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https://doi.org/10.22239/2317-269x.01695

Revista Visa em debate
sociedade, ciência & tecnologia

Production of proficiency test items containing bacteria in chicken matrix using freeze-drying technique

Produção de itens de ensaio de proficiência contendo bactérias em matriz frango utilizando a técnica de liofilização

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Received: 07 Jul 2020
Approved: 26 Oct 2020

ABSTRACT

Introduction: Participation in proficiency testing (PT) is used to evaluate the competence of testing and calibration laboratories. **Objective:** To evaluate the viability of the freeze-drying technique to produce six lots of PT items containing two *Escherichia coli*, two containing *Bacillus cereus* and *Staphylococcus aureus* concomitantly, and two containing *Salmonella* Enteritidis in baked shredded chicken matrix. **Method:** Homogeneity according to harmonized protocol and long and short-term stability testing according to ISO GUIDE 35 were carried out. **Results:** All lots produced were sufficiently homogeneous. In the long-term stability study, all lots were sufficiently stable at temperatures of -80 ± 10°C and -20 ± 4°C, except for the lot containing *B. cereus* and *S. aureus*. The other lots presented stability for at least 126 days at -80 ± 10°C and 84 days at -20 ± 4°C. The short-term stability was carried out only with the lots sufficiently stable in the long-term stability study. The lots were sufficiently stable at temperatures of 5 ± 3°C and at 35 ± 2°C, except for the lot containing *Salmonella* Enteritidis at 35 ± 2°C due to the significant decrease in cell concentration. **Conclusions:** The freeze-drying technique was satisfactory for the production of test items containing *E. coli* e *Salmonella* Enteritidis in the chicken matrix viable to use in a PT, but the lot containing *Salmonella* Enteritidis must be transported to the laboratories at the temperature ≤ 8°C for up to four days. Lots containing *S. aureus* e *B. cereus* simultaneously presented insufficient stability, indicating that the production of individual lots containing each bacteria individually is necessary.

KEYWORDS: Proficiency Test; Assay Laboratories; Test Items; Quality Control; Chicken Meat

RESUMO

Introdução: A participação em ensaios de proficiência (EP) é utilizada para avaliar a competência de laboratórios de ensaio e calibração. **Objetivo:** Avaliar a viabilidade da técnica de liofilização na produção de seis lotes de itens de EP, dois contendo *Escherichia coli*, dois contendo *Bacillus cereus* e *Staphylococcus aureus* concomitantemente, e dois contendo *Salmonella* Enteritidis em matriz frango desfiado cozido. **Método:** Foram realizados testes de homogeneidade segundo o protocolo harmonizado e de estabilidade em longo prazo pelo modelo clássico e curto prazo pelo modelo isócrono segundo a ISO Guide 35. **Resultados:** Todos os lotes produzidos foram considerados suficientemente homogêneos. No estudo de estabilidade em longo prazo, todos os lotes se apresentaram suficientemente estáveis nas temperaturas de -80 ± 10ºC e -20 ± 4ºC, exceto o lote contendo *B. cereus* e *S. aureus*. Os outros lotes apresentaram estabilidade por pelo menos 126 dias a -80 ± 10ºC e 84 dias a -20 ± 4ºC. Na avaliação da estabilidade em curto prazo, foram analisados apenas os lotes suficientemente estáveis no estudo em longo prazo. Os lotes foram suficientemente estáveis nas temperaturas de 5 ± 3ºC e 35 ± 2ºC, com exceção do lote contendo *Salmonella* Enteritidis a 35 ± 2ºC, devido ao decréscimo significativo da concentração celular. **Conclusões:** A técnica de liofilização foi satisfatória para produção de itens de ensaio contendo *E. coli* e *Salmonella* Enteritidis em matriz frango viáveis

para utilização em um EP, sendo que o lote contendo *Salmonella* Enteritidis deve ser transportado aos laboratórios participantes em temperatura ≤ 8°C por até quatro dias. Lotes contendo *S. aureus* e *B. cereus,* simultaneamente, apresentaram estabilidade insuficiente, indicando que a produção de lotes individuais contendo cada bactéria individualmente é necessária.

PALAVRAS-CHAVE: Ensaio de Proficiência; Laboratórios de Ensaio; Itens de Ensaio; Controle de Qualidade; Carne de Frango

INTRODUCTION

Brazil is in 2nd place in the world in the production of chicken cuts, with 12.9 million tons being produced and consumption of 41.1 kg of meat *per capita*, according to data from the Brazilian Association of Animal Protein (ABPA)[1]. The presence of pathogenic microorganisms in chicken meat sold in different countries, including Brazil, has already been reported and shows the importance of strict control in the production chain of this type of food[2,3].

Food-transmitted diseases (FTD) represent a significant impediment to the socioeconomic development of countries. According to estimates by the United Nations (UN), in 2015, the number of deaths per year from FTD in the world is 420,000. In America, for example, there are 9,000 deaths and it is estimated that 600 million people are affected annually[4]. In Brazil, data from the Ministry of Health from 2009 to 2018 report that *Salmonella* spp. is the second most identified etiologic agent in FTD outbreaks, with 11.2% of cases, just behind *Escherichia coli* (24,0%)[5]. Poultry meat *in natura*, processed meat, and offal were identified as the incriminated food in 84 of the total of 2,403 outbreaks that occurred in this period[5].

The reliability of the results issued by food quality control laboratories is of paramount importance to prevent risks to consumer health and avoid economic losses[6]. One of the most used quality assurance systems in testing laboratories is the ABNT NBR ISO/IEC 17025:2017 standard, which describes the general requirements for the competence of testing and calibration laboratories. One of these requirements is periodic participation in proficiency testing (PT). PT plays a valuable role as it provides objective proof of the competence of the participating laboratory. This evidence can be used for: improving the methodology used by the participant, increasing confidence in the results issued in the specific test, and as evidence of competence for accreditation and inspection bodies[8,9]. In addition, the standard allows mutual recognition between member countries of the *International Laboratory Accreditation Cooperation* (ILAC), which is an international forum that encompasses the accreditors of calibration and testing laboratories. Brazil is the only country in Latin America to obtain this recognition, an act that took place in November 2000, which gave the test reports (analytical reports) carried out in laboratories accredited by the National Institute of Metrology, Standardization and Industrial Quality (Inmetro) its acceptance by all countries that make up the forum. Accreditation facilitates international food trade practices and strengthens the country's sovereignty[10].

According to the Resolution of the Collegiate Board of Directors (RDC) No. 12, of January 12, 2001[11], which establishes the microbiological standards for foods, for ready-to-eat dishes (ready-to-eat foods from kitchens, restaurants and similar) based on meats, the search for *Salmonella* sp/25, is recommended, and coliform count at 45°C, coagulase positive staphylococci (CoPS), *Bacillus cereus*, and sulphite reducing clostridia at 46°C. As chicken meat is widely consumed and has great economic interest in Brazil, it is necessary that national testing laboratories that carry out microbiological control analyzes on these products produce reliable results. Given the need for quality and reliability of tests performed by these laboratories, the aim of this work was to evaluate the stability of *E. coli, B. cereus* and *Staphylococcus aureus* concomitantly, and *Salmonella* Enteritidis in chicken matrix aiming at the production of batches of test items (TI) to be used in PT.

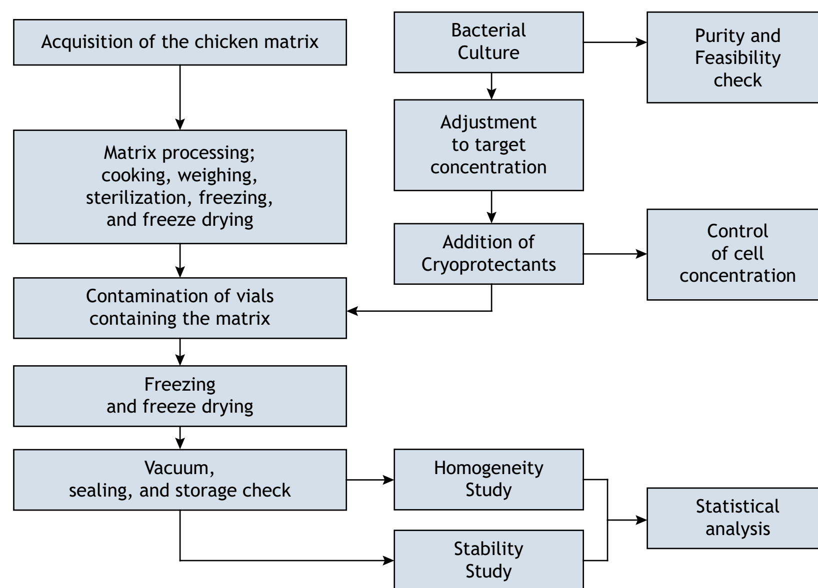
METHOD

Development of test item production methodology

Three pilot batches named EC-P, SALM-P, and BCSTA-P were produced containing *E. coli*; *Salmonella* Enteritidis; *B. cereus* + *S. aureus*, respectively, with 50 vials each. The production of these pilot batches aimed to evaluate the viability of microorganisms in this matrix before producing batches on a larger scale. Subsequently, three other final batches named EC-F, SALM-F, and BCSTA-F were produced, containing 150 vials each. The same procedures were used for all batches. The development of the methodology for the production of TI was based on the methodology described by Brandão et al.[12], who used the lyophilization technique to produce TI containing bacteria in a beef matrix. A diagram containing the steps of this study can be seen in Figure 1.

Matrix preparation

The chicken was obtained from a supermarket in the city of Rio de Janeiro-RJ in the form of frozen chicken fillets of the same brand and these were within the expiration date. The chicken fillets were immersed in water and pressure cooked for 20 min. At the end of cooking, the water was discarded, and the shredded chicken was fractionated into 5.0 g aliquots in glass jars with a capacity of 25 mL (Schott, Brazil) with rubber stoppers suitable for lyophilization (Schott, Brazil). In order to eliminate the intrinsic contamination, the vials containing the matrix were submitted to a sterilization cycle of 121°C/15 min. Then, they were frozen in an *ultrafreezer* at -80 ± 10°C (THERMO, USA) and subjected to a lyophilization cycle for 24 h at 200 μbar in lyophilizer model Minifast 4 (IMALIFE, China).



Source: Elaborated by the authors, 2020.

Figure 1. Schematic of production steps of test item batches containing microorganisms in chicken matrix.

Cultivation of bacterial strains

The choice of microorganisms followed the criteria of the Brazilian legislation in force at the time, RDC nº 12/2001[11], indicated for ready-to-eat chicken dishes. The strains of *E. coli* (P4328), *Salmonella* Enteritidis (P3440), *B. cereus* (P3441), and *S. aureus* (P4283) were isolated from: lettuce *in natura*, frozen chicken leg, cassava flour and ricotta cheese, respectively. These bacteria were isolated in the Food Sector of the National Institute for Quality Control in Health of the Oswaldo Cruz Foundation (INCQS/Fiocruz) and had their identity confirmed by phenotypic characterization using the Vitek 2.0 (bioMérieux, Durham NC, USA) semi-automated system and conventional biochemical tests[13].

All strains were grown in brain-heart infusion broth (BHI; Merck, Germany) at 35 ± 2ºC for 24 h. *E. coli* and *Salmonella* Enteritidis were then cultivated in Luria Bertani (LB) broth with 2.4% of NaCl (Difco, EUA) at 35 ± 2ºC for 28 h. Eight milliliters of each culture were centrifuged at 9,000 g for 10 min (Eppendorf, EUA) in sterile microtubes with a capacity of 2 mL each. The supernatants were discarded and the precipitate, resuspended in peptone saline solution (PSS) at 0,1%. Then, they were homogenized in a tube shaker apparatus. For *Salmonella* Enteritidis and *E. coli* cultures, supernatants were discarded, and the pellet was resuspended in 1 mL of 0,1% PSS (Merck, Germany) with 100 mM of cryoprotectant trehalose. Now for the *B. cereus* and *S. aureus* cultures, cryoprotectant sucrose (100 mM) was used. The concentration was adjusted in photocolorimeter (Libra S2, biochrom, England) at 520 nm wavelenght. Dilutions were performed in 0.1% PSS to reach the target concentration in each batch. For *Salmonella* Enteritidis and *E. coli* cultures, the bacterial suspension was held for approximately 30 min at 5 ± 3°C. In preparing the final batch of *E. coli* (EC-F), it was decided to prepare the batch with a higher concentration than the pilot batch (EC-P) so that the participating laboratories could use the plate counting methodology, which is recommended for foods that are expected to have a microbial load greater than 10^2/g[13].

Preparation of test items

A 1:100 dilution of the suspension containing the bacteria in 0.1% PSS containing cryoprotectant was carried out. This solution was homogenized on a shaker plate (Corning, USA), using a sterile magnet, for 30 min and kept in an ice bath, the latter having the function of maintaining the temperature of the solution around 4°C, so that the bacteria did not proliferate, not changing the desired target concentration before lyophilization. Still under agitation, volumes of 0.5 mL of the suspension were dispensed into vials containing 5 g of pre-lyophilized chicken using a peristaltic pump (Watson-Marlow, England).

In order to verify the number of cells to be used in the inoculum of chicken aliquots, serial dilutions of the microorganism suspensions and plated in plate count agar (PCA) (Merck, Germany) of *E. coli* and *Salmonella* Enteritidis. Plates were incubated at 35 ± 2°C for 48 h. For the other microorganisms, decimal dilutions were prepared in 0.1% PSS and 0.1 mL, was seeded, using the *spread-plate* technique, in duplicate, on Baird Parker agar (DIFCO, France) and mannitol egg yolk agar polymyxin (MYP, DIFCO, France) to count *S. aureus* and *B. cereus*, respectively. Baird Parker agar plates were incubated at 36 ± 1°C for 48 h, while MYP agar plates were incubated at 30 ± 2°C for 24 h. This quantification was performed after the homogenization of the solution, before the contamination of the vials, in order to verify if the concentration of cells present in the solution was similar to the planned one.

The vials were frozen in *ultrafreezer* at -80 ± 10ºC and subjected again to a lyophilization cycle for 26 h, as described above. After removing the closed vials from the liophilizer, a visual inspection was carried out in order to evaluate the appearance of the lyophilized samples. Bottles that presented the material with a liquefied or caramelized appearance would be discarded. The vials approved in this inspection were tested for the presence of a vacuum using an electric spark emitter (Tesla Coil, 2-12-8, Brazil). The vials that showed the presence of vacuum were sealed with metal lids, labeled and stored at -80 ± 10ºC and -20 ± 4ºC, those that did not show the presence of vacuum were discarded.

Homogeneity study

Ten units of each pilot batch and 16 units of each final batch, randomly selected, were quantified, using the sampling of the Microsoft Office Excel® 2010 program as a tool for this selection. Analyzes were performed one day after lyophilization and batch storage. The vials were removed from the freezer at -80 ± 10ºC and kept at room temperature for 30 min before analysis. The matrix was reconstituted with 20 mL 0,1% PSS. After 15 min, the matrix was transferred to a sterile *Whirl-Pak® Filter Bag* (Nasco, USA) plastic bag and an additional 25 mL of 0.1% PSS was added. Then, it was homogenized in a *Stomacher* apparatus (*Seward Fisher Scientific*, Canada), at normal speed level for 1 min, making up the 10^{-1} dilution. From the 10^{-1} dilution, serial decimal dilutions were prepared.

For the homogeneity studies for counting *E. coli*, the methodology of depth seeding in 10 mL of PCA agar was used. After 4 h of incubation at 35 ± 2˚C, 10 mL of an overlay of violet red bile lactose agar (VRBA, Difco, France) was added[12]. In addition, the most probable number technique (MPN) was performed in lauryl tryptose (LST) broth[14]. To count *Salmonella* Enteritidis, depth seeding was performed in 10 mL of PCA. After 4 h of incubation at 35 ± 2˚C, 10 mL of Crystal Violet, Neutral Red, Bile Agar with Glucose (VRBG, Difco, France) was added[12]. For the count of *S. aureus* and *B. cereus*, this was performed by the *spread-plate* technique on Baird Parker agar[15] and MYP agar[16], respectively. The count of colonies on the plates was performed taking into account the precision limit of the techniques mentioned above.

The statistical evaluation of homogeneity was performed according to the harmonized protocol[17], attributing the target standard deviation value (σp) to the cell concentration of 0.25 $\log_{10}$, which is a value already established and commonly used by providers in other PT rounds, including the INCQS/Fiocruz[12,18,19,20,21,22,23]. From the results obtained, the value of variance between samples (S^2am) was compared with the critical value of homogeneity (c). If $S^2am < c$, the batch is considered sufficiently homogeneous.

Long- and short-term stability study

Long-term stability studies were performed at storage temperatures -20 ± 4ºC, and reference temperatures - 80 ± 10ºC in the pilot and final batches; and short-term stability in the final batches. This last study mimics the temperatures that TIs may be exposed to during shipment to laboratories participating in the PT.

Long-term stability was analyzed following the classic model[24]. The period evaluated in this study is shown in the Chart, which demonstrates, at each point analyzed, two vials of the lot, using the methodology described in the homogeneity study.

To conduct the short-term stability study, the methodology of *Isochronous Design* or isochronous model was used[24]. Stability conditions were evaluated over a period of four days, at two temperatures: 5 ± 3ºC and 35 ± 2ºC. Each day, four bottles, which were stored at -80 ± 10ºC, were randomly selected and placed in two boxes suitable for transporting biological material (two vials in each box). The boxes were incubated at the temperatures tested for a total of four days. The incubation conditions of time and temperature were maintained until the date of analysis. Four days after the date of the first incubation, the vials were analyzed at the same time, under the same conditions, thus maximizing the repeatability of the analyses. The methodologies used for bacterial enumeration were the same as those described in the homogeneity study.

The statistical evaluation of the results was performed by the analysis of the residuals of the regression together with the Analysis of Variance (ANOVA) according to the ABNT ISO Guide 35[24].

RESULTS AND DISCUSSION

Control of cell concentration during the preparation of test items

Bacteria concentration did not show significant logarithmic differences after the lyophilization process, except for batch EC-F (Table 1). Despite the increase in the cellular concentration of the EC-F batch at the time of production, there was a prominent decrease after the lyophilization, which was not observed during

Chart. Outline of the long-term stability study of the batches produced in this study.

Batch	Temperature (°C)	Number of analyzes performed (period in which the analysis was performed in days)
SALM-P	- 80 ± 10	3 (0, 7, 21)
	-20 ± 4	4 (0, 7, 21, and 35)
SALM-F	- 80 ± 10	7 (0, 3, 31, 62, 90, 115, and 153)
	-20 ± 4	7 (0, 3, 17, 31, 45, 62, and 80)
EC-P	- 80 ± 10	3 (0, 8, 22)
	-20 ± 4	4 (0, 8, 22)
EC-F	- 80 ± 10	6 (0, 15, 35, 63, 93, and 126)
	-20 ± 4	7 (0, 11, 21, 35, 49, 63, and 84)
BCSTA-P	- 80 ± 10	3 (0, 7, 22)
	-20 ± 4	4 (0, 8, 22 e 38)
BCSTA-F	- 80 ± 10	3 (0, 7, and 35)
	-20 ± 4	5 (0, 7, 21, 38, and 49)

Source: Elaborated by the authors, 2020.
SALM: batches produced with *Salmonella* Enteritidis; EC: batches produced with *E. coli*; BCSTA: batches produced *B. cereus* and *S. aureus*; P: batches produced on a pilot scale; F: final batches produced on a larger scale.

the production of the EC-P pilot batch, even though the same production process was used. Therefore, the concentration of cryoprotectant (trehalose at 100 mM) may not have been sufficient to prevent cell death during lyophilization in this highest concentration of *E. coli*. Further studies are needed to understand the instability of this microorganism in the production of TI in this matrix. In the other batches, the results indicated that lyophilization did not significantly change the concentration of bacteria.

No vials failed after visual inspection. Regarding the presence of vacuum, there was a variation from 87.8% to 96.0% in the batches produced (Table 1). This result was similar to those obtained in other studies that used lyophilization in the preparation of batches of TI for PT and detected at least 83.3% of the vials with the presence of vacuum in the batches produced[12,18,19,21,22,23,25].

Homogeneity study

The results of the homogeneity study are shown in Table 2. Homogeneity guarantees that laboratories participating in a PT receive TI that do not present significant differences in the parameters to be measured[17,24]. In this study, all batches were sufficiently homogeneous, according to the Harmonized Protocol (Table 2). This result is consistent with previous studies that used the peristaltic pump to distribute the bacterial suspension under constant agitation and the lyophilization technique for preparing TI with microorganisms in matrices such as cheese and milk[19,20,25]. Brandão et al.[12] were successful in producing sufficiently homogeneous TI containing *Salmonella* Enteritidis in raw beef matrix.

Stability studies

The results of the homogeneity study are shown in Table 3.

The stability study verifies the possible variations that the TI of a batch may suffer, changing its homogeneity characteristic[24]. Initially, a trend analysis was performed by visualizing the data in the form of graphs (Figure 2). In this analysis, it was found that the BCSTA-F batch presented a vertiginous drop in the number

Table 1. Concentration of microorganisms in batches before and after the lyophilization process and percentage of vacuum.

Batches	Cell concentration (log_{10} CFU/g)			Vacuum presence (%)
	Expected final concentration	Before lyophilization	After lyophilization	
SALM-P	3.70	3.64	3.00	87.8
SALM-F	3.70	3.56	3.18	94.6
EC-P	2.00	2.04	2.26	91.6
EC-F	3.70	4.34	2.84	92.0
BCSTA-P - *B. cereus*	4.00	4.72	4.11	90.0
S. aureus	3.70	4.08	3.60	
BCSTA-F - *B. cereus*	4.00	4.04	3.92	95.9
S. aureus	3.70	4.28	3.67	

Source: Elaborated by the authors, 2020.
CFU: colony forming units; SALM: batches produced wit *Salmonella* Enteritidis; EC: batches produced with *E. coli*; BCSTA: batches produced *B. cereus* and *S. aureus*; P: batches produced on a pilot scale; F: final batches produced on a larger scale.

Table 2. Result of the homogeneity study of the pilot and final batches containing *Salmonella* Enteritidis, *E. coli*, and *B. cereus* + *S. aureus* produced in this study.

Batch	Microorganism	Method	Average (log_{10}/g)	S^2am	c	Result
SALM-P	*Salmonella* Enteritidis	Plate count	3.05	0.009	0.01	SH
SALM-F	*Salmonella* Enteritidis	Plate count	1.98	0.010	0.02	SH
EC-P	*E. coli*	Plate count	1.54	0.010	0,03	SH
		MPN	1.46	0.010	0.04	SH
EC-F	*E. coli*	Plate count	2.92	0.001	0.01	SH
		MPN	2.84	0.010	0,06	SH
BCSTA-P	*B. cereus*	Plate count	3.99	0.001	0,06	SH
	S. aureus	Plate count	3.70	0.010	0,36	SH
BCSTA-F	*B. cereus*	Plate count	3.98	0.010	0.02	SH
	S. aureus	Plate count	3.71	0.001	0.02	SH

Source: Elaborated by the authors, 2020.
SALM: batches produced with *Salmonella* Enteritidis; EC: batches produced with *E. coli*; BCSTA: batches produced *B. cereus* and *S. aureus*; P: batches produced on a pilot scale; F: final batches produced on a larger scale; SH: sufficiently homogeneous; MPN: Most probable number; S^2am: value of variance between samples; c: critical value of homogeneity.

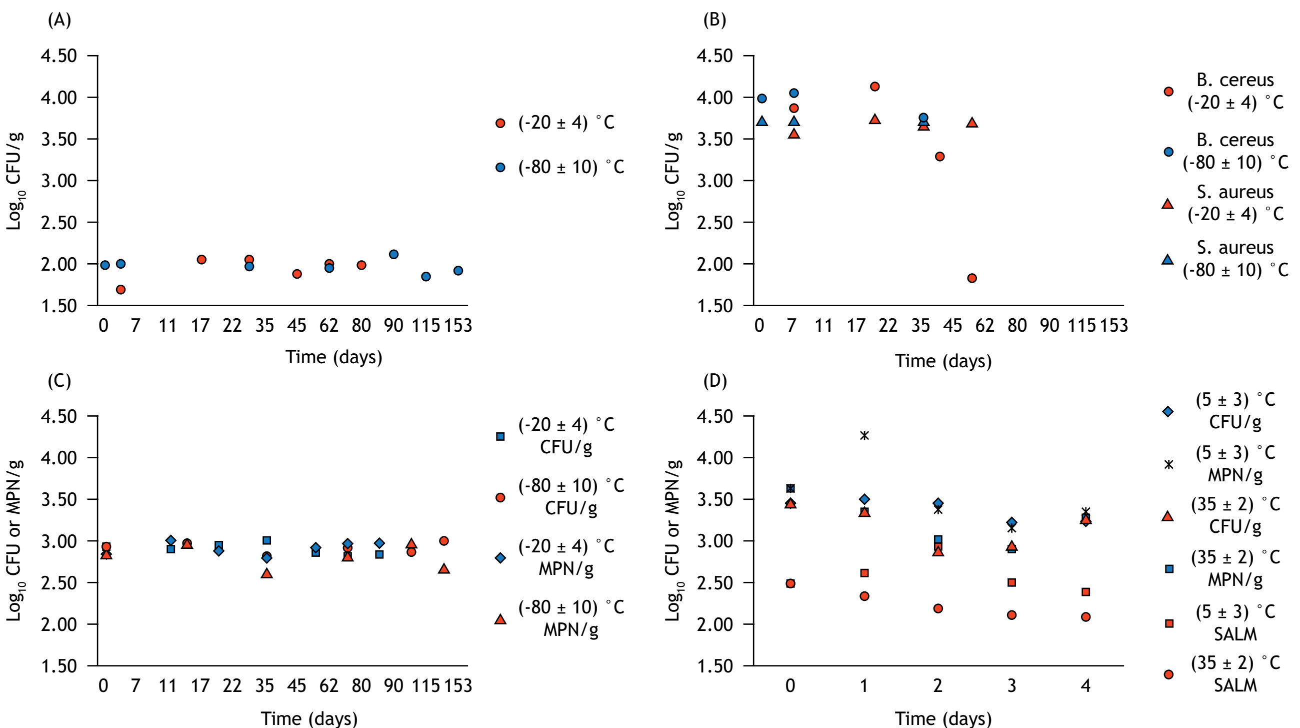
Table 3. Results of the stability study of the batches produced in this study.

Batch	Method	Study temperature (°C)	Angular coefficient	Lower limit (95%)	Upper limit (95%)	Result
SALM-P	Plate count	-80 ± 10	-0.00320	-0.05700	0.05000	SE
		-20 ± 4	-0.00355	-0.01399	0.00690	SE
SALM-F	Plate count	-80 ± 10	-0.00056	-0.00300	0.00180	SE
		-20 ± 4	0.00110	-0.00353	0.00574	SE
		5 ± 3	-0.03170	-0.26820	0.20490	SE
		35 ± 2	-0.10360	-0.15860	-0.04860	NE
EC-P	Plate count	-80 ± 10	-0.00540	-0.12000	0.11000	SE
		-20 ± 4	-0.01744	-0.03662	0.00174	SE
	MPN	-80 ± 10	-0.00057	-0.07200	0.07100	SE
		-20 ± 4	-0.01405	-0.03883	0.01073	SE
EC-F	Plate count	-80 ± 10	0.00036	-0.00140	0.00220	SE
		-20 ± 4	-0.00140	-0.00325	0.00046	SE
		5 ± 3	-0.06930	-0.13930	0.00080	SE
		35 ± 2	-0.07630	-0.34040	0.18780	SE
	MPN	-80 ± 10	0.00200	-0.00270	0.00670	SE
		-20 ± 4	0.00039	-0.00223	0.00300	SE
		5 ± 3	-0.16400	-0.55270	0.22470	SE
		35 ± 2	-0.11480	-0.36190	0.13240	SE
BCSTA-P (*B. cereus*)	Plate count	-80 ± 10	-0.00320	-0.07100	0.06400	SE
		-20 ± 4	0.00154	-0.00579	0.00887	SE
BCSTA-P (CoPS)	Plate count	-80 ± 10	0.00710	-0.00170	-0.12000	SE
		-20 ± 4	0.00707	-0.00169	0.01583	SE
BCSTA-F (*B. cereus*)	Plate count	-80 ± 10	-0.01000	-0.05200	0.03200	SE
		-20 ± 4	-0.05864	-0.13024	0.01296	SE
BCSTA-F (CoPS)	Plate count	-80 ± 10	-0.00160	-0.00650	0.00330	SE
		-20 ± 4	0.00166	-0.00485	0.00817	SE

Source: Elaborated by the authors, 2020.
SALM: batches produced with *Salmonella* Enteritidis; EC: batches produced with *E. coli*; BCSTA: batches produced *B. cereus* and *S. aureus*; P: batches produced on a pilot scale; F: final batches produced on a larger scale; MPN: most likely number; SE: stable enough; NE: not stable enough; CoPS: coagulase positive staphylococci.

of CFU/g of *B. cereus* after 49 days stored at -20 ± 4°C and, consequently, was considered insufficiently stable (Figure 2B). Subsequently, a linear regression analysis was performed, which showed that the TI were considered sufficiently stable in the other evaluated batches.

Although the concentration of CFU/g of *S. aureus* remained stable in the BCSTA-F lot, the sharp decline in the concentration of *B. cereus* led to the non-use of this batch in an PT. In contrast to the final batch, the pilot batch BCSTA-P showed stability at the two temperatures studied. This may have occurred because the stability analysis period of the pilot batch was shorter (only 38 days) when compared to the official batch (49 days). The great advantage of the long-term stability study carried out in the classic model is to know the concentration of the microorganism in the batch in each analysis performed in the determined terms intervals. In this way, it was possible to observe the instability behavior of the microorganisms in the matrix, before being sent to the laboratories participating in the PT. The option of producing this mixed batch containing the two microorganisms aimed to save costs in production and for the TI user, as they would use the same item for both analyses. The decrease in cell number of *B. cereus* no lote BCSTA-F in BCSTA-F batch may have occurred due to the competitiveness of the two microorganisms for nutrients over time[26], with prevalence of *S. aureus*, over *B. cereus*. MYP agar, used for counting *B. cereus*, has no inhibitor for *S. aureus*[15], therefore, this species can grow in this culture medium, although its colonies clearly differ from *B. cereus* because of the fermentation of mannitol and, consequently, the yellow coloration. Baird Parker medium, on the other hand, has a high concentration of salts, inhibiting the growth of *B. cereus*. Possibly, for this reason, no change in the *S. aureus* count was observed in the batches produced. It is necessary to deepen the knowledge about the interaction between the two species, *S. aureus* and *B. cereus*, in the chicken matrix, in addition to the relationship and influence of the matrix on these microorganisms



Source: Elaborated by the authors, 2020.

Figure 2. Stability study of the final batches produced. (A) Long-term stability of the SALM-F batch; (B) Long-term stability of BCSTA-F batch; (C) Long-term stability of the EC-F batch by plate counting techniques and most probable number; (D) Short-term stability of the SALM-F and EC-F batches by plate counting techniques and most probable number.

for a determined period of time. Thus, in the future, mixed batches may be produced with sufficient homogeneity and stability to be used in PT, reducing production costs and, consequently, the price of these TI offered to PT participants.

At the reference temperature - 80 ± 10ºC, all batches were stable for the period studied. In the final SALM-F batch, the TI were stable for 153 days at -80 ± 10°C and 80 days at -20 ± 4°C (Figure 2A). Regarding batches containing *E. coli*, the final batch EC-F was stable for 126 days at -80 ± 10°C and 84 days at -20 ± 4°C (Figure 2C). The stability of IE at a temperature of -80 ± 10°C for an expressive period demonstrates that they can be stored both by the producing laboratory and by the laboratories participating in the PT for a long period of time with the assurance that viability will not be lost. Other studies have reported stability of TI containing *E. coli*[19,20,25] and *Salmonella* Enteritidis[22,23] in different matrices, such as cheese, milk and chocolate, produced with lyophilization technique for a time similar to or longer than that evaluated in the present study. The production of stable IE at temperatures of -20 ± 4°C over an extended period is also important because not all laboratories have equipment to store IE at temperatures ≤ -70°C. Therefore, these laboratories can acquire the IE and stock them in usual equipment, such as common *freezers*, and use them in a longer period[19]. These results indicate that the lyophilization technique and the use of cryoprotectants in the production of batches in the chicken matrix was satisfactory for the preservation of bacteria throughout the long-term stock.

In the short-term stability study, the EC-F batch was sufficiently stable at the two temperatures evaluated both in the evaluation of the trend graph (Figure 2D) and in the linear regression analysis. However, the SALM-F batch was sufficiently stable only at a temperature of 5 ± 3ºC (Figure 2D). Previous studies have already reported insufficient stability in batches of TI containing Gram-negative bacteria in different matrices when stored at temperatures equal to or similar to 35°C[12,18,19]. Therefore, a temperature control during the transport of TI containing *Salmonella* Enteritidis in this matrix is mandatory so that laboratories do not run the risk of receiving compromised TI due to temperature excursions. As the transport of biological materials at refrigeration temperature is expensive, obtaining stable IE at high temperatures is important to lower the cost of shipping, consequently, reducing the total cost of the laboratory's participation in the PT[25]. Thus, further studies will be needed to obtain batches containing *Salmonella* spp. in chicken matrix stable at higher transport temperatures.

CONCLUSIONS

The shredded cooked chicken matrix associated with the freeze-drying production technique was considered satisfactory for the production of TI containing only *E. coli* or *Salmonella* Enteritidis, taking into account that they were sufficiently homogeneous and stable throughout the study period, a minimum of 80 days at -20 ± 4ºC and 126 days at -80 ± 10°C.

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Acknowledgment

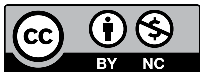
To the Multiprofessional Residency program in Health Surveillance with an emphasis on Quality at INCQS for the opportunity for professional improvement to the student Ingrid Camelo da Silva and to the Ministry of Health.

Author’s Contributions

Silva IC, Medeiros VM, Rosas CO, Lopes SMR, Brandão MLL –Conception, planning (study design), acquisition, analysis, data interpretation, and writing of the work. Costa PV, Vasconcellos L, Coimbra PT – Acquisition, analysis, data interpretation, and writing of the work. All authors approved the final version of the work.

Conflict of Interests

The authors inform that there is no potential conflict of interest with peers and institutions, politicians, or financial in this study.



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