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Original Article

Enterococcus Spp. Survival Through the Use of Standard Protocol for Clostridium Sp. Isolation

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ABSTRACT

The genera *Clostridium* and *Enterococcus* are very different from each other, both morphologically and physiologically. Due to the high resistance by the sporulation capacity of *Clostridium* species, the thermal shock is a characteristic tool used for the isolation and identification of these microorganisms, this way, it would eliminate any other bacteria that did not present spores. The objective of this work is to show that *Enterococcus* sp. resist the temperature treatment and grow in culture media used for the isolation of *Clostridium* sp. For this, the present study initially attempted to identify reducing sulfite clostridia in poultry products, through the use of specific culture media and heat shock treatment. However, the PCR did not detect the presence of *Clostridium* sp. Then, sequencing of the 16S rDNA region was performed, which showed that the reducing sulfite colonies that were being isolated were, actually, *Enterococcus* spp. With this, some tests were carried out using different temperature and time combinations in the thermal shock, as well as the use of five different selective and differential culture media, in an attempt to eliminate any contaminants, but all without success, because these bacteria resisted to all modification. Therefore, the standard protocol for the isolation of bacteria of the genus *Clostridium* does not eliminate *Enterococcus*, which can lead to failures in the quantification and qualification of sulfite reducing microorganisms, a fact that can significantly affect food safety and animal health.

INTRODUCTION

The microorganisms of the *Clostridium* genus are classified as bacilli, anaerobes, Gram-positive, capsulated and spore producing, the latter characteristic provides them with very high resistance to unfavorable conditions, being able to withstand extreme temperature variations (McClane, Robertson, & Li, 2013). While microorganisms of the *Enterococcus* genus are cocci, facultative anaerobes, Gram-positive, non-spore producing, but even without the production of spores, these microorganisms possess a high intrinsic resistance to unfavorable conditions in their surroundings, being able to grow in hypotonic, hypertonic, acid and alkaline environments. In addition, substances used to inhibit other microorganisms, such as concentrated bile and sodium azide are tolerated by *Enterococcus* and do not hinder its development (Miller, Munitas, & Arias, 2014).

Both genera of bacteria include some pathogenic species, however, in regards to poultry, the genus *Enterococcus* is seen as low risk, therefore it is not considered a limiting factor in the production of poultry products. The *Clostridium* genus however, does have pathogenic species that could pose a serious threat to animals and humans alike, generating economic losses in farms contaminated by *Clostridium perfringens*,

causing elevated animal mortality and prohibiting commercialization in the affected farm, since this microorganism also has the potential to cause diseases in humans (Agência Nacional de Vigilância Sanitária, 2001).

According to the International Commission on Microbiological Specifications for Foods, micro-organisms reflect the quality of animal products, therefore, microbiological tests are performed in order to verify the quality of those products and meet the quality control standards for the internal market and importing countries. The present study aimed to showcase the failure to eliminate *Enterococcus* using today's standard protocol for *Clostridium* isolation.

MATERIAL AND METHODS

Sample preparation

Three hundred samples of intestinal material from broiler chickens were used to isolate *C. perfringens*. Each sample was transferred into BHI broth tubes and submitted to thermal shock in a water bath at 80°C for 10 minutes followed by rapid cooling at 20°C (Duncan & Strong, 1968). After the thermal treatment, the samples were inoculated in Petri dishes containing Sulfite-Polymyxine-Sulfadiazine Agar (SPS). The plates were incubated in anaerobic jars using the Gas Pack system, at 37°C for up to 72 hours (Dave, 2017). After the incubation, sulfite reducing colonies, that presented a black pigmentation, were transferred into tubes and submitted for DNA extraction (Marmur, 1961). Following the extraction, multiplex PCR was performed for detection of *C. perfringens* (Baums *et al.*, 2004).

Molecular analysis

Colonies that showed similar morphology to *C. perfringens* but tested negative when using specific primers for that species were sequenced in order to establish their genus. The 16S rDNA region was amplified using the primers 8F/907R (Nercessian *et al.*, 2005). The amplified products were then sent to PCR sequencing using the Big Dye Terminator v3.1 Kit (Applied Biosystems). The resulting electropherograms were submitted to Phred, Phrap and Consed programs and the qualified DNA sequences were compared to other sequences in the database (GenBank, www.ncbi.nlm.nih.gov/Genbank) using BLAST in order to determine similarity. The acquired dendrogram using the Neighbor joining clustering algorithm and graphical distances between isolates were obtained using the software MEGA 4.0 (Tamura *et al.*, 2007). To assure the reliability of the cluster, a bootstrap validation was performed using 2000 repetitions.

Selective media and thermal treatment

Besides SPS, four other selective and differential media for *Clostridium* sp. were tested: Tryptose Sulfite Cycloserine Agar (TSCD); Shahidi-Ferguson Perfringens Agar (SFP); SFP agar supplemented with egg yolk and Differential Reinforced Clostridial Medium (DRCM) Broth. After inoculation, the plates were incubated at 46°C in anaerobic condition (Ministério da Agricultura, Pecuária e Abastecimento, 2003).

The final procedure was regarding the thermal shock, the standard temperature for eliminating non-sporulating bacteria is 80°C for 10 minutes, however, temperatures of 50ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC were tested, for 10, 20, 30, 40, 50 and 60 minutes respectively, all followed by a cooling phase at 20°C.

RESULTS

Regarding the bacterial growth from the intestinal material, from a total of 300 samples analyzed, 17 (6%) showed positive growth using selective *Clostridium* sp. media and thermal shock treatment. Phenotypically, 13 colonies (77%) were of similar morphology to *C. perfringens*, however, after Gram staining, it was observed that 4 colonies (23%) possessed coccobacillus-like morphology and no endospore production, and the sequencing of the 16S region from these colonies morphologically similar to *Clostridium* sp. revealed the bacteria to be *Enterococcus* sp., with the samples having between 97% to 99% similarity with database sequences of *Enterococcus faecalis*. As for the external group, two sequences of Gram-positive bacteria were added: *C. perfringens; Bacillus cereus* and *Bacillus subtilis,* represented in Figure 1.

All the selective *Clostridium* sp. media tested showed growth for *Enterococcus* sp., indicating the ability of the bacteria to resist the selective agents present in the media as well as the incubation under anaerobic conditions.

Regarding the thermal shock treatment, the results showed resistance of all four *Enterococcus* sp. strains to high temperatures, even when subjected to 100ºC for 20 minutes and 90ºC for 50 minutes, resisting well beyond the currently standard thermal protocol for eliminating non-sporulating bacteria, which is 80°C for 10 minutes, the detailed data for the thermal shock treatment is described in Table 1.

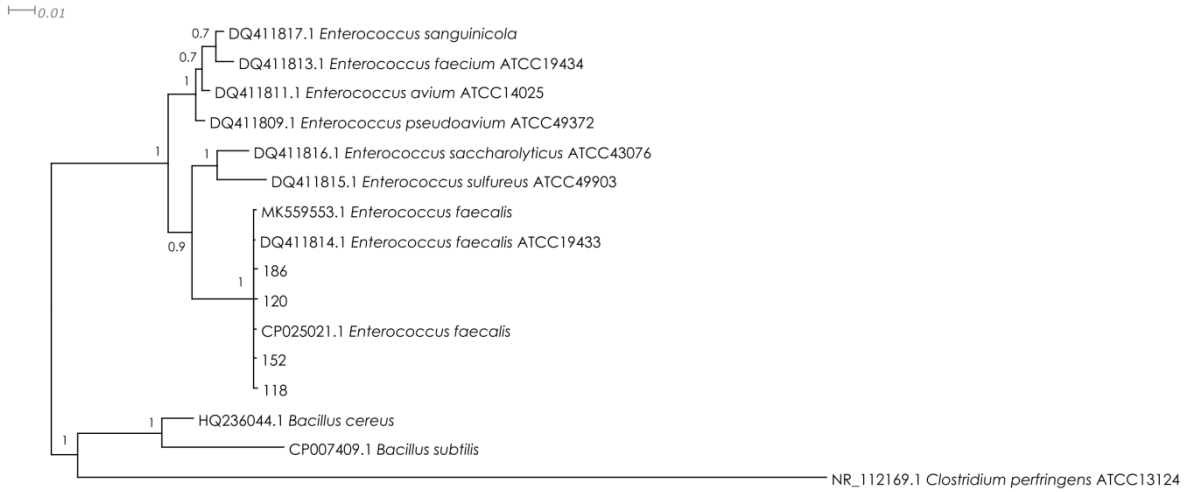

Figure 1 – Neighbor Joining clustering evidenciating homology between the tested samples and *Enterococcus faecalis*.
Notes: 186, 120, 152, 118: samples isolated via standard protocol.

Table 1 – Results regarding resistance under different temperatures and durations using 4 strains of Enterococcus spp. isolated and confirmed by sequencing.

Temperature (°C)	Time (minutes)					
	10	20	30	40	50	60
50	4	4	4	4	4	4
60	4	4	4	4	4	4
70	4	4	4	4	4	4
80	4	2	2	2	2	2
90	2	2	1	1	1	0
100	1	1	0	0	0	0

Notes: 1 – 4: number of surviving isolates post-treatment.

DISCUSSION

The Gram stain revealed pleomorphic morphology, making differentiation between coccus and bacillus not possible. Sequencing of the unidentified microorganism revealed it to be from the genus *Enterococcus* sp. Possibly, the pleomorphism occurred due to the culture medium not being ideal for *Enterococcus* sp. growth.

The presence of *Enterococcus* sp. in the present study calls for attention, since they are known to possess antibiotic resistance, specially towards antibiotics of the glycopeptide group (Miller *et al.*, 2014). However, the results observed in this study highlight a great nutritional adaptability and thermal resistance of *Enterococcus* sp., using non-specific culture media and temperatures up to 100°C for 20 minutes, which was previously unknown of, since Fisher and Philips (2009) reported the genus *Enterococcus* sp. being able to withstand a maximum of 60°C for 30 minutes.

Microbiological tests are mandatory for importation in Brazil as well as most European countries and many other regions, as they are decisive for the approval of poultry products to be consumed or exported (Cavani *et al.*, 2010). The regulation regarding animal products requires the absence of pathogenic and spore-producing bacteria once the samples have been thermally treated, meaning they should be free of *C. perfringens* (MAPA, 2003). However, the current regulation has no criteria regarding the presence of *Enterococcus* sp. and as presented in this study, the misidentification between the two microorganisms may result in the condemnation of the entire product batches.

Through the use of selective culture media and thermal shock, it was believed that only sporulating bacteria could survive, therefore, the quantification analysis uses differential culture media, classifying black-pigmented colonies as sulphite-reducing bacteria. The problem lies in the misjudgment of these black-pigmented bacteria being quantified and mistakenly determined as *C. perfringens.* Thus, the present study concludes possible mistakes in the interpretation of microbiologic analysis in the poultry industry, regarding the current regulations of selective media and sulphite-reducing colonies where, as shown in this study, *Enterococcus* sp could be misidentified as *Clostridium* sp by surviving the entire treatment protocol, resulting in condemnation of the products. A second analysis process would be suggested before submitting the bacteria to the thermal treatment, a simple Gram staining could reveal colonies that do not match *Clostridium*-like morphology and the absence of endospores, as well as biochemical tests for identification.

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CONCLUSIONS

Some strains of *Enterococcus* spp. are surviving under extreme conditions where only sporulating bacteria were thought to be able to. This is a concerning find, since these microorganisms are potentially harmful to humans and could go unnoticed in the industrialization process of food products.

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