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Efficiency of boiling and four other methods for genomic DNA extraction of deteriorating spore-forming bacteria from milk

Eficiência da fervura e outros quatro métodos para extração do DNA genômico de bactérias esporuladas deteriorantes do leite

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Abstract

The spore-forming microbiota is mainly responsible for the deterioration of pasteurized milk with long shelf life in the United States. The identification of these microorganisms, using molecular tools, is of particular importance for the maintenance of the quality of milk. However, these molecular techniques are not only costly but also labor-intensive and time-consuming. The aim of this study was to compare the efficiency of boiling in conjunction with four other methods for the genomic DNA extraction of sporulated bacteria with proteolytic and lipolytic potential isolated from raw milk in the states of Paraná and Maranhão, Brazil. Protocols based on cellular lysis by enzymatic digestion, phenolic extraction, microwave-heating, as well as the use of guanidine isothiocyanate were used. This study proposes a method involving simple boiling for the extraction of genomic DNA from these microorganisms. Variations in the quality and yield of the extracted DNA among these methods were observed. However, both the cell lysis protocol by enzymatic digestion (commercial kit) and the simple boiling method proposed in this study yielded sufficient DNA for successfully carrying out the Polymerase Chain Reaction (PCR) of the *rpoB* and *16S rRNA* genes for all 11 strains of microorganisms tested. Other protocols failed to yield sufficient quantity and quality of DNA from all microorganisms tested, since only a few strains have showed positive results by PCR, thereby hindering the search for new microorganisms. Thus, the simple boiling method for DNA extraction from sporulated bacteria in spoiled milk showed the same efficacy as that of the commercial kit. Moreover, the method is inexpensive, easy to perform, and much less time-consuming.

Key words: *Bacillus. Paenibacillus.* PCR. Spores.

Resumo

A microbiota esporulada é a principal responsável pela deterioração do leite pasteurizado de longa vida útil nos Estados Unidos. A identificação destes micro-organismos é de especial importância para a qualidade do leite e ferramentas moleculares são fundamentais nesse processo. No entanto, exigem a execução de etapas onerosas e laboriosas que podem inviabilizar algumas pesquisas. O objetivo do presente trabalho foi comparar a eficiência do método de extração de DNA por fervura com outros quatro métodos para extração de DNA genômico de bactérias esporuladas com potencial proteolítico e

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lipolítico isoladas do leite cru dos estados do Paraná e Maranhão, Brasil. Foram utilizados protocolos que se baseavam na lise celular por digestão enzimática, agitação com fenol, aquecimento em micro-ondas, tiocianato de guanidina e este trabalho propõe um método por fervura simples para o estudo desses micro-organismos. Observaram-se variações nos métodos de quantificação do DNA extraído e baixo coeficiente de correlação de Person entre esses métodos. No entanto, observou-se que tanto no protocolo de lise celular por digestão enzimática (kit comercial) quanto na fervura simples proposta pelo presente estudo, houve êxito na realização da Reação em Cadeia da Polimerase (PCR) para pesquisa dos genes *rpoB* e *16S rRNA* para todas as 11 cepas de micro-organismos testadas. Os outros protocolos não apresentaram sucesso na extração de DNA para a totalidade da microbiota avaliada, já que somente algumas amostras tiveram êxito nas reações de PCR, fato que os inviabiliza para a pesquisa desses micro-organismos. Dessa forma, o método de fervura simples das suspensões de bactérias esporuladas deteriorantes do leite demonstrou a mesma eficiência do kit comercial para a extração do DNA desses micro-organismos, sendo um método de baixo custo e de fácil e rápida execução.
Palavras-chave: *Bacillus.* Esporos. *Paenibacillus.* PCR.

Introduction

Several studies have shown that part of the spore-forming microbiota of raw milk are primarily composed of *Bacillus*, *Paenibacillus* (HUCK et al., 2007) and genera thereof, following reclassification. These spore-forming bacteria are capable of withstanding pasteurization (BUEHNER et al., 2014) and Ultra High Temperature (UHT) treatments (ESPEJO et al., 2014). These have been described as the major group of microorganisms responsible for reducing the shelf life of pasteurized milk in the United States (HUCK et al., 2007).

A preliminary Brazilian study showed that some microorganisms derived from germination of spores in milk exhibited deteriorating activity, due to their proteolytic and/or lipolytic activity (RIBEIRO JÚNIOR et al., 2016), thereby affecting the shelf-life and sensory characteristics of pasteurized milk and dairy products (BELOTI, 2015).

Molecular methods for identification and characterization of microorganisms are modern tools that allow for checking not only the composition and diversity of microbiota in milk (FUQUAY et al., 2011), but also genomic variations within the same species (BECKER et al., 2014), production of toxins (CHIEFARI et al., 2015), spoilage (SCATAMBURLO et al., 2015) and pathogenic (REDDY et al., 2016) potential. However, these methods are labor-intensive, time-consuming, and often costly.

The great diversity of microorganisms that compose the food microbiota is a limiting factor for the use of certain molecular methods. Some microorganisms require the execution of specific steps for genomic analysis, such as in cellular DNA extraction. It is known that simply boiling a suspension of *Escherichia coli* (MARTINS et al., 2015) or *Salmonella* spp. (ALVES et al., 2012), for example, is an effective method for inducing cellular lysis, for carrying out Polymerase Chain Reactions (PCR). However, for Gram-positive microorganisms, such as some *Bacillus* spp., specific methods must be carried out for DNA extraction.

Considering the importance of deteriorating spore-forming microbiota in milk and the use of molecular tools for identification and microbial characterization, the aim of present study was to verify the efficacy of boiling and four other methods for genomic DNA extraction of spore-forming bacteria that, previously, showed deteriorating activity.

Material and Methods

Selection of strains

Different strains of spore-forming bacteria were previously isolated from milk after heat treatment (80 ± 0.5 ºC for 12 minutes), according to the technique for aerobic spore count described in Standard Methods for Examination of Dairy

Products (FRANK; YOUSEF, 2004). These strains were identified by partial sequencing of the *16S rRNA* gene.

The proteolytic and lipolytic potential of each isolate were verified by plating the colony on milk agar and tributyrin agar, respectively. The strains and their respective spoilage activity are described in Table 1. *Bacillus sporothermodurans* strain does not exhibit any deteriorating activity. However, it was included in the study as it had been isolated from spore-forming microbiota of Brazilian UHT milk (PEREIRA et al., 2013).

Table 1. Strains of spore-forming bacteria from Brazilian raw milk.

Strains	16S rRNA GenBank accession number	Spoilage potential	Brazilian state of isolation
Bacillus sp. LIPOA/ UEL_83 [A]	KU377295	Proteolytic and Lipolytic	Paraná
Lysinibacillus massiliensis strain LIPOA/UEL_23	KT748553	Lipolytic	Maranhão
Bacillus licheniformis strain LIPOA/UEL_1 [B]	KP713760	Proteolytic and Lipolytic	Paraná
Bacillus sp. LIPOA/ UEL_86 [B]	KU377296	Proteolytic and Lipolytic	Paraná
Bacillus sporothermodurans strain LIPOA/UEL_87	KU377297	-	Paraná
Bacillus pumilus strain LIPOA/UEL_4	KP713763	Proteolytic and Lipolytic	Paraná
Paenibacillus sp. LIPOA/ UEL_7	KP713766	Lipolytic	Paraná
Paenibacillus sp. LIPOA/ UEL_88	KU377298	Lipolytic	Paraná
Bacillus circulans strain LIPOA/UEL_6	KP713765	Lipolytic	Paraná
Bacillus licheniformis strain LIPOA/UEL_3 [A]	KP713762	Proteolytic and Lipolytic	Paraná
Brevibacillus borstelensis strain LIPOA/UEL_29	KT784818	Proteolytic and Lipolytic	Maranhão

[A]Hemolytic strain on sheep blood agar
[B]No hemolytic strain

DNA extraction protocols

The strains were grown in Brain Heart Infusion Broth (BHI) for 48 hours at 35°C, distributed in 1-ml aliquots into microfuge tubes, and centrifuged for 5 minutes at 14,000 rpm. An estimated concentration of each microorganism after the incubation period was approximately 10^6 CFU ml $^{-1}$ of vegetative forms. The supernatant was discarded and the pellets were subjected to DNA extraction protocols.

Five bacterial DNA extraction protocols (A-E) used for microorganisms were compared; these are described in Table 2. The extraction method A, which was a commercial kit, gDNA ChargeSwitch® Bacteria Mini Kit (Invitrogen, Carlsbad, CA, USA) was used as standard.

Table 2. DNA extraction methods.

Method	Principle of the cellular lysis	Reference
A	Enzymatic digestion	ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, CA, USA)
B	Boiling	This study
C	Shaking with pure phenol	Cheng and Jiang (2006)
D	Microwave heating	Bollet et al. (1991)
E	Guanidine isothiocyanate	Boom et al. (1990)

This work proposes method B, i.e. cell lysis by simple boiling. Briefly, the bacterial pellets were suspended in 200 µl of TE buffer (Tris-HCl [10 mM]: EDTA [1 mM]) and subjected to 15 minutes of boiling. Immediately after boiling, the microfuge tubes were placed in an ice bath for 15 minutes and then centrifuged for 5 minutes at 14,000 rpm at room temperature. The supernatant containing DNA (100 µl) was transferred to another clean tube and stored at –20°C.

Quantification and quality assessment of DNA extraction products

Quantification of DNA extracted by different methods was performed spectrophotometrically (NanoDrop ND-2000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA), and the quality of the extracted DNA was estimated from the ratio of absorbance at 260 and 280 nm. A value of 1.8 was considered to indicate pure DNA.

All extraction of products was also quantified by Qubit® dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) which is not influenced by the presence of residual reactants and interfering chemicals. At this stage, the samples that gave a reading of "too low" were considered 0 ng μl^{-1} for quantification.

PCR Assays

The extracted DNA was subjected to PCR, which partially amplified the *rpoB* gene, as described by Durak et al. (2006). This gene allows for better identification and characterization of spore-forming microorganisms in milk.

In addition, DNA samples taken from protocol A and B the commercial kit and boiling were subjected to PCR amplification of the *16S rRNA* gene, which is widely used for sequencing and identification of bacteria. The primers and conditions for each assay are described in Table 3.

Table 3. Primers and PCR cycling conditions of genes *rpoB* and *16S rRNA*.

Gene	Primers (5' – 3')	Size (pb)	PCR cycling conditions	Reference
rpoB	AARYTIGGMCCTGAAGAAAT TGIARTTTRTCATCAACCATGTG	740	94ºC-3 min 20x (94ºC-30seg, TD[a]-30seg, 72ºC-1min) 20x (94ºC-1min, 50-30seg, 72ºC-1min) 72ºC-7min	Drancourt et al. (2004)
16S rRNA	GAGTTTGATCMTGGCTCAG GGYTACCTTGTTACGACTT	1465	94ºC-5min 35x (94ºC-1min, 58ºC-1min, 72ºC-1min) 72ºC-10min	Osborne et al. (2005)

[a]Touchdown PCR from 60ºC to 50ºC with temperature decrease of 0.5ºC per cycle.

The PCR was performed with approximately 50 ng DNA template, 100 nM of each deoxynucleotides, 5 µl 10X buffer, 75 mmol L^{-1} $MgCl_2$, 20 pmol L^{-1} of each primer and 2.5 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA).

Amplification was performed in a thermocycler (Aeris™ Thermal Cycler, Esco® Micro Pte. Ltd., Singapore) and the PCR amplified DNA samples were applied to 1% agarose gel (Invitrogen, Carlsbad, CA, USA) and subjected to electrophoresis for 1 hour at a constant voltage of 90 V. The gels were stained with ethidium bromide solution at 0.2 mg ml^{-1} for 20 minutes and visualized in UV transilluminator.

Statistical analysis

The Pearson's correlation coefficient between the DNA quantification methods was performed in Statistica software v. 6.0 (StatSoft, OK, USA).

Results and Discussion

The yield and quality of the extracted DNA by different methods is presented in Table 4. Large differences could be observed in both quantitative methods and the different DNA extraction protocols evaluated here.

Table 4. Quantification and quality of genomic DNA from spore-forming bacterial strains after different DNA extraction methods.

Extraction method		Qubit® DNA quantification (ng/µL)	NanoDrop® DNA quantification (ng/µL)	260/280	CC[b]
Enzymatic digestion[1]	Mean (± SD[a])	13.4 (10.6)	105.7 (121.4)	1.9 (0.2)	
	Maximum	33.3	406.3	2.1	-0.2
	Minimum	0.0	7.1	1.5	
Boiling[2]	Mean (± SD[a])	4.2 (5.7)	105.7 (38.3)	1.4 (0.7)	
	Maximum	20.6	196.4	1.7	0
	Minimum	1.1	66.8	-0.7	
Shaking with pure phenol[3]	Mean (± SD[a])	2.3 (2.5)	873.3 (118.9)	1.5 (0.1)	
	Maximum	7.8	1091.3	1.8	-0.1
	Minimum	0.0	604.3	1.5	
Microwave heating[4]	Mean (± SD[a])	0.1 (0.1)	415.3 (510.4)	1.5 (0.1)	
	Maximum	0.4	1263.8	1.8	-0.1
	Minimum	0.0	27.7	1.4	
Guanidine isothiocyanate[5]	Mean (± SD[a])	14.3 (14.4)	50.5 (44.6)	1.8 (0.2)	
	Maximum	41.9	156.9	2.1	0.7
	Minimum	1.6	15.2	1.6	

[a]SD = Standard derivation; [b]CC = Coefficient correlation between the methods of genomic DNA quantification
[1]ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, CA, USA); [2] This study; [3]Cheng and Jiang (2006); [4] Bollet et al. (1991); [5]Boom et al. (1990).

The absorbance readings can be changed by phenolic residues and/or chloroform, carbohydrates, proteins, and guanidine isothiocyanate, among others (CHENG; JIANG, 2006). Thus, DNA extraction methods involving purification steps are generally subject to spectrophotometric changes (Figure 1) that interfere with quantification, consequently affecting the 260/280 ratio, as was seen for methods B, C and D (Table 4). Precipitation and elution of DNA used in the methods A and B removed the interference of extraction matrix that led to lower spectrophotometric changes. However, the DNA purification step is not a limiting factor for the execution of PCR assay.

Figure 1. gDNA absorbance spectrums of spore forming bacteria extracted by methods ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, CA, USA) (A), boiling (this study) (B), Cheng e Jiang (2006) (C), Bollet et al. (1991) (D) and Boom et al. (1990) (E).

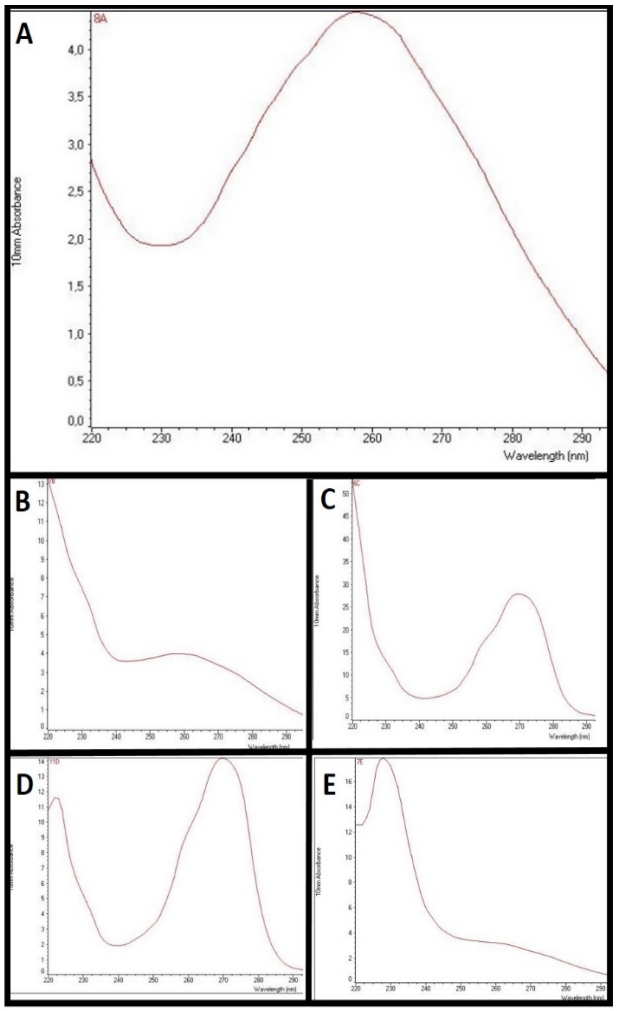


Table 4 shows the low coefficient of correlation between the methods of quantification of DNA, indicating that the quantification method can directly influence the PCR, since predetermined amounts of DNA template must be used in the reaction.

The same samples, where quantification showed near zero values in Qubit® (too low), showed positive results in the PCR for both genes in extraction protocols A and B (Figures 2 and 3). Thus, even at low quantities of DNA, the sensitivity of PCR is high enough to produce a satisfactory amount of amplicons for sequencing and further analysis, such as Restriction Fragment Length Polymorphism (RFLP), for example.

Considering the PCR results, it was observed that samples extracted by method A and B [gDNA ChargeSwitch® Bacteria Mini Kit (Invitrogen, Carlsbad, CA, USA) and simple boiling], respectively, consistently showed similar results, both for the *rpoB* gene (Figure 2), as well as the *16S rRNA* (Figure 3), in all samples tested. The methods C, D and E were not effective in extracting DNA from total sporulated microorganisms, as evidenced by amplification of the *rpoB* gene (Figure 4).

Figure 2. PCR results of *rpoB* (740pb) gene of strains from spore forming microbiota of the milk extract by commercial kit (A) (Invitrogen, CA, USA) and boiling (B) propose of this work. Line 1: DNA ladder; 2: *Bacillus sp*. LIPOA/UEL_83; 3: *Lysinibacillus massiliensis* strain LIPOA/UEL_23; 4: *Bacillus licheniformis* strain LIPOA/UEL_1; 5: *Bacillus sp*. LIPOA/UEL_86; 6: *Bacillus sporothermodurans* strain LIPOA/UEL_87; 7: *Bacillus pumilus* strain LIPOA/UEL_4; 8: *Paenibacillus sp.* LIPOA/UEL_7; 9: *Paenibacillus sp.* LIPOA/UEL_88; 10: *Bacillus circulans* strain LIPOA/UEL_6; 11: *Bacillus licheniformis* strain LIPOA/UEL_3; 12: *Brevibacillus borstelensis* strain LIPOA/UEL_29; 13: Negative control.

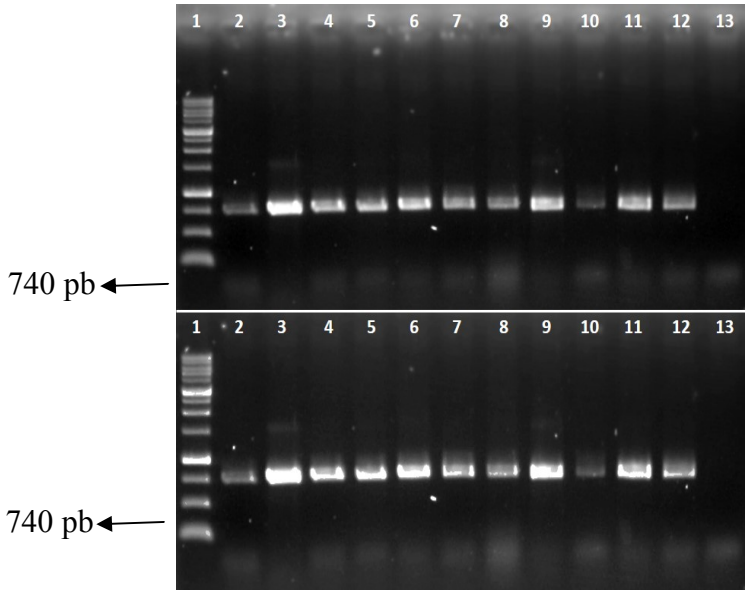


Figure 3. PCR results of *16S rRNA* (1465pb) gene of strains from spore forming microbiota of the milk extract by commercial kit (A) (Invitrogen, CA, USA) and boiling (B) propose of this work. Line 1: DNA ladder; 2: *Bacillus sp*. LIPOA/UEL_83; 3: *Lysinibacillus massiliensis* strain LIPOA/UEL_23; 4: *Bacillus licheniformis* strain LIPOA/UEL_1; 5: *Bacillus sp*. LIPOA/UEL_86; 6: *Bacillus sporothermodurans* strain LIPOA/UEL_87; 7: *Bacillus pumilus* strain LIPOA/UEL_4; 8: *Paenibacillus sp.* LIPOA/UEL_7; 9: *Paenibacillus sp.* LIPOA/UEL_88; 10: *Bacillus circulans* strain LIPOA/UEL_6; 11: *Bacillus licheniformis* strain LIPOA/UEL_3; 12: *Brevibacillus borstelensis* strain LIPOA/UEL_29; 13: Negative control.

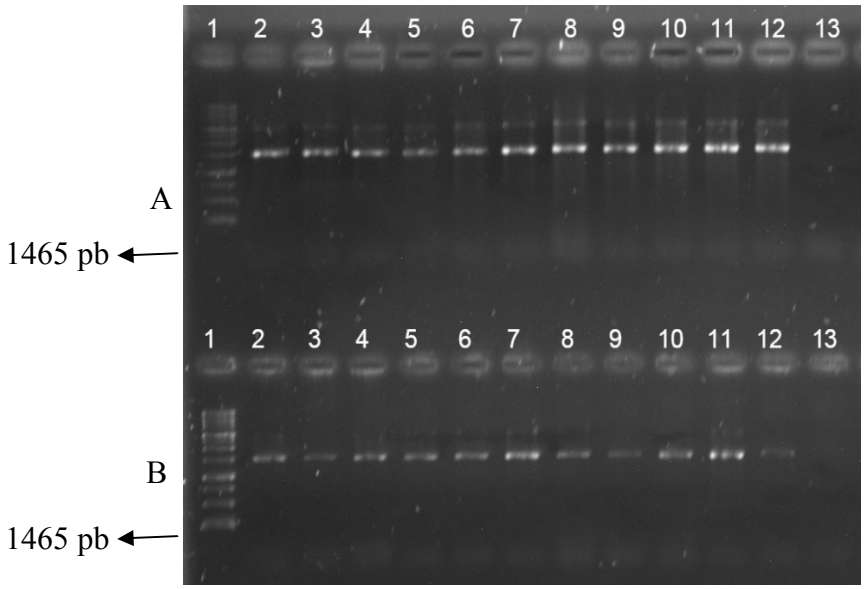
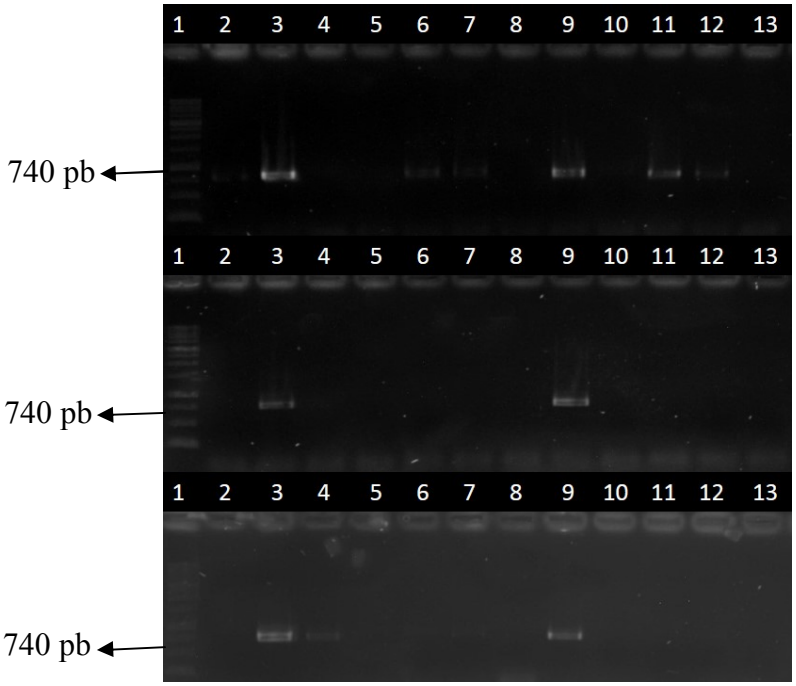


Figure 4. PCR results of *rpoB* (740pb) gene of strains of spore forming microbiota from milk extract by methods with shaking with pure phenol (CHENG; JIANG, 2006) (C), microwave heating (BOLLET et al., 1991) (D) and guanidine isothiocyanate (BOOM et al., 1990) (E). Line 1: DNA ladder; 2: *Bacillus sp*. LIPOA/UEL_83; 3: *Lysinibacillus massiliensis* strain LIPOA/UEL_23; 4: *Bacillus licheniformis* strain LIPOA/UEL_1; 5: *Bacillus sp*. LIPOA/UEL_86; 6: *Bacillus sporothermodurans* strain LIPOA/UEL_87; 7: *Bacillus pumilus* strain LIPOA/UEL_4; 8: *Paenibacillus sp.* LIPOA/UEL_7; 9: *Paenibacillus sp.* LIPOA/UEL_88; 10: *Bacillus circulans* strain LIPOA/UEL_6; 11: *Bacillus licheniformis* strain LIPOA/UEL_3; 12: *Brevibacillus borstelensis* strain LIPOA/UEL_29; 13: Negative control.



Ahmed et al. (2014), after comparing methods C and D for Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria, concluded that the lysis method by microwave showed better performance in the recovery of DNA. However, the present study demonstrated the limitations of these two methods in extracting gDNA from spore- forming bacteria in milk. Moreover, these methods are time consuming and require high quantities of inputs in relation to the method B, proposed in this study.

The extraction method based on cellular lysis with guanidine isothiocyanate (BOOM et al., 1990) (E) showed the best ratio 260/280 with respect to the purity of DNA obtained and the best Pearson's correlation coefficient between the quantification methods. However, the results of the PCR (Figure 4) indicate that the method was not capable of extracting DNA from all milk deteriorating spore-forming microbiota, being effective only for *Lysinibacillus massiliensis*, *Bacillus licheniformis*, and *Paenibacillus* spp., among those tested. Futhermore, the method involves multiple steps and reagents and it is more expensive and time consuming.

This may be related to the composition of the cell wall of Gram-positive microorganisms of spore-forming microbiota of milk, which has peptidoglycan responsible for increased rigidity to the wall of Gram-negative microorganisms (FUQUAY et al., 2011) that easily have their cell walls disrupted by boiling (MARTINS et al., 2015).

It was observed that for Gram-positive bacteria that comprise the milk- deteriorating spore-forming bacteria, boiling was sufficient for DNA extraction and PCR amplification of both *16S rRNA* and *rpoB*

genes. As the method is fast, cheap, and easy to perform, the boiling of suspensions of spore-forming microbiota from milk is an effective method for molecular studies of these microorganisms.

Conclusion

The DNA extraction by boiling proposed in this work was as efficient as the commercial kit for the PCR detection of rpoB and 16S rRNA genes of all the spoilage microorganisms evaluated.

The other gDNA extraction protocols evaluated were found to be ineffective. Thus, the simple boiling method of spoilage bacteria sporulated suspension is an important alternative for carrying out molecular studies of this microbiota, since it is as efficient as the commercial kit, but much less costly and laborious.

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