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Genetic Diversity Quantification and Characterization of Spore-Forming Bacteria Isolated from Milk Powder Produced in Rio Grande Do Sul, Brazil

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HIGHLIGHTS

- Genetic varieties of spore-forming bacteria in milk.
- Spore-forming bacteria can affect the quality and safety of dairy products.
- Spores support stressful environmental conditions.
- Sporulated microflora of powdered milk in Brazil.

Abstract: Heat treatment used in milk powder processing cannot always inactivate spore-forming microorganisms. Toxin deterioration and production by some species are significant concerns for the dairy industry, making the identification of these microorganisms essential for solving problems with the quality of the products. Given this scenario, this study sought to quantify and identify the predominant species in milk powder, aiming to know the isolated genetic varieties. Fourteen samples were analyzed for aerobic/anaerobic, mesophilic, and thermophilic bacterial spore presence, totaling 52 analyses. Spore contamination was verified in all samples analyzed, and the thermophilic spores had the highest contamination levels. 16S rRNA sequencing revealed 47 isolates: *Weizmannia coagulans* (21%), *Anoxybacillus flavithermus* (5.3%), *Clostridium butyricum* (5.3%), *Bacillus amyloliquefaciens* (2.6%), *Geobacillus stearothermophilus* (2.6%), *Clostridium pabulibutyricum* (2.6%), *Clostridium sporosphaeroides* (2.6%), *Bacillus licheniformis* (2.6%), *Bacillus* sp. (34%), *Geobacillus* sp. (15.8%), and *Clostridium* sp. (5.3%). The results indicate the need to establish control measures to improve the hygiene and processing conditions of powdered milk, thereby ensuring product quality and consumer safety.

Keywords: 16S rRNA; Spores; Milk powder; Sequencing.

INTRODUCTION

Milk powder began to be produced to increase the shelf life, reduce transportation and storage costs, and offer a product with a higher concentration of milk solids, serving as an ingredient for the ice cream, confectionery, and infant formula industries, among others [1]. The microbiological quality of milk is related to the stability of the raw material and processing conditions. As soon as it is taken from the udder of healthy animals, raw milk shows contamination by microorganisms, generally with a total colony-forming unit (CFU) of $<10^3$ $\log_{10}$ CFU/mL. Therefore, apart from the milking, it is subject to bacterial and spore contamination from the production system. If hygiene conditions are neglected, the microbial load can reach 10^6 $\log_{10}$ CFU/mL [2]. According to Brazilian Normative Instruction n. 76, Art. 7th, of November 26, 2018, raw milk from individual or communal tanks must present quarterly geometric averages of standard count plates of a maximum of 3.0×10^5 $\log_{10}$ CFU/mL [3].

Spore-forming bacteria are important contaminants in the dairy industry and can significantly affect the quality and safety of dairy products, reducing the shelf life during storage. However, effective control of these bacteria is still challenging due to the high resistance of the spores and the ability of some species to form biofilms in processing lines [4, 5]. Christiansson and coauthors (1999) [6] identified soil as the primary source of spore contamination in raw milk, mainly due to the contact between cow udders and the soil during grazing. Additionally, other sources of contamination include residual water from washing milking equipment, feed, and the type of bedding material used for confined animals [7].

Burgess and coauthors (2014) [8] studied ten *G. stearothermophilus* strains and a species of *Anoxybacillus* isolated from a milk factory and concluded that these strains could produce spores from biofilms. Another study showed that spores of *A. flavithermus* and *Geobacillus* were detected (>1 $\log_{10}$ CFU/g) in milk powder after 9 h of processing and reached levels of up to 10^4 $\log_{10}$ CFU/g in the evaporated after 18 h [9].

The spores support stressful environmental conditions such as exposure to high temperatures, freezing, dehydration, irradiation, preservatives, and disinfectants. Once formed, they remain dormant, and under favorable conditions, they germinate and produce vegetative cells with all their metabolic activities [10, 11]. According to Setlow (2003) [12], the acids released during the germination of one spore can stimulate the germination of two other spores in the same environment.

Some studies have shown that the primary main sporulated microorganisms isolated from dehydrated dairy products (e.g., powdered milk) were *Anoxybacillus flavithermus*, *Geobacillus* sp., *Paenibacillus* sp., and the genera *Bacillus* and *Clostridium*, which have pathogenic and toxin-producing species [13]. Among the deteriorating ones, *G. stearothermophilus* and *Bacillus sporothermodurans* stand out as they do not present a risk to consumer health, although they are indicators of unsatisfactory hygiene conditions and poor manufacturing practices [14]; Scott and coauthors, 2007 [9]; nevertheless, further research is needed to shed more light on the species present.

Some sporulate species are enzyme producers, such as proteases and lipases, influencing the quality of dairy products by modifying the aroma, texture, and flavor [15–17]. Heat-resistant lipases can hydrolyze the fat in products to which powdered milk is added as an ingredient [18]. In one study, [19] identified the species *B. licheniformis* in refrigerated raw milk, where 63% of the isolated strains showed proteolytic and lipolytic activity. Junior (2015) [20] found relatively low aerobic spore contaminants in milk samples; for instance, 30% of the isolated species showed proteolytic and/or lipolytic activity.

To date, there have been no reported outbreaks involving spore-forming bacteria in powdered milk, either in Brazil or internationally. However, such outbreaks have been documented with other food products. For instance, *B. licheniformis* has been associated with outbreaks linked to meat, cooked vegetables, bread, raw milk, and processed foods for infants. This is due to the thermal stability of its toxin, which is similar to the emetic toxin produced by *Bacillus cereus* [21]. In Brazil, *B. cereus* is frequently involved in outbreaks, most of which are related to the consumption of cereals or sauces that have been improperly stored at ambient temperatures [22].

In Brazil, no legislation contemplates quantifying bacterial spores in powdered milk, and each company must establish its limit. In the United States, the US Dairy Export Council implements spore limits of $<5 \times 10^2$ and $<10^3$ $\log_{10}$ CFU/g for thermophilic and mesophilic aerobics, respectively, in powdered milk for foreign customers. In Ireland, the Food Safety Authority of Ireland (FSAI) states that aerobic spore counts in dairy powder products should ideally be $<10^4$ log10 CFU/g. Additionally, China has set a limit of 10^3 $\log_{10}$ CFU/g for aerobic spores in infant powdered milk formulas [23, 24].

Characterizing the microorganism at the species level is crucial for the food industry as it requires constant monitoring. Various molecular techniques have emerged recently, offering rapid detection, specificity, and sensitivity [25]. For instance, [13] analyzed 22 milk samples from Ireland using the 16S rRNA sequencing method; of the 269 isolates, 68% were identified as *B. licheniformis*, 16% were other *Bacillus* species, and others were identified as other genera including *Clostridium*, *Geobacillus*, and *Anoxybacillus*.

Considering the problem of sporulates in powdered milk, this study sought to quantify and identify the predominant species to know the genetic varieties of the isolates through 16S rRNA gene sequencing.

MATERIAL AND METHODS

Samples

Fourteen samples of whole milk powder at the beginning of the shelf life and from six different commercial brands produced in Rio Grande do Sul State (southern Brazil) were analyzed between September 2020 and September 2021 in terms of aerobic/anaerobic, mesophilic, and thermophilic bacterial spore presence.

Sample preparation

Aliquots (10 g) were aseptically weighed and transferred to the sterile stomacher bag, followed by adding distilled water (90 mL) or a sodium hydroxide solution (0.02 N) for thermophilic aerobic spores; a 10^{-1} dilution was considered. This initial dilution was transferred to a 250 mL Schott flask and subjected to the appropriate heat treatment for each type of spore.

Analysis of bacterial spores

Aerobic mesophilic

The initial dilution was subjected to heat treatment in a water bath at 80.0 ± 1.0 °C for 12 min, with subsequent cooling in an ice bath until reaching room temperature. Serial decimal dilutions of up to 10^{-3} were carried out, and plate count agar (Oxoid) was supplemented with 0.1% soluble starch (Synth) added to the plates and incubated at 35 °C for 48 h [26].

Aerobic thermophilic

The initial dilution was subjected to heat treatment in an autoclave at 108.4 ± 1.0 °C for 10 min and then cooled in an ice bath until reaching room temperature. Serial decimal dilutions of up to 10^{-3} were carried out. To obtain the dilution 10^{0}, an aliquot of 2 mL of the initial dilution were seeded in 5 Petri dishes; dextrose tryptone agar (Oxoid) was then added to the plates incubated at 55 ± 1.0 °C for 48 ± 3 h [27].

Anaerobic mesophilic

This analysis was modified to count plates instead of using the most probable number technique (MPN). The initial dilution was subjected to heat treatment in a water bath at 80.0 ± 1.0 °C for 10 min with subsequent cooling in an ice bath until reaching room temperature. Serial decimal dilutions of up to 10^{-3} were carried out. To obtain the dilution 10^{0}, an aliquot of 2 mL of the initial dilution were seeded in 5 Petri dishes, and reinforced clostridial agar (Oxoid) was added to the plates incubated under an anaerobic generator (Biomérieux) in a jar at 35 °C for 120 h [28].

Anaerobic thermophilic

Analysis was conducted with methodological modifications to carry out counting in plates. The initial dilution was subjected to heat treatment in a bain-marie at boiling point for 5 min, with subsequent cooling in an ice bath until reaching room temperature. Serial decimal dilutions of up to 10^{-3} were carried out. To obtain the dilution 10^{0}, an aliquot of 2 mL initial dilution were immediately seeded in 5 Petri dishes, and reinforced clostridial agar (Oxoid) was added to the plates incubated with an anaerobic generator (Biomérieux) in a jar at 55 °C for 72 h [29].

Quality assurance

The reference material (RM) Spore Reference (RM4290) was used as a standard. It was from IFM Quality Services Pty Ltd (Ingleburn, Australia). It consisted of aliquots of milk contaminated with bacterial spores, with the first ten samples analyzed. To verify a significant difference between the reference value provided by the supplier and the value obtained in the analyzed reference samples, analysis of variance (ANOVA) was used with 95% confidence using the Microsoft Excel software (version 12.0).

Colony isolation and purification

Whenever possible, three colonies of variable morphology were selected by visual inspection. For purification, the aerobics were streaked on the slanted nutrient agar, and later, growth was transferred to 2 mL cryotubes containing brain heart infusion broth (Oxoid). The anaerobic spores were placed in 2 mL cryotubes with reinforced clostridium medium (Oxoid), adding a drop of sterile mineral oil (Laborclin) to generate anaerobiosis. The incubation conditions were the same as the initial stage for each type of sporulation. The tubes that showed growth received 0.3 mL of glycerol (Synth) and were stored in the freezer at ≤ -18 °C.

DNA extraction and purification

All isolates were activated on nutrient agar plates. After growth on the plates, the DNA extraction and purification stages were carried out with the commercial bacteria DNA kit (DPK-116S) (Cellco Biotec do Brasil Ltda., São Paulo, Brazil), according to the manufacturer's instructions.

16S rRNA amplification and sequencing

Polymerase chain reaction (PCR) analysis was carried out using the MasterMix kit from Ludwig Biotecnologia. The primers were 27F/1492R (27F - GAGTTTGATCATGCTCAG, 1492R – GGTTACCTTGTTACGACTT). The reactions contained 1.5 µL of DNA, 1.25 µL of each primer, and 12.5 µL of MasterMix completed with 8.5 µL of free water of DNases in a thermocycler (QuantStudio 5, ThermoFisher Scientific) under the following conditions: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and, 72 °C for 5 min, as described by [30]. The PCR products were purified and sequenced according to the method of Sanger [31]. The quality of the sequences was analyzed by the Bioedit and Chromas software, and a genetic database search was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Only isolates where the 16S rRNA sequences presented similarities ≥97% with the sequences of the databases were considered to belong to a certain species [32].

Phylogenetic analysis

A DNA sequence corresponding to the 16S ribosomal subunit of the organism *Aquifex aeolicus* strain VF5 (a Gram-negative and hyperthermophilic bacterium) was obtained from the NCBI database (access number NR_075056.2) for use as an outgroup in phylogenetic analyses. A sequence of the outgroup with the sequences of all samples was aligned with the ClustalW algorithm available in the MEGA v.11 software; the default parameters of the tool were maintained. A tree was built from the neighbor-joining method. The Kimura-2-parameter nucleotide substitution model was used for the analysis, and the nodes' reliability was evaluated through 1000 bootstrap replications.

RESULTS

Spore quantification

The quantitative results of the spores found in the milk powder are listed in Table 1, and Figure 1 shows the contamination levels in the 14 samples.

Table 1. Population (log_{10} CFU/g) of the different bacteria sporulates in the 14 samples.

Spores	Minimum	Maximum
Aerobic mesophilic	0.78	2.82
Anaerobic mesophilic	0.30	2.45
Aerobic termophilic	1.30	4.56
Anaerobic termophilic	0.30	3.95

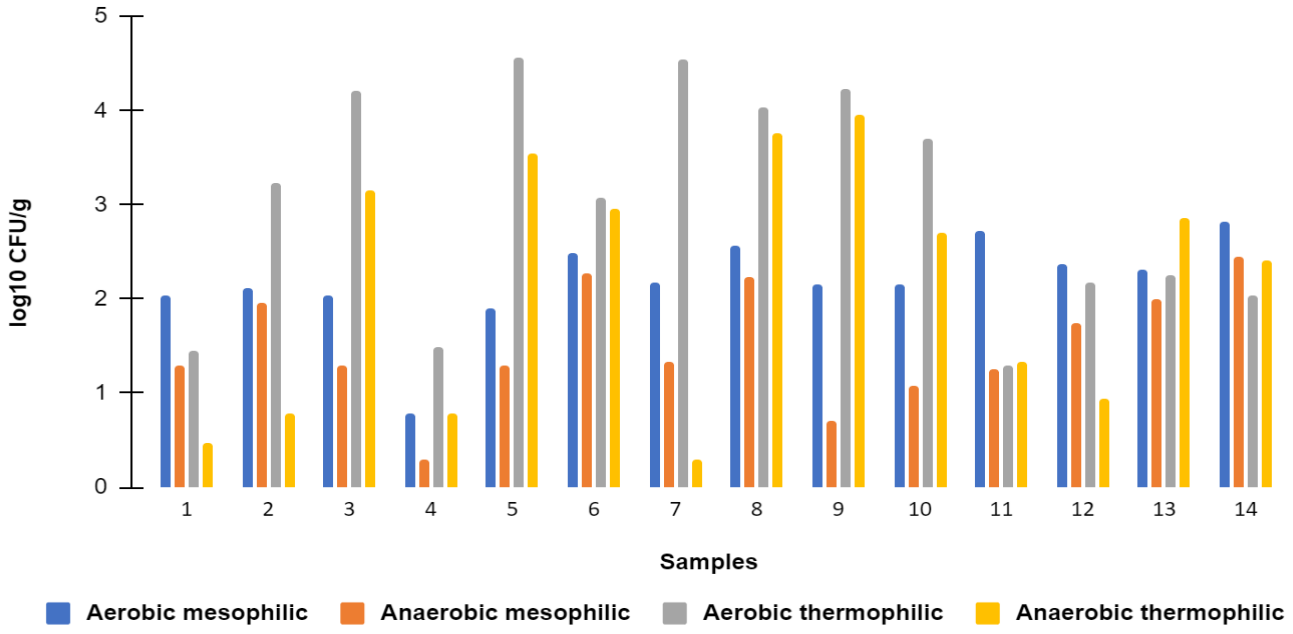

Figure 1. Population in log_{10} CFU/g of the spores in the milk powder.

Reference material quantification

To guarantee the quality of the analysis in the recovery of the spores in the samples, the RM spore reference was used, submitted to the same analytical procedure of the samples, and compared to the value obtained with the one provided by the supplier for each spore type. The average values of the populations are provided in Table 2.

Table 2. Average values of the reference material (RM) and average values obtained for 10 aliquots of RM submitted to the same analytical procedure of milk powder samples for each spore type.

Spore type	Mean values of RM (log_{10} CFU/g)*	Mean values of RM (log_{10} CFU/g)
Aerobic mesophilic	4.36	4.26
Anaerobic mesophilic	3.11	3.98
Aerobic thermophilic	3.04	3.08
Anaerobic thermophilic	3.18	3.23

*Values provided by the supplier.

In order to compare if there was a difference between the reference counts and counts obtained by the analytical procedure for each spore type, ANOVA with a significance level of 95% was conducted for a single factor. The F-value (6.468828) was less than the F-critical (6.591382), revealing no significant difference between the counts.

Isolate identification

A total of 47 isolates of spore-forming bacteria were sequenced. The 16S rRNA gene enabled us to identify 17 (36.17%) species of the 47 isolates. Other 21 (44.68%) strains were identified at the genus level, and 9 (19.15%) showed low quality in the electropherogram since these sequences were not used for identification (Table 3).

Table 3. Identification by sequencing of the 16S rRNA gene of spore-forming bacteria.

Identification	Nucleotide similarity (%)*	(n)	(%)
Weizmannia coagulans	98.38–100	8	21
A. flavithermus	100	2	5.3
C. butyricum	99.2 and 99.73	2	5.3
B. amyloliquefaciens	99.30	1	2.6
G. stearothermophilus	100	1	2.6
C. pabulibutyricum	100	1	2.6
C. sporosphaeroides	100	1	2.6
Bacillus licheniformis	100	1	2.6
Bacillus sp.	97.14–100	13	34.2
Geobacillus sp.	97.85–100	6	15.8
Clostridium sp.	100	2	5.3

*Percentage of identity based on non-feeding of sequences obtained from those deposited in GenBank using the BLAST tool; n = total number of identified.

Phylogenetic tree

Based on the phylogenetic tree generated by the software (Figure 2), we observed that starting from the node (branching point) of the common ancestral, there was a bifurcation in two new nodes: the first one was a group involving the genera *Bacillus*, *Weizmannia*, *Anoxybacillus*, and *Geobacillus,* and the second one comprised the *Clostridiaceae* family. The first grouping of the family *Bacillaceae* had a new bifurcation separating the genera *Bacillus* from *Weizmannia*. As many isolates were only identified at the genus level, it is likely that some of the isolates grouped with *B. licheniformis* and *B. amyloliquefaciens* belonged to the *B. subtilis* group because they are closely related to the identified species. The other species belonging to the group were *B. subtilis*, *B. atrophaeus*, *B. mojavensis*, *B. sonorensis*, *B. vallismortis*, *B. pumilus*, *B. tequilensis*, and *B. velezensis* [33]. Samples 31 and 5 were closely related to the species *W. coagulans*, and this is probably due to *W. coagulans* previously being classified in the similar genus *Bacillus* [34]. At the other bifurcation node, there was a separation of the species *A. flavithermus* from the genus *Geobacillus*, both being thermophiles.

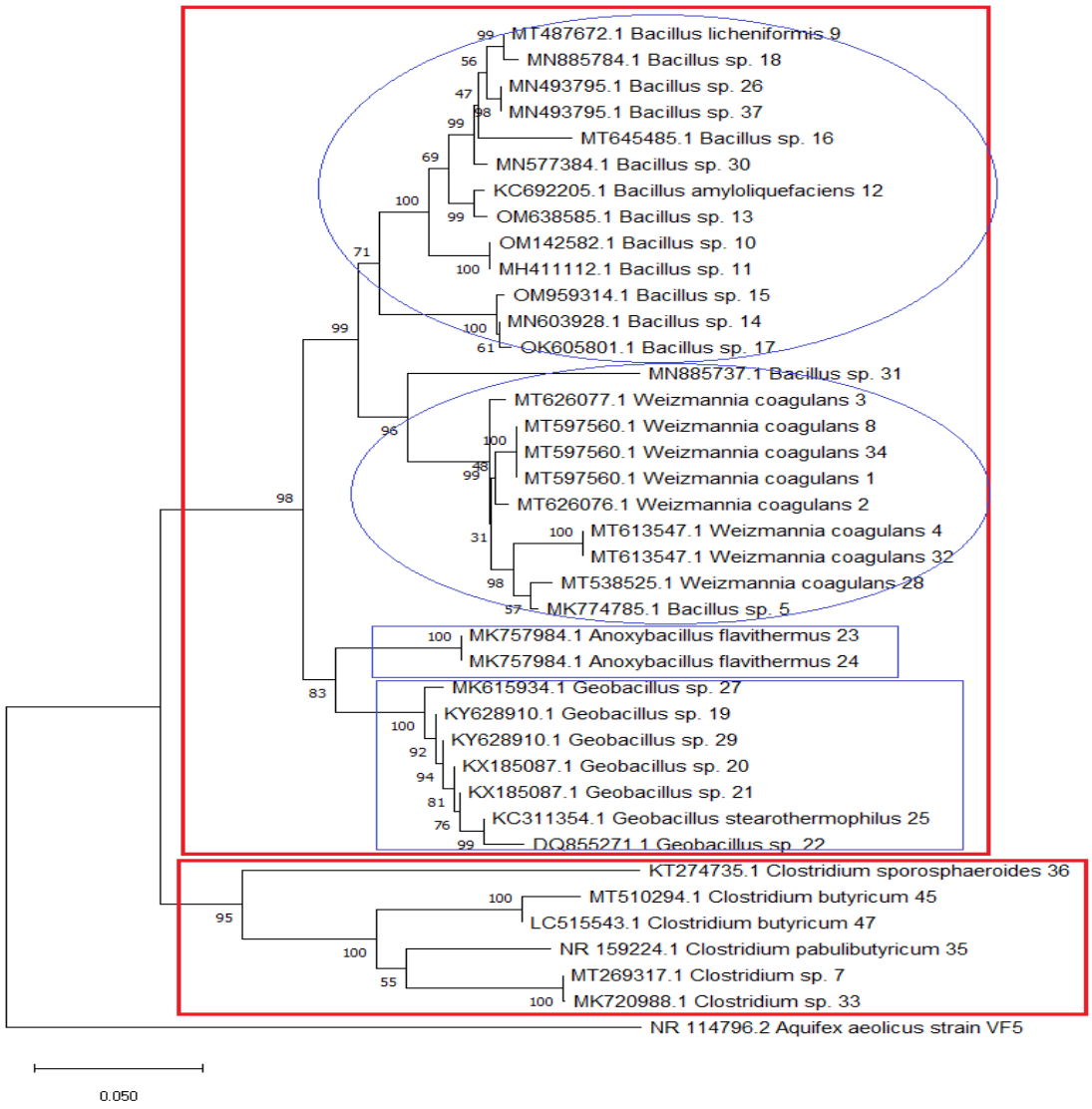

Figure 2. Phylogenetic tree obtained from 16S ribosomal sequences with the neighbor-joining method. The values in the branches represent the bootstrap support for a subsequent division.

DISCUSSION

Our findings showed that the population levels of spores were obtained in all the analyzed samples, indicating that contamination is frequent by spore-forming bacteria; therefore, the thermophiles present the highest contamination rates (Table 4). By comparing the values obtained for aerobic thermophilic spores in milk powder samples with internationally established limits, 8 of the 14 samples exceeded these standards.

Table 4. Results of the aerobic thermophilic spore count for 8 samples of powdered milk compared to internationally established tolerance limits.

Sample	Count	Council (USA)	FSAI (Ireland)	China
LP2	$1.7 \times 10^{3*}$			
LP3	1.6×10^{4}			
LP5	3.6×10^{4}			
LP6	$1.2 \times 10^{3*}$	$<5 \times 10^{2}$	$<10^{4}$	10^{3}
LP7	3.5×10^{4}			
LP8	1.1×10^{4}			
LP9	1.7×10^{4}			
LP10	$5.0 \times 10^{3*}$			

*For Council and China only. Source: The authors; Watterson et al. (2014); Yuan et al. (2012).

Considering that most samples presented unsatisfactory results for aerobic thermophilic spores according to international standards, this study indicates a need for specific targeting for greater control of thermophilic spores in processing plants. Due to the high heat resistance of this type of spore, a greater understanding of the proliferation, survival, and structure of biofilms is needed to develop control measures and reduce high counts. This study contributes to compiling data considering a future proposal to establish a standard in the Brazilian legislation for aerobic thermophilic spores. However, further research must be conducted in different regions with a larger sample.

Similar results have also been reported elsewhere, including [24], who analyzed 22 milk powder samples manufactured in China and found populations varying from 2.18 to 4.59 $\log_{10}$ CFU/g. Additionally, [35] analyzed 25 milk powder samples, including infant formula, and reported that contaminants varied from 1.00 to 4.30 $\log_{10}$ CFU/g.

This condition is explained by the proliferation of thermophilic bacilli being selected due to a sequence of thermal treatments, especially during pasteurization, evaporation, and drying, where temperatures vary from 80 to 180 °C.

Some species of mesophilic bacilli (e.g., *B. licheniformis*) grow in a wide temperature range and can be detected under ideal conditions for thermophilic microorganism growth (55 °C). In this study, *B. licheniformis* was identified using the mesophilic method, so isolates counted by the thermophilic method not identified at the species level by 16S rRNA sequencing may be of some mesophilic species inserted in the growth condition in a wide range of temperatures that can contribute to the higher rates of thermophiles found in the samples. This condition can be indicated as a limitation of the method employed and described herein.

Considering the treatment temperature, the variation between the applied methods can lead to differences in sample spore counts. Examples of other methods for thermophilic spores not applied in this study include those for highly heat-resistant spores $\log_{10}$ CFU/g; these methods include heat treatment at 100 °C for 30 min and 106 °C for 30 min [36], in which thermoresistant spores of thermophilic bacteria are selected [37]. All the methods present similar strategies (i.e., heat shock for a specific time to eliminate vegetative cells) with subsequent plating in non-selective recovery agar. Selecting the agar allied with the incubation can contribute to the variability of the spore count [23] analyzed milk samples to count thermophilic spores by applying heat treatments $\log_{10}$ CFU/g: 80 °C for 12 min and 100 °C for 30 min with incubation of the plates at 55 °C. Their findings showed that the treatment at 80 °C for 12 min presented almost two times more thermophilic spore growth, indicating that these tests do not yield the same information and are therefore not comparable. This is probably because the spores present different resistances between the species, in addition to the thermal treatment used in the analysis directly interfering in selecting the species that will be counted and identified.

Another condition for the high counts of thermophilic spores is the ability of some species to form biofilms that can protect both spores and vegetative cells during industrial sanitation. The thermophilic spores tend to increase in number during the cleaning process, mainly where there is continuous use of the equipment and where sanitation is not performed adequately. Scott and coauthors (2007) [9] verified that the bacteria present in the incrustations, after non-local cleaning, were predominantly in the spore form, suggesting that the incrustations could be a possible source of contamination in the final products of dairy concentrates. Burgess and coauthors (2014) [8] studied 10 strains of *G. stearothermophilus* and one species of *Anoxybacillus* isolated from a milk powder factory and concluded that these strains could produce spores from biofilms, both species being thermophilic.

The presence of thermophilic bacilli in dairy products indicates poor hygiene, and high contamination rates are unacceptable as they can lead to product defects caused by enzymes (e.g., proteases, lipases, and acids) capable of destroying the final product.

No other study was found in the literature investigating anaerobic spore counts in milk. In this study, the American Public Health Association (APHA) [28, 29] was used, adapted for seeding on plates, and the most probable number (MPN) technique was not used as a reference to the official method, in which the population density is estimated through positive tubes. The official technique makes the semi-quantitative method, or it seems, difficult to interpret given that the client needs quantitative data instead of percentages of positive tubes. Due to methodological differences, the two techniques are not comparable. The MPN technique is considered more sensitive because it does not require colony formation, which can be a limitation for injured microorganisms.

To guarantee the quality of two results of adapted methods, the use of RM is one of the main tools used to guarantee the reliability of the tests by comparing the measurements of the value obtained and the expected value. There was no significant difference between the populations (Table 2), indicating that the adaptation to the method for anaerobic spores reached the same logarithmic order as the reference value.

The ability to detect and identify the spores present represents an economic advantage for the industry and a guarantee of hygiene for consumers. The 38 isolates tested in this study that were identified by 16S rRNA sequencing were found in the presence of five genera, among which we identified the species *B. licheniformis*, *Weizmannia coagulans*, *B. amyloliquefaciens*, *G. stearothermophilus*, *A. flavithermus*, *C. butyricum*, *C. pabulibutyricum*, and *C. sporosphaeroides*. The genus *Bacillus* predominated in the isolates identified, followed by the genera *Weizmannia*, *Geobacillus*, *Clostridium*, and *Anoxybacillus* (Table 3). Suppose a greater number of colonies were selected during visual inspection of each agar plate or under different incubation conditions, then the variety of microorganisms identified may have been different; if the method used for the heat treatment were different, the variety of microorganisms identified might have changed. Although the species *B. licheniformis* and *B. amyloliquefaciens* belong to the *Bacillus subtilis* group, in which identification at the species level within the group is difficult due to high evolutionary proximity, in the present study, it was possible to differentiate.

The high prevalence of the genus *Bacillus* sp. can be attributed to contamination by agricultural sources, including the silage and internal sources during the dairy process, that allows these microorganisms to remain and multiply. Similar results were reported by [38], in which 75% of two isolates in milk powder samples belong to the genus *Bacillus*, followed by *Geobacillus*. The dominant species identified are *B. licheniformis*, *B. subtilis*, and *G. stearothermophilus*. Li and coauthors (2019) [13] isolated 269 powder milk-sporulated bacteria, of which the predominant ones were *B. licheniformis*, being 68% of the isolates, 16% of other *Bacillus* species, 5% of *Clostridium* spp., 3% *Geobacillus* spp., and others with lower percentages (genera such as *Lysinibacillus* spp., *Brevibacillus* spp., *Anoxybacillus*, and *Aneurinibacillus*).

The identified species *W. coagulans*, *G. stearothermophilus*, *A. flavithermus*, *C. pabulibutyricum*, and *C. sporosphaeroides* have already been mentioned as spoiling dairy products, among other foods, that potentially cause economic losses for food facilities [39–43].

The species *B. licheniformis*, *B. amyloliquefaciens*, and *C. botulinum* have been associated with dairy product deterioration; however, some strains produce toxins that can cause food poisoning. According to [21], the toxins produced by *B. licheniformis* are thermally stable and similar to the emetic toxin produced by *B. cereus* since *B. amyloliquefaciens* produces cereulide toxin, although it has lower levels than *B. cereus* [44, 45]. The anaerobic *C. botulinum* was cited as the source of two cases of type E infant botulism in Italy; the toxin was purified and considered similar to the toxin produced by *C. botulinum* [46]. The presence of these species deserves attention because milk powder is an ingredient for infant formulas, among other products, making toxic production highly concerning.

CONCLUSIONS

The results revealed that contamination by spore-forming bacteria is frequent in powdered milk samples, with both aerobic and anaerobic thermophiles showing high contamination levels. The adaptation performed in the American Public Health Association [24, 25] method showed satisfactory results, showing no significant difference between the traditional method and the one adapted for the quantitative technique. The presence of both spoilage and toxin-producing species was identified. This study described for the first time the detection of the species *W. coagulans*, *B. amyloliquefaciens*, *C. pabulibutyricum*, *C. sporosphaeroides*, and *C. butyricum*, which are part of the sporulated microflora of powdered milk in Brazil. Therefore, the results indicate the need to establish control measures to improve the hygiene and processing conditions of powdered milk, thus guaranteeing the quality of the product as well as consumers' safety.

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