, the microbiological RMs prepared with 10 to 14% of these matrices were not homogeneous [3, 7]. However, these authors assessed the homogeneity of the microbiological RMs by different analyzes from those used in this study.

Although not all mixed microbiological RMs developed in the present study meet the criteria for homogeneity, it was possible to detect *Salmonella* in all of them by the conventional method, immunomagnetic separation, and PCR. Thus, the competing microbiota in our mixed microbiological RMs did not interfere in the detection of *Salmonella* by the methods used, meeting an interesting criterion for microbiological RMs [1]. The *invA* gene was also used by Múrtula et al. [9] to quantify by quantitative PCR (qPCR) *Salmonella* spp. present in RM developed in tablet format.

Finally, Rambach Agar was allowed to differentiate *Salmonella* and coliforms colonies, as well as greater recovery of coliforms from mixed microbiological MRs when performed on PCA with an overlay of Rambach Agar. The highest count on Rambach Agar may be due to the recovery of the injured cells in a non-selective medium such as PCA for 4 h at 37 °C before adding an overlay of Rambach Agar, a selective and differential medium. In the VRBA, the previous recovery in PCA was also performed, but the count was statistically lower than in Rambach Agar. This difference can be attributed to the short time and inadequate temperature of recovery, which were room temperature and 2 h, respectively. In Petrifilm EC, the injured cells are directly plated in the selective medium without previous recovery, which may justify the lower enumeration of coliforms. Smith

et al. [88] also showed that the number of cells recovered from *E. coli* O157:H7 using a non-selective medium with an overlay of the selective medium was significantly higher relative to direct inoculation in Petrifilm EC. However, most microbiological analyses of foods may involve direct inoculation of the samples into a specific selective medium for the subsequent enumeration of microorganisms. Most injured bacteria do not grow in a selective medium, taking a count to underestimate and threatening consumers' health [88, 89].

Therefore, the production of stable, homogeneous, and recoverable mixed microbiological RMs, containing a higher number of culturable cells and a lower number of injured cells, remains an important issue that needs to be further investigated as it is the critical point [1]. These challenges must be overcome until scientific knowledge can be transformed into innovative, affordable and low-cost products [1, 90, 91], as there is a shortage of microbiological RMs available [1].

Conclusion

The cell cultivation conditions to increase bacterial resistance to the stresses inherent in the production process of the microbiological RMs is necessary due to the importance of the cell viability in microbiological RMs for food microbiology. Therefore, *Salmonella* survival to freeze-drying was successfully obtained when cells were previously cultivated in MMS until the stationary phase, added with Mn and submitted to osmotic stress. This pathogen was also successfully recovered from the mixed microbiological MRs containing coliforms using different methods, and the Rambach Agar allowed the differentiation of colonies of *Salmonella* and coliforms. Thus, this knowledge generated will contribute to the development of other more stable and homogeneous mixed microbiological RMs in the dehydrated form.

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Author contribution Maria Roméria da Silva: conceptualization, methodology, investigation, data curation, writing-original draft, writing-review, and editing. Felipe Alves de Almeida: investigation, data curation, writing-original draft, writing-review, and editing. Ana Íris Mendes Coelho: methodology and investigation. Fernanda Lopes da Silva: investigation. Maria Cristina Dantas Vanetti: conceptualization, supervision, project administration, funding acquisition, resources, writing-original draft, writing-review, and editing.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Not applicable.

Consent for publication The authors Maria Roméria da Silva, Felipe Alves de Almeida, Ana Íris Mendes Coelho, Fernanda Lopes da Silva, and Maria Cristina Dantas Vanetti are in accordance with the submission of this manuscript to the Brazilian Journal of Microbiology.

Conflict of interest The authors declare that they have no conflict of interest.

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