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ANA PAULA MACIEL PEREIRA

"Spore forming bacteria throughout cocoa powder processing: incidence, genotypic characterization and impact of the process"

"Bactérias esporuladas na cadeia produtiva do cacau em pó: incidência, caracterização genotípica e impacto do processo"

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Orientador: **PROF. DR. ANDERSON DE SOUZA SANT'ANA**

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RESUMO

Assegurar-se o uso de matérias-primas e ingredientes com baixas populações iniciais de micro-organismos esporulados, constitui-se uma das principais premissas para obter-se produtos alimentícios estáveis durante a vida de prateleira e microbiologicamente seguros. A nível industrial, são comuns os relatos de recolhimento de produtos submetidos ao tratamento ultra-high temperature (UHT) deteriorados, como leites achocolatados. Em muitos destes casos, a causa da deterioração são micro-organismos pertencentes ao gênero *Bacillus* spp. e a fonte da contaminação é o cacau em pó. Apesar de sua importância como matéria-prima industrial, existem poucos estudos cujos objetivos tenham sido estudar a ecologia microbiana com referência à micro-organismos esporulados em diferentes unidades produtoras (fazendas de cacau) localizadas em zonas tropicais, como o Brasil. Portanto, o principal objetivo deste trabalho foi avaliar as populações de bactérias esporuladas durante todo o processamento do cacau em pó. 618 amostras foram coletadas de duas fazendas e de três linhas industriais, localizados no estado da Bahia. Além disso, avaliou-se o impacto da fermentação e da torrefação sobre cepas de *B. cereus* e *G. stearothermophilus*. Os resultados mostraram que a contaminação por bactérias esporuladas ocorre durante a fermentação, e pode chegar a industria com contagens altas (5.6 log esporos/g). Etapas de torrefação e alcalinização do processo industrial reduziram a população de esporulados em alguns casos (3-6 log esporos/g), porém houve resistência ao processo. Na simulação da fermentação do cacau em laboratório, a população de *B. cereus* e *G. stearothermophilus* permaneceu estável (3-4 log esporos/g), com exceção aos dois últimos dias, quando ocorreu um ligeiro aumento. Na simulação do processo de torrefação observou-se que esta pode ter um efeito limitado na inativação de esporos. Estes resultados são importantes pois o conhecimento dos efeitos do processamento do cacau sobre os micro-organismos esporulados, é essencial não somente para a melhoria da qualidade microbiológica, mas também para a melhoria dos métodos de preservação visando-se garantir a segurança e estabilidade microbiológica de produtos formulados com cacau em pó.

Palavras-chave: cacau em pó, bactérias esporuladas, processamento do cacau, segurança microbiológica.

ABSTRACT

Ensuring the use of raw materials and ingredients with low initial populations of spore forming bacteria is one of the main assumptions for obtaining shelf-stable and microbiological safety food products. At industrial level, reports of spoilage of ultra-high temperature (UHT) products such as chocolate milks are common. In many of these cases, the cause of the spoilage is spore forming bacteria belonging to the genus *Bacillus* spp. and the source of contamination is cocoa powder. Despite its importance as an industrial raw material, there are few studies whose objectives have been to study microbial ecology with reference to spore forming bacteria in different production units (cocoa farms) located in tropical zones, such as Brazil. Therefore, the main objective of this work was to evaluate populations of spore forming bacteria along the processing of cocoa powder. 618 samples were collected from two farms and three industrial lines, located in the state of Bahia, Brazil. In addition, the impact of fermentation and roasting on *B. cereus* and *G. stearothermophilus* strains was evaluated. The results showed that contamination by spore forming bacteria occurs during fermentation, and this contamination can reach industry with high counts (5.6 log spore/g). Roasting and alkalization stages of the industrial process reduced the spore population in some cases (3-6 log spore/g), but there was resistance to the process. In the simulation of cocoa fermentation in the laboratory, the population of *B. cereus* and *G. stearothermophilus* remained stable (3-4 log spore/g), except for the last two days, when a small increase occurred. In the simulation of the roasting process it was observed that this may have a limited effect on the inactivation of spores. These results are important because knowledge of the effects of cocoa processing on spore population is essential not only for the improvement of microbiological quality but also for the improvement of preservation methods in order to guarantee microbiological safety and stability of products formulated with cocoa powder.

Keywords: cocoa powder, spore forming bacteria, cocoa processing, microbiological safety.

SUMÁRIO

INTRODUÇÃO GERAL

O cacaueiro pertence à família Sterculiaceae, gênero *Theobroma*, espécie *Theobroma cacao* L., e consiste na principal matéria-prima para a produção de cacau em pó, chocolate e outros produtos derivados do cacau (Oracz et al., 2014; Belscak et al., 2009). De acordo com as suas características morfológicas e geográficas, o cacaueiro pode ser classificado em dois grupos: Criollo e Forastero. O grupo Criollo, encontrado principalmente na América Central, corresponde a somente 5% da produção mundial de cacau. Este grupo é caracterizado por apresentar menor tempo de fermentação da polpa, produção de cacau de melhor qualidade e maior susceptibilidade à pragas e doenças. Já o grupo Forastero, típico da região amazônica, produz cacau com aroma mais suave e corresponde a maior parte do cacau produzido no mundo. Um terceiro grupo denominado Trinitário, foi obtido a partir da hibridização dos grupos anteriores e apresenta resistência à pragas e doenças, além de cacau com suaves atributos sensoriais (Saltini et al., 2013; Motamayor et al., 2003; Afoakwa et al., 2008). Apesar disso, sabe-se que as propriedades do chocolate podem variar de acordo com a origem do cacaueiro, composição do fruto e condições de processamento. Tais fatores afetam a aceitabilidade do produto final pelo consumidor (Torres-Moreno et al., 2012, Copetti et al., 2011).

As plantações de cacau são cultivadas em diversas regiões tropicais do mundo, sendo Costa do Marfim, Gana, Nigéria, Camarões, Indonésia e Brasil, os principais produtores (Afoakwa et al., 2008; Schwan and Wheals, 2004; Moreira et al., 2013). No Brasil, sua faixa ideal para cultivo fica entre os estados do Espírito Santo, Bahia e Rondônia (ABICAB, 2014). Costa do Marfim no continente africano, lidera a produção mundial de cacau com 1,3 milhão de toneladas. O Brasil ocupa o quinto lugar, com 220 mil toneladas produzidas em 2010 (ABICAB, 2014), 60.074 produtores, numa área de cultivo total de 729.676 ha (Neves et al., 2013). A produção e comercialização do cacau representa grande importância econômica para alguns estados brasileiros, particularmente, Bahia (Moreira et al., 2013).

Assim como outros ingredientes e matérias-primas, o cacau e seus derivados são susceptíveis à contaminação por micro-organismos patogênicos e deterioradores em toda cadeia produtiva. As frutas (cacau) in natura, incluindo sementes e polpa, podem ser consideradas estéreis até o início do processamento. Na etapa de corte do cacau, sementes e polpa são contaminadas por uma ampla variedade de micro-organismos. Poeira, água, ar, animais, utensílios, manipulação, e equipamentos são alguns exemplos de fontes de contaminação do

cacau nos estágios iniciais do processamento. Parte destes micro-organismos é responsável pela fermentação natural do cacau (Nascimento et al., 2010).

Os principais grupos microbianos presentes durante o processamento do cacau são leveduras, bactérias lácticas, bactérias acéticas, fungos, Enterobacteriaceae e espécies do gênero *Bacillus*, dentre outras (Nascimento et al., 2010, Copetti et al., 2011, Lima et al., 2012). Contudo, a presença de compostos antimicrobianos, redução da atividade de água, mudanças no pH e tratamentos térmicos durante o processamento do cacau e chocolate, levam à seleção de micro-organismos resistentes, principalmente os formadores de esporos.

Os esporos são estruturas dormentes, refratárias, não reprodutivas, de grande resistência térmica e química, constituídas principalmente por DNA, uma capa proteica, uma camada de peptidoglicano, ácido dipicolínico, e sais de cálcio (Abee et al., 2011; Indest et al., 2009). Em virtude de sua resistência térmica e química, os micro-organismos esporulados sobreviventes ao processamento podem ser isolados de matérias-primas, ingredientes, ambientes, e a partir destes, podem contaminar produtos processados (Carlin, 2011; Meer et al., 2011). A contaminação por esporos bacterianos é relatada em quase todos os tipos de produtos e processos (Carlin, 2011). A principal fonte de bactérias esporuladas é o solo, mas estes micro-organismos podem também ser encontrados no trato gastrointestinal de insetos e animais de sangue quente. Além disso, equipamentos e utensílios de processamento também podem ser fontes de contaminação por micro-organismos esporulados, como *Bacillus* spp., que possuem habilidade de aderir à superfície de equipamentos, formar biofilmes e/ou resistir aos procedimentos de limpeza e sanificação (Postollec et al., 2012; Carlin, 2011). A presença destes micro-organismos em matérias-primas e produtos processados pode levar a perdas econômicas significativas e a danos na reputação da indústria (Lücking et al., 2013). Algumas espécies podem ser patogênicas e outras deterioradoras. Por exemplo, *B. cereus* é conhecido por causar dois tipos de doenças de origem alimentar, as síndromes diarréica (infecção) e emética (intoxicação). A síndrome diarréica é causada por células vegetativas, ingeridas como células ou esporos viáveis, que produzem enterotoxinas no intestino delgado. Os sintomas típicos incluem dores abdominais, diarreia aquosa, náuseas e vômitos. Já a síndrome emética, considerada mais grave, é causada pela toxina emética denominada cereulide, produzida no alimento antes da ingestão. Os sintomas da síndrome emética incluem principalmente náuseas e vômitos (Abee et al., 2011; Andersson et al., 1995). Já micro-organismos como *Geobacillus stearothermophilus, Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus pumilus*, dentre outros, podem causar diferentes tipos de deterioração dos alimentos, devido à produção de ácidos e/ou enzimas (Pereira et al., 2018; Caspers et al., 2011; Lucking et al., 2013). Apesar

de sua importância, poucos estudos tem reportado a presença de bactérias esporuladas em diferentes etapas do processamento de cacau e nos produtos derivados, como cacau em pó.

De acordo com o exposto, o objetivo deste estudo foi investigar a incidência de bactérias esporuladas ao longo da cadeia produtiva do cacau em pó, da fazenda até a indústria, assim como analisar a persistência de *B. cereus* no processamento. Além disso, foi investigado o impacto de etapas do processamento do cacau (fermentação e torrefação) sobre o comportamento (sobrevivência) de *B. cereus* e *G. stearothermophilus.*

CAPÍTULO 1

REVISÃO DA LITERATURA

Diversity and fate of spore forming bacteria in cocoa powder, milk powder, starch and sugar during processing: a review

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Review

Diversity and fate of spore forming bacteria in cocoa powder, milk powder, starch and sugar during processing: A review

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ABSTRACT

Background: Raw materials such as cocoa, milk powder, sugar, and starch are widely used in the formulation of a wide variety of processed foods. The unit operations applied during their production vary according to their specific properties, technological advances and needs, which undoubtedly affect the microbial composition found in these raw materials. The microbial composition of these raw materials is known to consist mainly of spores of bacteria, which are known to withstand harsh food processing conditions.

Scope and research: This article aims to discuss data available on the diversity of spore forming bacteria in selected raw materials (cocoa, milk powder, sugar, starch). These raw materials are contaminated mainly by spore forming bacteria, which can germinate and be a concern in products made with these ingredients. In addition, this review presents data gaps and studies needed to establish the fate of spore forming bacteria throughout the production chain of specific raw materials.

Key findings and conclusions: The review of literature conducted in this study indicates that data on the effects of processing and diversity of spore forming bacteria in sugar and starch are much scarcer compared with cocoa and milk powders. Thus, cutting-edge approaches combining quantitative data with metagenomics could be used to improve our knowledge on the fate and diversity of spore forming bacteria in raw materials. These approaches can be employed to guide further developments that are aimed at enhancing food safety and controlling food spoilage caused by spore forming bacteria.

1. Introduction

Every food product has its characteristic microbial composition, depending on processing conditions and raw material used (Gram et al., 2002). Raw materials are important for the quality of final products (Zugarramurdi, Parin, Gadaleta, Carrizo, & Lupin, 2004) as even adequately designed preservation processes are not able to ensure the microbiological quality and safety of foods if low-quality raw materials are used in food formulations (Inteaz, 1989). Thus, proper handling and processing of raw materials is necessary to guarantee a microbiological load that does not negatively affect food safety and stability of final products.

Cocoa, milk powder, sugar, and starch are raw materials widely used in the food industry for the manufacturing of a wide variety of products. Cocoa is the primary raw material for the production of cocoa powder, chocolate, and other cocoa products (Moreira, Miguel, Duarte, Dias, & Schwan, 2013). Milk powder is used in the manufacturing of various dairy foods such as ice cream, reconstituted dairy products and infant formula, in addition to bakery and confectionery products (Medeiros, 2010). Sugar is used as an ingredient in multiple desserts such as jams, jellies, puddings and other sweetened products (Hoffmann, Cruz, & Vinturim, 1992). Starch is commonly used as thickening agent in soups and meat sauces, binding agent in sausages, gel-forming in candies and puddings, stabilizer in salad dressings, etc. (Amorim, 2011). Spore forming bacteria are the primary contaminants of these raw materials due to the processing characteristics which favor the elimination of vegetative bacteria and may allow for survival of spores, or even outgrowth of sporeformers that subsequently produce spores (Amorim, 2011; Lima et al., 2012; Walstra, Wouters, & Geurts, 2005; Wojtczak, Biernasiak, & Papiewska, 2012).

Both soil and the gastrointestinal tract of insects and warm-blooded animals are considered the main sources of spore forming bacteria (Hong et al., 2009; Nicholson, 2002; Postollec et al., 2012). The soil is the primary habitat of these microorganisms and can be a direct source of food contamination (Carlin, 2011). The counts of spore forming microorganisms such as *Bacillus cereus* and *Clostridium* spp. can reach 10^5-10^6 spores per g of soil (Carlin, 2011; Lund, 1986; te Giffel, Beumer, Slaghuis, & Rombouts, 1995). Counts of aerobic spore forming

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bacteria of approximately 10^6 and 10^4 spores per g were found in soil and human feces, respectively (Hong et al., 2009). Other sources of bacterial spores include silage, which can be a primary source of spores of *Clostridium tyrobutyricum* that may be present in milk (Carlin, 2011; Vissers, Driehuis, Giffel, Jong, & Lankveld, 2007) and compost, which can be a source of highly resistant spores (Cliff et al., 2005). *Bacillus* species dominate the microbial composition of arable land and therefore are found in animals, plants and various types of food (Hong et al., 2009). In addition, processing facilities may also serve as a source of spore forming bacteria. Certain spore forming are known to have the ability to adhere and form biofilms on food contact surfaces as well as their resistance to cleaning procedures (Andersson, Ronner, & Granum, 1995; Auger, Ramarao, & Faille, 2009; Carlin, 2011).

Spore forming bacteria such as *Bacillus cereus* (Drobniewski, 1993), *Clostridium botulinum* (Hatheway, 1990, 1998; Villar, Elliott, & Davenport, 2006) and *C. perfringens* (Songer, 1996, 2010) have been linked with foodborne disease outbreaks in several countries (Drobniewski, 1993; Otuki, 2010; Peck, 2006; Schoeni & Wong, 2005). Moreover, germination and outgrowth of spores of non-pathogenic spore forming species may result in food spoilage, leading to substantial financial losses and damage to reputation (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-schulz, 2013). Thus, the use of raw materials and ingredients containing low populations (2 $\log_{10}$ CFU per g/mL) of spore forming bacteria is crucial to obtain shelf-stable and microbiologically safe products.

Given the above, this article aims to present the diversity of spore forming bacteria reported until now in selected raw materials - cocoa, milk powder, sugar and starch, commonly used in the food industry. This review also identifies data gaps on the fate of spore forming bacteria present throughout the production chain of these raw materials.

2. Spore forming bacteria: implications for food safety and quality

Bacterial spores can survive in foods and persist in food processing facilities for a long time. After germination, the growth of the vegetative cells may occur under various conditions of temperature, pH, and water activity (Carlin, 2011).

Among aerobic spore forming bacteria, *Bacillus cereus* is known to cause two types of foodborne diseases, the emetic and diarrheal syndromes (Fogele, Granta, Valcina, & Berzins, 2018). In general, both syndromes are relatively mild, but fatal incidences have been associated with consumption of food containing high levels of emetic toxin (Ehling-Schulz, Fricker, & Scherer, 2004; Lund, Buyser, & Granum, 2000) and cytotoxin (CytK) (Lund et al., 2000). Foodborne disease (FBD) caused by *Bacillus cereus* has been reported worldwide (Drobniewski, 1993; Ehling-Schulz et al., 2004; Schoeni & Wong, 2005). Other species of *Bacillus* such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus* can also produce toxins playing a role in food safety (Ehling-schulz & Messelhäusser, 2013; Logan, 2011; Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-schulz, 2013).

Among the anaerobic spore forming bacteria, *C. botulinum* stands out because it causes foodborne botulism (Proverbio, Lamba, Rossi, & Siani, 2016). Botulism is a severe disease with a high fatality rate (Tseng et al., 2009). Another relevant anaerobic spore forming bacteria is *C. perfringens*, capable of causing intestinal illness in humans and animals (Allaart, Asten, Vernooij, & Gröne, 2014; Martin & Smyth, 2010). This bacterium has frequently been involved in food poisoning (Allaart, Asten, & Gröne, 2013; Paredes-Sabja, Udompijitkul, & Sarker, 2009; Sarker, Shivers, Sparks, Juneja, & McClane, 2000). Food poisoning caused by *C. perfringens* is highly prevalent in the United States and elsewhere (Udompijitkul, Alnoman, Banawas, Paredes-Sabja, & Sarker, 2014; Xiao, Wagendorp, Moezelaar, Abee, & Wells-Bennik, 2012). Foods most commonly associated with this food poisoning are beef, poultry and pork; to a lesser extent eggs, vegetables and grains (Lynch, Painter, Woodruff, & Braden, 2006). The cost of the disease caused by this bacterium was estimated at $ 309.4 million per year (Hoffmann, Batz, & Morris, 2012).

In addition to causing disease, spore forming bacteria in foods also cause food spoilage (Caspers et al., 2011). Spoilage is characterized by changes in foods that makes them unacceptable for consumption (Gram et al., 2002). Consequences of the growth of spore forming bacteria in foods include changes in pH, color, nutritional composition, potentially formation of toxic components, off-odors, off-flavors, gas, and slime (Veld, 1996). The ingredients or raw materials and the environment are considered the main sources of spore forming bacteria that can cause spoilage (Fabian & Wethington, 1950; Waite, Jones, & Yousef, 2009). Microorganisms such as *Geobacillus stearothermophilus*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus pumilus*, among others, can cause different types of food spoilage (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013).

3. Survival strategies of spore forming bacteria during food processing

The capacity to form spores enables certain bacteria to survive food processing as well as to persist for extended periods in foods (Moeller et al., 2008; Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000; Postollec et al., 2012). Species within *Bacillus, Clostridium*, *Geobacillus, Brevibacillus, Paenibacillus* genera can produce highly resistant spores under unfavorable growth conditions. Spores are resilient to adverse environmental conditions such as pressure, heat, bacteriocides, UV radiation, absence of water and nutrients, and may remain inactive for centuries (Nicholson et al., 2000). The resistance of spores is a consequence of their structure (Fig. 1), consisting of a dehydrated core surrounded by several protective layers: intermembrane, cortex, outer membrane, spore coat and exosporium (when characteristic of a bacterium) (Abee et al., 2011; Indest, Buchholz, Faeder, & Setlow, 2009). Therefore, knowledge about their features is crucial for improving inactivation and preservation methods (Abee et al., 2011; Wells-Bennik et al., 2016a,b).

The germination process comprises four steps: activation, commitment, stage I, stage II and outgrowth (Setlow, 2014). The activation occurs through the addition of nutrients, sublethal heat shock, high pressure or other agents, such as lysozyme, salts, Ca^{2+} - DPA (dipicolinic acid) and cationic surfactants (Gould, 1969; Setlow, 2003). Upon commitment to germinate, there is a release of H^+, monovalent cations and Zn^{2+} from the core of the spore (Setlow, 2013; Setlow, Cowan, & Setlow, 2003; Swerdlow, Setlow, & Setlow, 1981; van Beilen & Brul, 2013). Stage I is characterized by release of DPA and associated divalent cations (Ca^{2+}). The release of DPA, which is replaced by water, increases core hydration, leading to partial decrease in heat resistance (Moir & Smith, 1990; Paidhungat & Setlow, 2002; Setlow, Melly, & Setlow, 2001a). Stage II is characterized by hydrolysis of the cortex and core hydration, leading to enzymatic activity and a more significant loss of resistance (Setlow, Melly, & Setlow, 2001b). These events allow for the initiation of spore metabolism, followed by the synthesis of macromolecules that transform germinated spores into vegetative cells that initiate outgrowth (Paidhungat & Setlow, 2002; Wells-Bennik et al.,

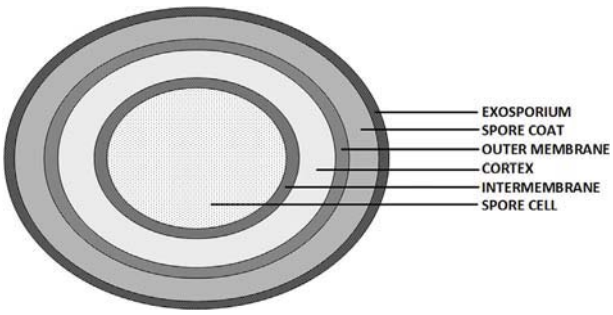

Fig. 1. Spore structure.

2016a,b). Spores are metabolically inactive but can recognize their surrounding environment and respond fast when appropriate nutrients for germination and outgrowth become available (Paidhungat & Setlow, 2002). However, it should be highlighted that germination and outgrowth can be highly variable given the inter- and intra-species variability. Particularly, the estimation of lag times may be characterized by large differences due to heterogeneity of individual spores, which is challenging for accurate assessment of sporeforming bacteria germination and outgrowth. The heterogeneity can be even higher when bacterial spores are injured or when the environmental conditions for outgrowth are not optimal (Wells-Bennik et al., 2016a,b).

Besides their ability to form resistant spores, such microorganisms can adhere to surfaces of industrial equipment, forming biofilms (Carlin, 2011; Postollec et al., 2012), which further aides their survival and persistence (Shi & Zhu, 2009). Biofilms are complex multi-cellular structures attached to surfaces, surrounded by a highly hydrated matrix of extracellular polysaccharides (Faille et al., 2014; Shi & Zhu, 2009; Simões, Simões, & Vieira, 2010). Biofilms can also be comprised of proteins, fats, minerals and corrosive materials (Teh et al., 2014). Due to the adherence capacity and resistance to cleaning procedures targeted at bacteria in food processing surfaces, biofilms comprise a significant concern in the food industry (Faille et al., 2013). Individual cells are less resistant to antimicrobial treatment than bacterial biofilms due to reduced diffusion, physiological changes due to reduced growth rates and the production of enzymes degrading antimicrobial substances (Kumar & Anand, 1998). Stainless steel, aluminium, glass, Buna-N and Teflon seals and nylon are example of surfaces used in the food industry on which microorganisms can form biofilm (Kumar & Anand, 1998; Mafu, Roy, Goulet, & Hagny, 1990). The surface of stainless steel has cracks and crevices allowing the escape of bacteria from the cleaning process (Kumar & Anand, 1998; Wirtanen, Husmark, & Mattila-Sandholm, 1996). The brewing, dairy, poultry and meat sectors are examples of segments that require effective control methods to prevent biofilms formation (Chen, Rossman, & Pawar, 2007; Frank, Ehlers, & Wicker, 2003; Jessen & Lammert, 2003; Somers & Wong, 2004). In addition to potentially affecting the operation of some equipment, biofilms may result in cross-contamination, and post-processing contamination of foods (Kumar & Anand, 1998; Shi & Zhu, 2009). Spore forming bacteria can sporulate within biofilms in low-nutrient conditions, leading to spore contamination of food (Kumar & Anand, 1998; Scott, Brooks, Rakonjac, Walker, & Flint, 2007).

4. Recovery of spore forming bacteria

Recovery of spore forming bacteria depends on two factors: nutrients and heat activation, both used in laboratory methods. Spore germination is triggered by presence of nutrients and non-nutrients agents. Nutrient agents called germinants, such as glucose, amino acids and purine ribosides, bind to germinating receptors located on the inner membrane of the spore, triggering germination (Ghosh & Setlow, 2009; Paidhungat & Setlow, 2002; Setlow, 2003). *B. subtilis* spores respond to L-alanine, L-valine, mixture of L-asparagine, D-glucose, D-frutose and K^+ ions (Atluri, Ragkousi, Cortezzo, & Setlow, 2006; Paidhungat & Setlow, 2002; Setlow, 2003). *B. megateruim* spores respond to D-glucose, L-proline, L-leucine, L-valine or salts (Christie & Lowe, 2007; Ghosh & Setlow, 2009). Non-nutrient agents such as Ca-DPA and cationic surfactant may trigger germination as well (Ghosh & Setlow, 2009; Paidhungat & Setlow, 2002).

Most of the spores germinate rapidly (30–60 min) (Ghosh, Scotland, & Setlow, 2012) in the presence of the nutrients, but a small percentage of spores remain dormant and can take hours or days to germinate, called super dormant (SD) spores (Gould, 1970; Wei et al., 2010). SD spores present a higher optimal temperature for heat activation (Ghosh, Zhang, Li, & Setlow, 2009), and while they germinate poorly in the presence of nutrients, they germinate normally in the presence of dodecylamine and Ca-DPA (Ghosh & Setlow, 2009, 2010; Wei et al., 2010). When a whole population of spores germinates and environmental conditions change, this population can eliminated, so spore super dormancy can be a strategy to protect the species against extinction (Ghosh et al., 2012; Setlow, Liu, & Faeder, 2012; Veening, Klaas Smits, & Kuipers, 2008). SD spores have high levels of wet heat resistance and its population is a concern for the food industry (Ghosh et al., 2012, 2009).

Table 1
Heat activation conditions for spore forming bacteria.

	Microbial group	Heat activation conditions T (°C)/t (min)	Food category
Aerobic spore forming	Thermophilic	100/5	Sugar
		108.4/10	Starch, milk powder
		90/5	Tomato, tomato pulp, concentrate milk
		121/20	Milk cream
	Mesophilic	80/10	Foods in general
		80/12	Milk
		75/15	Water
Anaerobic spore forming	Thermophilic	100/5	Sugar, milk powder
		100/15	Starch, flour, cereals
	Thermophilic H_2S producer	100/5	Sugar
		100/15	Starch
		108.4/10	Milk powder
	Mesophilic	100/5	Sugar
		100/20	Starch, flour, milk powder
		80/12	Milk, cheese

Reference: Downes and Ito (2001).

The sublethal heat process used to germinate spores of spore forming bacteria is termed heat activation. Temperature and time of heat activation may vary among different groups of spore forming bacteria (Keynan, Evenchik, Halvorson, & Hastings, 1964). Table 1 shows the conditions for heat activation for each spore forming group in different category of foods (Downes & Ito, 2001). The optimal temperature of heat activation for dormant and SD spores decreases when spores are in conditions with a higher amount of germinants, but here is no answer about how spores are activated by heat or any other activation treatment (Ghosh et al., 2009).

5. Diversity and fate of spore forming bacteria during the processing of raw materials

Due to their ubiquitous and resistant nature, spore forming bacteria can contaminate many raw materials used in the food industry. The inherent characteristics of raw materials and their processing steps and conditions are known to be critical factors affecting qualitatively and quantitatively the microbial composition. Below, the aspects of processing and the fate and diversity of spore forming bacteria along the processing chain of cocoa, milk powder, sugar and starch are discussed.

5.1. Cocoa powder processing

Pre-processing of cocoa held on the farm consists of harvesting and breaking the fruit, fermentation and drying (Fig. 2A). Such steps occur under limited hygienic conditions, leading to contamination of cocoa seeds by various microorganisms from the environment, handlers, and utensils (Nascimento et al., 2010). The newly harvested fruits are opened manually to remove the seeds. Thus, knives and hands of workers used for collection and transportation are examples of contamination sources. Once the seeds are removed, they are placed in wooden boxes, baskets, trays or platforms, which are naturally fermented for about six days (Copetti, Iamanaka, Frisvad, Pereira, & Taniwaki, 2011; Wood, 1985).

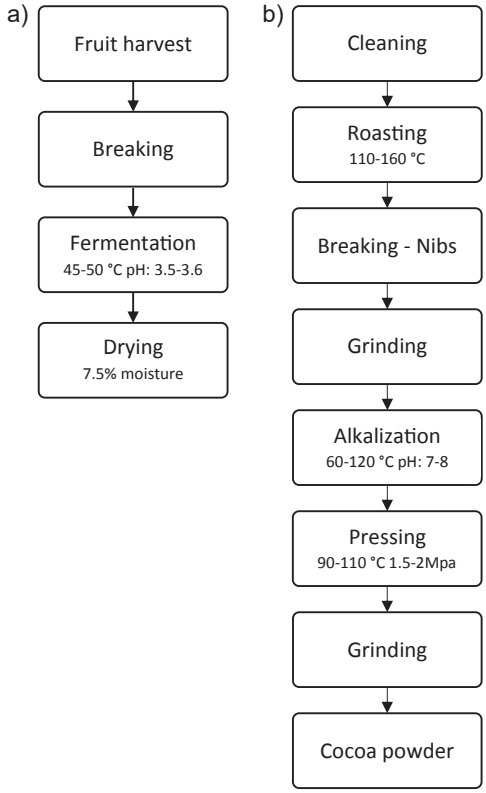

Fig. 2. A. Flowchart of pre-processing of cocoa. B. Flowchart of cocoa processing.

Fermentation is a consequence of the action of several microorganisms (yeasts, lactic acid bacteria, acetic acid bacteria and filamentous fungi) that contaminate the environment surrounding the pulp of cocoa beans after opening. As can be seen in Table 2, although several *Bacillus* spp. can be recovered during the fermentation of cocoa, primarily at the end of this fermentation, their role has not been fully elucidated yet. Some species are capable of producing pectinolytic enzymes, such as polygalacturonase and pectin lyase, during the fermentation, which can cooperate with yeast in depectinization of the pulp (Ouattara et al., 2008; Schwan & Wheals, 2004). A study of 98 *Bacillus* strains isolated from fermenting cocoa found 90 strains capable of pectin degradation, and of these, 80% had detectable pectinolytic activity during fermentation. The production of polygalacturonase and pectin lyase was detected in 48 and 47 strains respectively, and 23 strains showed production of both enzymes (Ouattara et al., 2008). In another study, it was found that *B. fusiformis, B. subtilis* and *B. pumilus* showed high levels of production of pectin lyase, with maximum production in the stationary phase of growth (Ouattara, Reverchon, Niamke, & Nasser, 2011).

Besides the production of pectinolytic enzymes, spore forming bacteria produce various compounds, which can contribute to the acidity and may lead to the formation of undesirable flavors in fermented beans. Acetic and lactic acids, 2,3-butanediol and tetramethylpyrazine produced by *Bacillus* spp. can be detrimental to the chocolate flavor. Furthermore, it has been described that short-chain free fatty acids encountered during the aerobic phase of fermentation and accountable for the formation of off-flavors are produced by *B. subtilis, B. cereus* and *B. megaterium* (Lopez & Quesnel, 1971; Zak, Ostovar, & Keeney, 1972). Alcohols and acids produced through fermentation affect the sensory properties of the final product. These compounds contribute in a positive manner, however, when they are present at high concentrations, they can result in off-flavors, making the products unsuitable for the preparation of good quality chocolate (Schwan, Vanetti, Silva, Lopez, & Moraes, 1986). Spore forming

Table 2
Sporeforming bacteria isolated during cocoa fermentation in different countries [a].

Origin	Number of sample	Counts (log cfu/g)	Prevalence (%)	Number of isolates	Species	Method	Reference
Brazil	15	[a]ND	100	115	*B. brevis, B. cereus, B. circulans, B. coagulans, B. firmus, B. laterosporus, B. licheniformis, B. macerans, B. megaterium, B. pasteurii, B. polymyxa, B. pumilus, B. atrophaeus, Geobacillus stearothermophilus*	Heating to 80 °C/20 min. Plating on TYGKCP (0.5% tryptone, 0.5% yeast extract, 0.1% glucose, 0.1% K_2HPO_4, 0.1% $CaCO_3$, 1% pulp extract and 2% agar) with 1% cocoa pulp. Incubation at 37 °C for 48 h.	Schwan et al. (1986)
Trinidad (Centeno and San Louis estate)	[a]ND	[a]ND	[a]ND	[a]ND	Centeno - *B. cereus, B. subtilis, G. stearothermophilus, B. coagulans, B. mycoides;* San Louis - *B. cereus, B. megaterium, B. subtilis, G. stearothermophilus, B. coagulans, B. pumilus*	Plating on TYGKCP. Incubation at 28 °C, 37 °C and 45 °C for 72 h.	Ostovar and Keeney (1973)
Ivory Coast	[a]ND	5.0–7.5	[a]ND	98	*B. atrophaeus, B. pumilus, B. sphaericus, B. cereus, B. thuringiensis, B. fusiformis*	Plating on Nutrient Agar containing 0.01% cycloheximide	Ouattara et al. (2008); Ouattara et al. (2011)
Ghana	[a]ND	3.6–7.99	[a]ND	526	*Bacillus licheniformis, B. megaterium, B. pumilus, B. atrophaeus, B. cereus, B. sphaericus*	Plating on Nutrient Agar containing 0.1% cycloheximide. Incubation at 30 °C for 3 days	Nielsen et al. (2007)
Indonesia	[a]ND	4.0–8.0	[a]ND	[a]ND	*Bacillus pumilus, B. licheniformis, B. atrophaeus, B. cereus, B. sphaericus, B. coagulans*	Plating on Tryptone Yeast Extract Agar. Incubation at 28 °C for 1–4 days	Ardhana and Fleet (2003)
Brazil	[a]ND	4.48–7.58	[a]ND	[a]ND	*Bacillus atrophaeus, B. megaterium, B. flexus, Paenibacillus* sp.	Plating on Nutrient Agar containing 0.1% cycloheximide. Incubation at 30 °C for 3–4 days	Pereira, Magalhães, Almeida, Coelho, and Schwan (2013)

[a] ND – Not determined.

Table 3
Sporeforming bacteria isolated from cocoa powder[a].

Origin	N° of sample	Prevalence (%)	Counts (log cfu/g)	Number of isolates	Species/prevalence (%)	Method	Reference
[a]ND	36	100	[a]ND	509	*Bacillus licheniformis*/46, *B. cereus*/20, *B. megaterium*/10, *B. atrophaeus*/8, *B. alvei*/2, *B. badius*/2, *B. larvae*/2, *B. pumilus*/1.6, *B. pantothenticus*/1.4, *B. lentus*/1.2, *B. circulans*/1, *B. firmus*/1, *B. polymyxa*/0.8, *B. laterosporus*/0.6, *B. brevis*/0.4, *B. coagulans*/0,4, *B. macerans*/0.4, *B. pulvifaciens*/0.2, *B. sphaericus*/0,2, *Geobacillus stearothermophilus*/0.2	Standard Plate Count Agar (PCA)	Gabis, Langlois, and Rudnick (1970)
Europe, South America, Africa, Asia	25	92	0.4–3.7	[a]ND	[a]ND	Heat treatment of 80 °C/10 min. Plating on PCA. Incubation at 30 °C for 72 h.	Lima, Almeida, et al. (2011) and Lima, Kamphuis, et al. (2011)
		52	0.4–3.1	[a]ND	[a]ND	Heat treatment of 80 °C/10 min. Plating on PCA. Incubation at 55 °C for 48 h.	
		36	0.7–1.8 0.6–2.2	61	*Bacillus atrophaeus*/51, *B. amyloliquefaciens*/14, *B. licheniformis*/22, *B. cereus*/10, *B. simplex*/2, *Geobacillus* spp./2	Heat treatment of 100 °C/10 min, 100 °C/30 min, 108 °C/30 min, 110 °C/10 min, 120 °C/10 min, 150 °C/10 min, 170 °C/10 min. Plating on PCA. Incubation at 30 °C for 5 days	
		12	1.0–1.9 0.6–1.6			Heat treatment of 100 °C/10 min, 100 °C/30 min, 108 °C/30 min, 110 °C/10 min, 120 °C/10 min, 150 °C/10 min, 170 °C/10 min. Plating on PCA. Incubation at 55 °C for 3 days	
Netherlands	[a]ND	[a]ND	2.5–2.8	[a]ND	[a]ND	Heat treatment of 80 °C/10 min. Plating on PCA. Incubation ate 30 °C/72 h.	Lima et al. (2012)
			1.0–1.8			Heat treatment of 80 °C/10 min. Plating on PCA. Incubation ate 55 °C/48 h.	
			1.0–2.0	10	*Bacillus licheniformis*/50, *B. cereus* group/10, *B. atrophaeus*/40	Heat treatment of 100 °C/10 min. Plating on PCA. Incubation ate 30 °C/5 days	
			0.0–0.5	1	*Bacillus licheniformis*/100	Heat treatment of 100 °C/10 min. Plating on PCA. Incubation ate 55 °C/3 days	
[a]ND	13	62	[a]ND	11	*B. amyloliquefaciens, B. thermoamylovorans, G. pallidus*	Heat treatment of 100 °C/20 min. Enrichment on BHI broth for 24 h and plating on BHI agar at 30 °C and 55 °C	Lücking et al. (2013)
[a]ND	[a]ND	[a]ND	[a]ND	[a]ND	*G. pallidus, B. thermoamylovorans*	Heat treatment of 100 °C/20 min. Enrichment on BHI broth for 24 h and plating on BHI agar at 30 °C and 55 °C	Witthuhn, Lücking, Atamer, Ehling-Schulz, and Hinrichs (2011)

[a] ND – not determined.

bacteria and filamentous fungi can lead to spoilage during the final stage of fermentation, when pH is appropriate for their growth (Schwan & Wheals, 2004). After fermentation, the drying procedure takes place, aiming to diminish the moisture content to about 7.5%. Artificial methods can be used, but the most commonly employed method is sun drying (Hashim, Selamat, Muhammad, & Ali, 1998).

After the pre-processing steps, the fermented and dried beans are roasted, ground, stored and marketed. During roasting, temperatures of 110–160 °C are employed (Farah, Zaibunnisa, & Misnawi, 2012). This process plays an essential role in the development of the typical flavor of chocolate, such as a more intense brown color and textural changes (Oliviero, Capuano, Cämmerer, & Fogliano, 2009; Oracz & Nebesny, 2014). The roasted beans are broken into fragments called nibs which are ground to form a mass or liquor (Fig. 2B) (Copetti, 2009; Wood, 1985).

In cocoa powder processing, alkalization is carried out using alkali solutions or suspensions, such as sodium or potassium carbonates. The concentration of alkali may range from 1 to 6%, whereas the temperature may vary from 60 °C to 120 °C, and the process can occur for 30–150 min (Bispo, 1999; Li et al., 2014; Minifie, 1999). This process neutralizes the acidity of the cocoa by raising the pH from 5.0 to 5.6 to 7.0–8.0 (Kostic, 1997; Li et al., 2012).

In the pressing step, the liquor or mass is subjected to high pressure (1.5–2 MPa) at temperatures between 90 and 110 °C when the separation of cocoa butter and cake occurs (Fang, Tiu, Wu, & Dong, 1995; Minifie, 1999; Venter, Schouten, Hink, Kuipers, & Haan, 2007). The cake is subjected to a breaking and milling process to obtain the cocoa powder. Cocoa powder can be used in chocolate-drinks and various confectionery and bakery products (Bispo, 1999).

5.1.1. Diversity and fate of spore forming bacteria during cocoa processing

The presence of antimicrobial compounds, reduction of water activity, pH changes and heat treatments during cocoa processing lead to the selection of resistant microorganisms, especially spore forming bacteria. Despite its importance, few studies have reported the presence and diversity of spore forming bacteria at different stages of cocoa processing.

Spore forming bacteria can be isolated through the initial three days of fermentation with counts of about 4 log CFU per g of pulp. Subsequently, as the aeration temperature (about 45 °C) and pH (3.5–5.0) increases, these bacteria dominate the microbial composition, reaching up to 7.74 log CFU per g (Schwan & Wheals, 2004). *Bacillus megaterium, B. cereus*, and *Bacillus firmus* have been isolated from the hands of workers, and the last two species have also been isolated from machetes used for fruit opening (Ostovar & Keeney, 1973). Cocoa seeds are microbiologically sterile until the opening of the fruit. Thus, machetes, manipulator's hands, dirty carriers used for seeds transportation, leaves used in the heap fermentation, residuals found in the fermentation boxes and utensils employed during processing are the primary source of microorganisms (Schwan & Wheals, 2004). *B. coagulans, B. pumilus* and *G. stearothermophilus*, have been isolated from boxes used in cocoa fermentation (Ostovar & Keeney, 1973). A marked decrease in the microbial population is observed after drying, and only spore forming bacteria (2 $\log_{10}$ CFU per g) and filamentous fungi (1 $\log_{10}$ CFU per g) can survive (Schwan & Wheals, 2004). This decrease is also observed for microbial composition after roasting, and only spore forming bacteria survive (Barrile, Ostovar, & Keeney, 1971; Lima et al., 2012). Anaerobic spore forming bacteria seems to be less prevalent in cocoa processing (Lima et al., 2012).

Lima et al. (2012) studied the impact of cocoa powder processing on the microbial populations. High counts of aerobic spores in raw nibs ranging from 4.3 to 5.5 log CFU per g were observed. After alkalization and pre-drying of the nibs, it was found that the number of aerobic spores and thermophilic spores was significantly reduced in different batches. A further reduction was observed after the roasting step, but this number increased slightly during the subsequent processing steps (liquor, cake, and cocoa powder). In the same study, the wet-heat resistance of 22 strains (comprising *B. subtilis complex*, *Geobacillus* spp, *B. licheniformis* and *B. cereus* group) isolated from cocoa powder process, after alkalization, roasting and milling was evaluated by subjecting the spores of these isolates a treatment of 110 °C for 5 min in 10 mM phosphate buffer. Reductions of 3.5–7 log were observed for *B. subtilis* strains (Lima et al., 2012).

Different species of spore forming bacteria were isolated from the final product, cocoa powder (Table 3). The wet heat resistance of strains isolated from cocoa powder obtained from various countries in Europe, South America, Africa and Asia was determined as described above (110 °C/5 min) (Lima, Kamphuis, Nout, & Zwietering, 2011). 24 strains out of 60 showed low heat-resistance (reduction of 5.5–7 log), and eight strains showed high heat-resistance (decrease of < 1 log). The thermal kinetic inactivation parameters were determined for six resistant strains belonging to the *B. subtilis* complex. Strains M1 and M112 had *D*-values of 9.51 and 111 min at 110 °C, respectively. The remaining strains had *D*-values varying from 0.07 to 2.49 min at 110 °C. Thus, spores of *B. subtilis* complex can survive thermal treatments applied on food industry. A study shows that certain strains of *B. subtilis* produce heat resistant spores that survive sterilization, but others not. One group of strains presents $D_{120°C}$ of 0.33 s while heat resistant group presents $D_{120°C}$ of 45.7 s (Berendsen, Zwietering, Kuipers, & Wells-Bennik, 2015). This heat resistant is due to the presence of a mobile genetic element (Tn1546-like) carrying operon that confer high-level heat resistance to spores (Berendsen et al., 2015).

It becomes clear that limited information exists on the impact of cocoa processing steps such as roasting, alkalization, fermentation, and pressing, on the diversity and fate of spore forming bacteria. For instance, during cocoa processing high temperatures are applied in different stages, ranging from 60 to 160 °C (roasting, alkalization and pressing steps). However, as spore forming bacteria prevalence and counts may vary among seasons, harvesting/processing conditions, storage and transportation practices, facilities, etc., the responses of these microorganisms to processing may affect qualitatively and quantitatively the microbial composition found in cocoa powder.

At the industrial level, reports on either spoiled or contaminated products, that were subjected to ultra-high temperature (UHT) treatment, such as chocolate milk, are frequent. In many of these cases, spoilage is caused by microorganisms belonging to the genus *Bacillus* spp. that are present in cocoa powder. Also, these microorganisms may be introduced in the process and form biofilms and further contaminate foods. Thus, knowing the diversity of spore forming bacteria throughout cocoa powder processing and in the final product is critical for the establishment of effective controlling measures to ensure safety and microbiological stability of products formulated with these ingredients. Then, the occurrence and importance of these microorganisms in raw materials with diverse applications such as cocoa powder should not be ignored, as they present a risk to the stability and safety of the formulated products (Byrer, Rainey, & Wiegel, 2000; Lima et al., 2012).

5.2. Milk powder processing

Milk powder is obtained through the following steps: standardization, homogenization, pasteurization, concentration, drying and packaging (Fig. 3). The fat content is adjusted during the standardization step. Centrifuges are used to separate milk into cream and skimmed milk, and then both are standardized to obtain the desired fat content (Schuck, 2011). The separation can occur at high (50–52 °C) or low (4–20 °C) temperatures. Cold separation improves the quality of product because it inhibits microbial growth (GEA Westfalia, n.d.; Moejes & Boxtel, 2017). After that, milk is heat treated by pasteurization (72 °C for 15 s or 63 °C for 30 min) (Medeiros, 2010). Sterilization at 110–125 °C for 10–45 min or UHT at 135 °C for 0.4–4 s can be used to produce commercially sterile foods (GEA Westfalia, n.d.; Moejes &

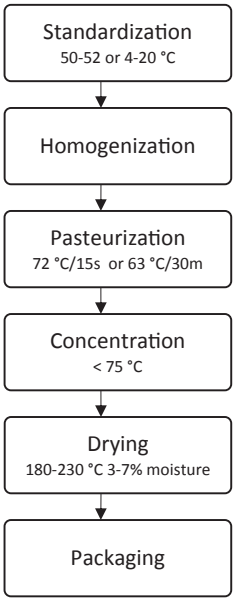

Fig. 3. Flowchart of milk powder processing.

Boxtel, 2017). Heat treatments aim to destroy microorganisms and inactivate enzymes, assuring long shelf-life of final product (Schuck, 2011).

Concentration of milk can be achieved by evaporation processes, which removes the water by multiple effect vacuum evaporators at low temperatures (< 75 °C). Evaporation minimizes the energy cost of the subsequent drying process (Medeiros, 2010). This process can be combined with reverse osmosis (Schuck, 2011). After evaporation, the drying process takes place, where the concentrated milk emulsion is injected into the spray dryer through a spinning disk atomizer. The emulsion is transformed into droplets that come into direct and immediate contact with the downward current of hot air (Medeiros, 2010). The temperature range applied is 180–230 °C, and the powder leaves the drying step with a water content of 3–7% (Fellows, 2009; Kessler, 1981; Moejes & Boxtel, 2017).

The powder formed in the spray dryer still has a high moisture, and further, it is dried to the desired moisture (2.5–3% for whole milk powder and 3.5–4% for skimmed milk powder) on a vibrofluidized bed system. Air is injected into two strategic positions of the equipment, first, at 80–100 °C and then at room temperature; if necessary, a third jet of air in injected at a temperature 5–8 °C below room temperature. Thereafter, the milk powder is packaged, stored and distributed (Doyle et al., 2015; Gopal et al., 2015; Medeiros, 2010).

5.2.1. Diversity and fate of spore forming bacteria during milk powder processing

The manufacturing process of milk powder usually reduces the number of bacteria by 5–6 log (Oliveira et al., 2000). The microbial composition present in raw milk is influenced by high temperature and reduced water activity during processing, which only enables the survival of the most resistant micro-organisms, such as spore forming bacteria (Walstra et al., 2005). The contamination of raw milk by spores occurs mainly on the farm, arising from soil, air, pasture, silage, fecal material, udder, storage and transportation tanks (Coorevits et al., 2008). Specks of dirt present on the udder are considered one of the most important contamination factors (Waes, 1976). Several species of spore forming bacteria have been recovered from equipment, the environment and raw milk (Table 4). The method used to detect spores may influence the types and levels of spores found (Kent, Chauhan, Boor, Wiedmann, & Martin, 2016). The number of spores of these microorganisms in raw milk is low (1–2 $\log_{10}$ CFU per mL). However, counts of aerobic spores ranging from 5 to 8 $\log_{10}$ CFU per g can be found in the soil, silage, used bedding and feces (Slaghuis, Giffel, Beumer, & Andre, 1997). The quality of milk powder with respect to spore forming bacteria is not necessarily related to the quality of raw milk, because production practices can affect contamination in addition to spores of spore forming bacteria found in raw milk (Walstra et al., 2005). In dairy plants, the contamination with spores is also associated with the processing conditions. Many bacteria including spore forming bacteria form biofilms which serve as constant point of contamination, releasing cells and thus compromising the quality of the final products (Ronimus et al., 2003; Scott et al., 2007; Seale, Bremer, Flint, & McQuillan, 2010).

Preheaters and evaporators operate at temperatures from 45 to 75 °C, providing a suitable environment for growth and sporulation of some spore forming bacteria species (mesophilic and thermophilic). This growth and sporulation ability allows for the survival of these bacteria in adverse conditions, resulting in long-term persistence and continuous contamination of final products. Scott et al. (2007) determined the counts of thermophilic bacteria throughout a milk powder processing line in a company located in New Zealand. There was no significant variation in the counts of these microorganisms in raw milk and standardized milk, but there was an increase in thermophilic spores counts in the balance tank and pre-evaporator. Therefore, this stage allowed for the growth of vegetative cells and their sporulation. A similar increase in spore counts was observed in the evaporator sampling point. The species isolated at these stages of processing milk powder were *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus*. This fact confirms that spore formation may occur during processing and that it is not only the result of external contamination. Murphy, Lynch, and Kelly (1999) also observed growth and sporulation of thermophilic bacteria at the pre-heating and evaporation steps.

Bacillus species or those which previously belonged to this genus, as *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus*, constitute a significant concern to producers of milk powder because of their ability to survive high temperatures. Other species of the genus *Bacillus* as *Bacillus licheniformis, Bacillus coagulans,* and *Bacillus subtilis* can also be found in milk powder (Table 5) (Gopal et al., 2015; Rueckert, Ronimus, & Morgan, 2005; Yuan et al., 2012). Spores of *B. licheniformis, A. flavithermus* and *G. stearothermophilus* strains isolated from milk powder were characterized for their thermal resistance. Spores of all strains survived heat treatments of 72 °C/15 s, 85 °C/1 min and 93 °C/3 min (pasteurization treatments) and only *G. stearothermophilus* strains survived the treatment at 121 °C/15 s (UHT treatment). Three out of five *B. licheniformis* strains and two out of three *G. stearothermophilus* strains presented high heat resistance surviving the pasteurization treatments with no decrease in counts of thermophilic spores. Besides surviving three pasteurization treatments, with no decrease in the thermophilic spore counts, some strains showed increase (1.6–2 $\log_{10}$ CFU per g) in the count during the treatment of 85 °C and 93 °C (Yuan et al., 2012). The presence of heat-resistant spores in milk powder is of concern since this product is used as ingredient for production of other heat treated products (Burgess, Lindsay, & Flint, 2010).

Even though drying of milk takes place with inlet air temperature from 180 to 230 °C (Fellows, 2009; Kessler, 1981; Moejes & Boxtel, 2017), the temperature experienced by the product is much lower, approaching 60–100 °C in most cases. Bacterial spores may vary in their responses to heat and mass transfer that take place during spray drying. Thus, knowing the effects of temperature and water activity changes during spray drying and their effects on survival or destruction of spores is needed. While it is clear that spray drying is not designed to kill microorganisms, if microbial inactivation occurs, it must be characterized and quantified, so the microbiological quality of final raw materials are well-known.

Heat resistance of spores of mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powder (Sadiq et al., 2016) was determined at temperatures ranging from 100 °C to 125 °C. All

Table 4
Sporeforming bacteria isolated from different sources in farms, dairy environment and milk[a].

Origin	Source (N° of sample)	Prevalence (%)	Counts	Number of isolates	Species/prevalence (%)	Method	Reference
Belgium	Raw milk (n = 18)	100	≤10-≤10^5 cfu/g	[a]ND	[a]ND	Heat treatment of 80 °C/10min. Plating on Milk Plate Count Agar. Incubation at 30 °C/72 h.	Scheldeman, Pil, Herman, De Vos, and Heyndrickx (2005)
			≤10-≤10^2 cfu/g	186	*Bacillus. barbaricus* (1.2), *B. fordii* (1.2), *B. licheniformis* (22.3), *B. pallidus* (15.1), *B. smithii* (1.2), *B. atrophaeus* (*B. subtilis* group) (1.2), *Bacillus* sp. nov. B (3.0), C (6.0) e D (0.6), *Aneurinibacillus thermoaerophilus* (1.2), *Brevibacillus agri* (4.8), *Brevibacillus borstelensis* (7.2), *Brevibacillus* spp. (4.8), *Paenibacillus lactis* (4.2), *Paenibacillus* sp. nov. J (3.0), L (0.6) e M (2.4), *Virgibacillus proomii* (3.6), *Virgibacillus* sp. nov. F (5.4), *Ureibacillus thermosphaericus* (6.6)	Heat treatment of 100 °C/30min. Plating on BHI agar. Incubation at 20 °C/72 h, 37 °C/48 h and 55 °C/48 h	
	Milking equipment (n = 128)	100	≤10-≤10^5 cfu/swab	[a]ND	[a]ND	Heat treatment of 80 °C/10min. Plating on PCA. Incubation at 30 °C/72 h.	
			≤10-≤10^3 cfu/swab	158	*Bacillus circulans* (1.4), *B. farraginis* (2.9), *B. fordii* (5.8), *B. fortis* (0.7), *B. licheniformis* (11.6), *B. oleronius* (4.3), *B. pallidus* (22.5), *B. smithii* (7.2), *B. sphaericus* (0.7), *B. atrophaeus* (*B. subtilis* group) (2.9), *B. thermoamylovorans* (2.9), *Bacillus* sp. nov. C (1.4) e E (0.7), *Aneurinibacillus aneurinilyticus* (1.4), *Brevibacillus agri* (7.2), Brevibacillus brevis (0.7), *Brevibacillus* spp. (5.8), *Paenibacillus lactis* (2.9), *P. thiaminolyticus* (2.2), *Paenibacillus* sp. (2.9), *Paenibacillus* sp. nov. I (1.4), *Ureibacillus thermosphaericus* (0.7)	Heat treatment of 100 °C/30min. Plating on BHI agar. Incubation at 20 °C/72 h, 37 °C/48 h and 55 °C/48 h.	
	Green crop (n = 72)	100	≤10-≤10^7 cfu/g	[a]ND	[a]ND	Heat treatment of 80 °C/10min. Plating on PCA. Incubation at 30 °C/72 h.	
			≤10-10^3 cfu/g	56	*Bacillus cereus* group (2.3), *B. circulans* (2.3), *B. farraginis* (16.3), *B. licheniformis* (9.3), *B. pallidus* (11.6), *B. smithii* (4.7), *B. sporothermodurans* (2.3), *B. atrophaeus* (*B. subtilis* group) (4.7), *B. thermoamylovorans* (4.7), *Bacillus* sp. nov. K (4.7), *Brevibacillus agri* (11.6), *Brevibacillus* spp. (4.7), *Geobacillus* spp. (11.6), *Paenibacillus* sp. nov. G (4.7)	Heat treatment of 100 °C/30min. Plating on BHI agar. Incubation at 20 °C/72 h, 37 °C/48 h and 55 °C/48 h.	
	Fodder (n = 64)	100	≤10-≤10^7 cfu/g	[a]ND	[a]ND	Heat treatment of 80 °C/10min. Plating on PCA. Incubation at 30 °C/72 h.	
			≤10-10^3 cfu/g	301	*Bacillus farraginis* (8.6), *B. flexus* (1.2), *B. fordii* (2.0), *B. fortis* (2.0), *B. licheniformis* (5.5), *B. oleronius* (1.2), *B. pallidus* (15.2), *B. smithii* (2.7), *B. sporothermodurans* (5.9), *B. atrophaeus* (*B. subtilis* group) (25.4), *B. thermoamylovorans* (0.4), *Bacillus* sp. (0.4), *Bacillus* sp. nov. B (3.9) e D (1.2), *Aneurinibacillus aneurinilyticus* (0.4), *Aneurinibacillus thermoaerophilus* (2.0), *Brevibacillus agri* (2.0), *Brevibacillus borstelensis* (2.3), *Brevibacillus* spp. (1.2), *Brevibacillus* sp. nov. H (2.7), *Geobacillus* spp. (5.9), *Paenibacillus* sp. nov. G (1.2), *Paenibacillus* sp. nov. J (2.0) e N (0.4), *Virgibacillus* sp. nov. A (0.8)	Heat treatment of 100 °C/30min. Plating on BHI agar. Incubation at 20 °C/72 h, 37 °C/48 h and 55 °C/48 h.	

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Table 4 (*continued*)

Origin	Source (N° of sample)	Prevalence (%)	Counts	Number of isolates	Species/prevalence (%)	Method	Reference
ND	Raw milk ([a]ND)	[a]ND	[a]ND	1	*Geobacillus pallidus (100)*	Heat treatment of 100 °C/20min. Plating BHI agar. Incubation at 30 °C and 55 °C	Witthuhn et al. (2011)
Belgium	Raw milk (n = 20)	80	< 2 spores/ml - > 2 log spores/ml	794	*B. licheniformis* (28.8), *B. pumilus* (12.5), *U. thermosphaericus* (7.2), *B. clausii* (3.6), *B. circulans* (2.6), *B. amyloliquefaciens* (3.1), *B. cereus* group (6.0), *Bacillus* sp. (3.5), *B. oleronius* (1.6), *L. fusiformis* (1.6), *B. atrophaeus* (0.6), *P. lactis* (0.6), *B. silvestris* (0.6), *B. thermoamylovorans* (0.5), *B. simplex* (0.4), *Paenibacillus* sp. (0.6), *P. polymyxa* (2.4), *B. badius* (0.2), *P. lautus* (0,1), *Ornithinibacillus* sp. (0.2), *Oceanobacillus* sp. (1.4), *L. sphaericus* (0.2), *B. galactosidilyticus* (0.1), *B. sporothermodurans* (0.1), *Br. invocatus* (0.1), *O. profundus* (0.1), *P. macerans* (0.1), *P. odorifer* (0.1), *Sporosarcina* sp. (0.1), *P. terrae* (0.1), *Brevibacillus* sp. (0.1)	Heat treatment of 80 °C/10min. Plating on BHI agar. Incubation at 37 °C/72 h.	Coorevits et al. (2008)
Belgium	Feed concentrate (n = 6)	100	4.0×10^3–1.1×10^6 cfu/g	106	*B. amyloliquefaciens* (4.7), *B. cereus* group (0.9), *B. circulans* (1.9), *B. clausii* (7.5), *B. flexus* (0.9), *B. licheniformis* (7.5), *B. oleronius* (0.9), *B. pallidus* (3.8), *B. psychrophilus/B. globisporus* (0.9), *B. pumilus* (23.6), *Bacillus* sp. (2.8), *B. sphaericus* (0.9), *B. sporothermodurans* (2.8), *B. atrophaeus* (*B. subtilis* group) (30.2), *Brevibacillus borstelensis* (1.9), *Paenibacillus illinoisensis* (0.9), *Paenibacillus thiaminolyticus* (1.9), *B. cereus* (5.6)	Heat treatment of 80 °C/10min. Plating on Milk Plate Count Agar. Incubation at 30 °C.	Vaerewijck et al. (2001)
Republic of Ireland	Skim milk ([a]ND)	[a]ND	200-2000 cfu/g	[a]ND	*B. lincheniformis, Geobacillus stearothermophilus*		Murphy et al. (1999)
New York	Raw milk (n = 99)	63	0.75–5.20 log cfu/ml	444	*B. cereus* (0.5), *B. cereus* sensu lato (1.1), *B. cf. aerophilus* (2.3), *B. cf. badius* (0.2), *B. cf. nealsonii* (0.5), *B. clausii* (0.2), *B. licheniformis* (32.9), *B. megaterium* (0.9), *B. muralis* (0.2), *B. pumilus* (12.8), *B. safensis* (2.7), *Bacillus* sp. (0.2), *B. atrophaeus* (*B. subtilis* sensu lato) (5.0), *B. weihenstephanensis* (11.7), *P. amylolyticus* (0.2), *P. amylolyticus* sensu lato (2.9), *P. borealis* (0.2), *P. cf. cookii* (1.4), *P. cf. pabuli* (0.9), *P. cf. peoriae* (7.0), *P. graminis* (2.7), *P. lactis* (0.2), *P. macerans* (0.5), *P. odorifer* (9.0), *Paenibacillus* sp. (1.4), *Lysinibacillus* sp. (1.1), *Planococcaceae* sp. (0.2), *Psychrobacillus* sp. (0.2), *Viridibacillus arvi/arenosi* (0.7)	Heat treatment of 80 °C/12min. Plating on SPC agar.	Masiello et al. (2014)
USA	Raw milk Corn silage Milking clusters	[a]ND [a]ND [a]ND	0.50–2.39 log cfu/mL 4.45–13.10 log cfu/mL 0.32–4.38 log cfu/cm^2	142	*Bacillus safensis* (2.0), *Bacillus cereus* (1.4), *Bacillus licheniformis* (66.2), *Bacillus sonorensis* (12), *Bacillus pumilus* (9.8), *Bacillus altitudinis* (1.4), *B. atrophaeus* (7.0)	Heat treatment of 80 °C/10min. Plating on PCA. Incubation at 37 °C and 55 °C for 48 h.	Buehner, Anand, and Garcia (2014)
Tunísia	Raw milk (n = 30)	[a]ND	[a]ND	40	*Bacillus sporothermodurans* (5.0), *Bacillus cereus* (10.0), *B. atrophaeus* (10.0), *Bacillus licheniformis* (7.5), *Brevibacillus brevis* (5.0), *Bacillus sphaericus* (2.5), *Bacillus pumilus* (7.5)	Heat treatment of 100 °C/30min. Plating on BHI agar. Incubation at 37 °C/48 h.	Aouadhi, Maaroufi, and Mejri (2014)
	Pasteurized milk (n = 20)	[a]ND	[a]ND		*Bacillus sporothermodurans* (5.0), *Bacillus cereus* (7.5), *B. atrophaeus* (2.5), *Bacillus licheniformis* (2.5), *Brevibacillus brevis* (5.0), *Bacillus sphaericus* (2.5), *Bacillus pumilus* (2.5)		
	UHT milk (n = 30)	[a]ND	[a]ND		*Bacillus sporothermodurans* (15), *Bacillus cereus* (5.0), *Bacillus licheniformis* (2,5), *Bacillus sphaericus* (2.5)		

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Table 4 (*continued*)

Origin	Source (N° of sample)	Prevalence (%)	Counts	Number of isolates	Species/prevalence (%)	Method	Reference
Australia	Raw milk	[a]ND	[a]ND	38	*B. licheniformis group* (60.5), *B. atrophaeus (B. subtilis* group*)* (15.8), *B. pumilus group* (5.3), *B. cereus* group (13.2), *B. megaterium* (5.3)	Heat treatment of 80 °C/10min. Plating on NA with 2% starch. Incubation at 30 °C and 55 °C for 24–48 h.	Chauhan et al. (2013)
	Pasteurized milk			61	*B. licheniformis* group (37.7), *B. atrophaeus* (*B. subtilis* group) (16.4), *B. pumilus* group (13.1), *B. cereus* group (21.3), *A. flavithermus* (1.6), *B. megaterium* (8.2), *B. clausii* (1.6)	Plating on NA with 2% starch. Incubation at 30 °C and 55 °C for 24–48 h.	
Netherlands	Raw milk (n = 25)	[a]ND	$< 1\text{-}10^2$ spores/mL	25	*B. cereus, B. sphaericus, B. licheniformis, B. pumilus, B. atrophaeus, Paenibacillus, Aneurinibacillus, Brevibacillus*	Heat treatment of 80 °C/10min. Plating on PCMA. Incubation at 30 °C/48 h.	te Giffel et al. (2002)
	Grass silage (n = 25)	100	$10^2\text{-} > 10^5$ spores/g		*B. cereus, Aneurinibacillus, B. atrophaeus, B. licheniformis, Paenibacillus*		
	Maize silage (n = 24)	100	$10\text{-}10^3$ spores/g		*Aneurinibacillus, B. licheniformis, B. oleronius/ sporothermodurans*		
Northern Ireland	Raw milk (n = 50)	[a]ND	0.02–3.5 log spores/mL	[a]ND	[a]ND	Heat treatment of 80 °C/10min. Skimmed milk medium with 0.1% L-alanine. Incubation at 6.5 °C/15 days.	Mcguiggan, McCleery, Hannan, and Gilmour (2002)
		[a]ND	1.4×10^1–2.4×10^5 spores/mL	[a]ND	[a]ND	Heat treatment of 80 °C/10min. Skimmed milk medium with 0.1% L-alanine. Incubation at 30 °C/15 days.	
		[a]ND	0.08–54 spores/mL	[a]ND	[a]ND	Heat treatment of 80 °C/20 and 30 min. Skimmed milk medium with 0.1% L-alanine. Incubation at 55 °C/ 7 days.	
Italy	Milk pasteurized (n = 90)	67	1.31 log cfu/g*	132	*B. cereus* (6.8), *B. circulans* (2.3), *B. coagulans* (18.2), *B. laterosporus* (2.3), *B. lentus* (2.3), *B. licheniformis* (22.7), *B. macerans* (4.5), *B. megaterium* (2.3), *B. mycoides* (2.3), *B. pumilus* (4.5), *B. sphaericus* (6.8), *Geobacillus stearothermophilus* (2.3), *B. atrophaeus* (18.2)	Heat treatment of 80 °C/15min. Plating on TSA. Incubation: 55 °C/ 48 h, 30 °C/72 h, 6 °C/14 days.	Cosentino, Mulargia, Pisano, Tuveri, and Palmas (1997)
	UHT milk (n = 60)	30	0.57 log cfu/g*	27	*B. brevis* (22.2), *B. coagulans* (11.1), *B. laterosporus* (22.2), *B. licheniformis* (11.1), *B. pumilus* (11.1), *B. sphaericus* (22.2)		
Netherlands	Raw bulk tank milk (n = 288)	[a]ND	1.20 log cfu/ml*	5	*B. cereus*	Heat treatment of 80 °C/10min. Plating on PCA. Incubation at 30 °C/ 3 days.	Slaghuis et al. (1997)
	First milk, installation (n = 60)	[a]ND	1.48 log cfu/ml*	15			
	First milk individual cows (n = 91)	[a]ND	1.61 log cfu/ml*	21			
	Udder, teats (n = 86)	[a]ND	2.82 log cfu/swab*	11			
	Faeces (n = 87)	[a]ND	5.20 log cfu/g*	63			
	Bedding used (n = 78)	[a]ND	5.15 log cfu/g*	53			
	Bedding unused (n = 73)	[a]ND	4.08 log cfu/g*	6			
	Maize silage (n = 20)	[a]ND	5.18 log cfu/g*	45			
	Silage (n = 70)	[a]ND	4.71 log cfu/g*	67			
	Concentrate (n = 82)	[a]ND	4.10 log cfu/g*	24			
	Grass (n = 20)	[a]ND	3.80 log cfu/g	50			
	Soil (n = 20)	[a]ND	5.52 log cfu/g	65			

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Table 4 (*continued*)

Origin	Source (N° of sample)	Prevalence (%)	Counts	Number of isolates	Species/prevalence (%)	Method	Reference
Scotland	Raw milk (n = 162)	58	$< 10^2$- $> 10^6$ cfu/ml	35	*B. amyloliquefaciens* (2.8), *B. cereus* (37.3), *B. cereus var mycoides* (8.6), *B. circulans* (28.6), *B. lentus* (2.8), *B. mycoides* (8.6), *B. pasteurii/sphaericus* (2.8), *B. polymyxa* (2.8), *B. thuringiensis* (5.7)	Heat treatment of 80 °C/10min. Plating on Milk agar. Incubation at 6 °C/14 days	Griffiths and Phillips (1990)
	Pasteurized milk (n = 80)	68.8	< 0.5–170 spores/l	90	*B. brevis* (2.2), *B. carotarum* (2.2), *B. cereus* (36.7), *B. cereus var mycoides* (1.1), *B. circulans* (7.8), *B. firmus* (2.2), *B. lentus* (5.6), *B. mycoides* (32.2), *B. polymyxa* (1.1), *B. pumilus* (2.2), *Geobacillus stearothermophilus* (1.1), *B. thuringiensis* (5.6)		
India	Comercial plant -	[a]ND	[a]ND	39	*B. cereus* (33.3), *B. atrophaeus* (33.3), *Bacillus* spp. (33.3)	Plating on TGYE. Incubation at 37 °C/24 h.	Sharma and Anand (2002)
	Experimental dairy plant -	[a]ND	[a]ND	16	*B. cereus* (6.3), *B. atrophaeus* (37.5), *Bacillus* spp. (56.2)		

[a] ND – not determined.

spores tested survived 100 °C for 30 min. Spores of a total of 15 strains survived, and spores of 12 strains were inactivated at 115 °C for 30 min. Log reduction varied from 0.39 (*G. stearothermophilus*) to 7.44 log (*B. licheniformis*). Only four strains survived the treatment at 125 °C/30 min, namely: *G. thermoleovorans* (2 strains), *G. stearothermophilus* (1 strain), *Br. brevis* (1 strain). This study demonstrated high resistance of *G. sterothermophilus* with reduction of only 1.4 $\log_{10}$ units after being exposed at 125 °C for 30 min. In an extensive meta-analytic study, Rigaux, Denis, Albert, and Carlin (2013) reported high variability on $D_{121°C}$ (3.3 min, 95% CI = [0.8; 9.6]) and z values (9.1 °C, 95% CI = [5.4; 13.1]) for *G. stearothermophilus* strains. The results shown by these studies reveal that bacterial spores can survive the processing of milk into milk powder. When milk powder is used for the manufacturing of food products in which conditions allow for spore germination, outgrowth and multiplication of vegetative cells. The production of microbial enzymes by spore forming bacteria, such as proteases, lipases, and phospholipases may cause spoilage, quality defects, and reduced shelf life of dairy products. Thus, sporulation combined with ineffective food preservation strategies can lead to significant economic losses (De Jonghe et al., 2010; Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-schulz, 2013; Seale et al., 2010).

5.3. Sugar processing

Fig. 4 shows the schematic processing for the production of conventional sugar from sugarcane. In the preliminary operations, sugarcane harvesting can be done manually or mechanically and is carried out on the farm, followed by transportation to the factories. Sugarcane can be stored for a maximum two days to prevent sugar loss by bacterial decomposition and plant metabolism. Upon receipt the cane is washed and chopped. Breaking the cells that contain sugar-rich materials facilitates sugarcane juice extraction. At the pressing stage, a set of rotating blades or grinder may be used. Conventional mills consist of three rolls arranged in a triangular form, so the fiber is pressed twice. As the pressure alone cannot extract more than 90% of the juice from the fibers, a re-soaking process in water is required to extract larger volumes of sugarcane juice. After grinding, coarse impurities such as bagasse, sugarcane pieces, sand, and soil are separated from juice. The sugarcane juice is then weighed and sent to the clarification step (Hamerski, 2009).

In the clarification step, non-sugar impurities are removed. The clarification of white crystal sugar is typically carried out by sulphitation. This process consists of addition of gaseous sulfur dioxide (SO_2) to the pre-heated juice (60 °C) until reaching pH 3.8 to 4.2, followed by alkalization with lime to achieve pH values of 7–7.2. The neutralization of the sulphited juice leads to formation of slightly soluble precipitate of calcium sulfite, which acts on the adsorption of colored compounds and other impurities (Hamerski, 2009).

In the production of raw sugar (the raw material of refineries), the clarification is carried out only by liming of the juice. The clarified juice is then heated to 90-105 °C, and this process is ineffective in removing colored compounds and polysaccharides. Therefore the addition of polyelectrolytes, acrylamide copolymers, and sodium acrylate improves treatment efficiency (Hamerski, 2009). Brown sugar does not undergo intense treatment, so the cane must be clean and free of impurities, and freshly harvested (Verruma-Bernardi, Borges, Lopes, Della-Modesta, & Ceccato-Antonini, 2007).

After the clarification step, the juice is evaporated, yielding a syrup with a concentration of about 65° Brix. The syrup is sent to the crystallization stage (58–65 °C) performed in vacuum calendar type evaporating crystallizers. Typically, this process takes from 3 to 5 h, and the crystal mass obtained is sent to horizontal crystallizer equipped with cooling jacket until room temperature. The final mass is then subjected to a spin cycle, in which the crystals are washed by applying water and steam, and then subjected to drying (35–40 °C) and packaging (Mantelatto, 2005). The sugarbeet process is quite similar to

Table 5
Sporeforming bacteria isolated from milk powder samples[a].

Origin	Number of sample	Prevalence (%)	Counts (cfu/g)	Number of isolates	Species/prevalence (%)	Method	Reference
Uruguay	22	100	1.0×10^2–1.0×10^4	207	*Bacillus licheniformis* (52.8), *B. megaterium* (8.3), *B. pumilus* (5.7), *B. atrophaeus (B. subtilis group)* (9.3), *Anoxybacillus flavithermus* (18.7)	Heat treatment of 80 °C/10 min. Plating on PCA. Incubation at 37 °C/24 h.	Reginensi et al. (2011)
			8.0×10^1–3.0×10^4	193	*Bacillus licheniformis* (42.5), *B. megaterium* (4.8), *B. pumilus* (3.0), *B. atrophaeus (B. subtilis group)* (4.8), *Anoxybacillus flavithermus* (41.5)	C Heat treatment of 80 °C/10 min. Plating on PCA. Incubation at 55 °C/24 h.	
			2.2×10^2–2.6×10^4	201	*Bacillus licheniformis* (44.3), *B. megaterium* (6.4), *B. pumilus* (4.0), *B. atrophaeus (B. subtilis group)* (7.0), *Anoxybacillus flavithermus* (34.3)	Plating on PCA. Incubation at 55 °C/24 h.	
China	22	100	5.5×10^1–3.9×10^4	752	*Bacillus licheniformis* (39.2), *Anoxybacillus flavithermus* (25.2), *Geobacillus stearothermophilus* (21.3), *Bacillus* sp. JM4 (1.6), *Bacillus* sp. R-6484 (2.3), *B. atrophaeus* (3.0), *Brevibacillus borstelensis* (2.3), *Aneurinibacillus thermoaerophilus* (1.8), *B. thermoamylovorans* (3.3).	Heat treatment of 80 °C/10 min. Plating on PCA. Incubation at 55 °C/48 h.	Yuan et al. (2012)
New Zealand	[a]ND	[a]ND	[a]ND	[a]ND	*Geobacillus stearothermophilus, Anoxybacillus. flavithermus, B. licheniformis e B. atrophaeus*	Plating on TSA with 0.2% potato starch. Incubation at 55 °C/24 h.	Ronimus et al. (2003)
New Zealand	4	100	7.1×10^1–3.6×10^2	147	*Bacillus atrophaeus, B. licheniformis, B. coagulans, Geobacillus stearothermophilus, Ureibacillus thermosphaericus*	Plating on TSA with 0.2% potato starch. Incubation at 55 °C/16–24 h.	
			1.7×10^1–3.3×10^2			Heat treatment of 80 °C/20 min. Plating on TSA with 0.2% potato starch. Incubation at 55 °C/16–24 h.	
Different countries	28	100	8.0×10^0–5.0×10^4	742	*Anoxybacillus flavithermus (43.8), Bacillus licheniformis (39.2), Geobacillus stearothermophilus (10.8), B. atrophaeus (2.9), B. circulans (1.2), Ureibacillus thermosphaericus (1.2), B. coagulans (0.7), B. pumilus (0.1)*	Heat treatment of 80 °C/10 min.	Rückert, Ronimus, and Morgan (2004)
Germany	11	73	[a]ND	13	*Bacillus atrophaeus, B. licheniformis*	Heat treatment of 100 °C/20 min. Enrichment on BHI broth (24 h) and plating on BHI agar. Incubation at 30 °C and 55 °C	Lücking et al. (2013)
Germany	[a]ND	[a]ND	[a]ND	2	*Anoxybacillus flavithermus (50), Geobacillus stearothermophilus (50)*	Heat treatment of 100 °C/20 min. Enrichment on BHI broth (24 h) and plating on BHI agar. Incubation at 30 °C and 55 °C	Witthuhn et al. (2011)
Australia	[a]ND	[a]ND	[a]ND	66	*Bacillus licheniformis* group (18.2), *Geobacillus* spp. (45.4), *B. atrophaeus* (*B. subtilis* group) (7.6), *B. pumilus* group (7.6), *B. cereus* group (1.5), *Anoxybacillus flavithermus* (18.2), *Paenibacillus polymyxa* (1.5)	Heat treatment of 80 °C/10 min. Plating on NA with 2% starch. Incubation at 30 °C and 55 °C/24–48 h.	Chauhan et al. (2013)

[a] ND – not determined.

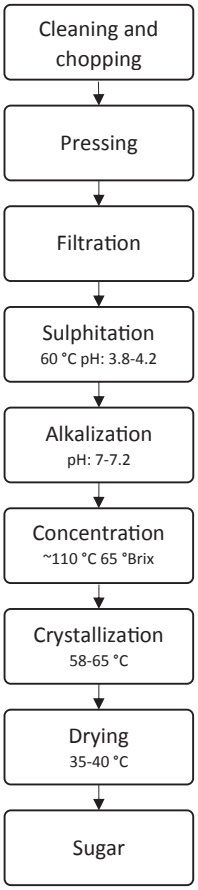

Fig. 4. Flowchart of white sugar processing.

sugarcane process. The main difference is the extraction stage. The beetroot is washed and cut into thin strips and diffused into a tank of hot water and shaking. The clarifying and concentrating steps are performed in similar way of sugarcane process (Moore, 2017).

5.3.1. Diversity and fate of spore forming bacteria during sugar production

The microbial composition of sugarcane consists of several types of microorganisms. The bacteria often found in sugarcane include *Bacillus* spp., *Flavobacterium* spp., *Pseudomonas* spp., *Xanthomonas* spp., *Lactobacillus* spp. and *Enterobacteriaceae* spp., in addition to the *Saccharomyces* spp., *Torula* spp. and *Pichia* spp, *Penicillium* spp., *Actinomyces* spp. and *Streptomyces* spp (Klaushofer, Clarke, Rein, & Mauch, 1998; Lucca, Kitchen, Clarke, & Goynes, 1992; Martini, Margarido, & Ceccato-Antonini, 2010; Nunez & Colmer, 1968). Shortly after sugarcane cutting, the microbial composition is comprised mainly of yeasts and bacteria as *Leuconostoc* spp., *Xanthomonas* spp. and *Aerobacter* spp (Cerutti de Guglielmone, Diez, Cardenas, & Olivier, 2002; Eggleston, 2001). Microbial growth is related to the sugar content and pH as well as the humidity and temperature of the environment. The crude juice obtained by grinding has a pH of 5.0–5.6 and a sugar content of 10–18% RDS (refractometric dry substance). Such conditions, combined with the presence of organic and inorganic salts, proteins and other nutrients provide ideal conditions for bacterial growth and thus the counts of bacteria in the juice commonly vary from 5 to 7 log CFU/mL. Some microorganisms can grow during extraction and refining stages, or contaminate the product after processing (Mackrory, Cazalet, & Smith, 1984; Wojtczak et al., 2012).

While microbial counts in sugar are typically less than 2 $\log_{10}$ CFU per g, higher counts may lead to spoilage of foods containing sugar as an ingredient (Table 6). Examples of such microorganisms include *Geobacillus stearothermophilus* and *Bacillus coagulans*, which cause flat-

Table 6
Occurrence of sporeforming bacteria in different types of sugar.

Origin	Type of sugar	Number of samples	Prevalence (%)	Aerobic thermophilic sporeforming bacteria	"Flat sour"	H_2S producing sporeforming bacteria	Non- H_2S producing sporeforming bacteria	Method	Reference
Europe	Raw sugar	14	100	2.4–2.8				Plating on Camerona agar. Incubation at 30 °C/48 h.	Wojtczak et al. (2012)
	Refined white cane sugar	14	29	2–4					
Brazil	Brown sugar	9	78	1–4	10^1 - 8.9 × 10^3 spores/10 g				Verruma-Bernardi et al. (2007)
			67		5 - 430 spores/10 g				
Brazil	Refined sugar	10	60					Heat treatment of 100 °C/5 min. Plating on DTA. Incubation at 55 °C/48 h.	Hoffmann et al. (1992)
			100			10–72.5 spores/10 g		Heat treatment of 100 °C/5 min. Plating on sulphite agar. Incubation at 55 °C/48 h.	
Brazil	Brown sugar A	12	25		5-73 spores/10 g			Heat treatment of 100 °C/5 min. Plating on GTA. Incubation at 55 °C/48 h.	Parazzi et al. (2009)
			0		N.D.			Heat treatment of 100 °C/5 min. Plating on Sulphite agar. Incubation at 55 °C/48 h.	
			50				1–6	Heat treatment of 100 °C/5 min. Tubes with PE-2 sealed with 2% agar. Incubation at 55 °C/72 h.	
	Brown sugar B	11	18	4 - 783 spores/10 g				Heat treatment of 100 °C/5 min. Plating on GTA. Incubation at 55 °C/48 h.	
			36		5 - 283 spores/10 g			Heat treatment of 100 °C/5 min. Plating on Sulphite agar. Incubation at 55 °C/48 h.	

sour spoilage; *Clostridium thermosaccharolyticum*, which is known to produce acid in canned foods; *Desulfotomaculum nigrificans*, which causes spoilage by H_2S production, and mesophilic bacteria, yeasts, and molds (Hoffmann et al., 1992). In the liming step, the vegetative cells are eliminated due to the increase and temperature (100–105 °C) resulting in the survival of only mesophilic and thermophilic spore forming microorganisms (Hoffmann et al., 1992; International Commission on Microbiological specifications for foods, 1978). The presence of bacterial spores in sugar is frequent as they can be found in high loads in the raw materials employed for sugar production (cane and beetroots, for instance, which have close contact with soil) as well as due to their resistance to the processing conditions (Parazzi, Jesus, Lopes, & Valsechi, 2009).

Few studies have assessed the occurrence of spore forming bacteria in sugarcane (Table 6) and none evaluated sugar beets. Despite the high importance of these data to serve as a base for improving the quality of this ingredient, there are no studies on the thermal resistance of spore forming bacteria isolated from sugar processing. In Japan, the presence of *Clostridium botulinum* type A spores was reported in raw sugar and molasses, and in 2 of 41 brown sugar lump samples. Despite this, spores of this pathogen were not found in any of 56 samples of refined white sugar and 13 samples of intermediate products of the sugar processing plant (Nakano, Yoshikuni, Hashimoto, & Sakaguchi, 1992). Lucca et al. (1992) isolated *Alicyclobacillus acidocaldarius* and *Clostridium* spp. from sugar refining process and found that the bacilli were able to grow at 70 °C. These authors concluded that spores of mesophilic and strict thermophilic spore forming bacteria could survive the refining process, which may potentially cause great losses of final processed products. The employment of contaminated refined sugar as a raw material in the processing of different products can significantly contribute to an increase in the microbial load in the product to be thermally processed (Hoffmann et al., 1992). The demand for high-quality white sugar has increased in recent years, because besides its nutritional contribution to daily diet, it is used as raw material in the food and beverage industry (Hamerski, 2009). Beverage producers stand out on microbiological criteria of ingredients and hygiene during processing to produce shelf-stable beverages (Hamerski, 2009).

5.4. Starch/fecula production

Commercial starch is extracted mainly from five raw materials, as follows: corn, wheat, cassava, potato and sweet potato (Cereda, 2005). The amylaceous materials are classified as starches, which are extracted from the edible aerial parts of plants such as corn and wheat starch; and fecula, which are obtained from the edible underground parts of plants, such as potato and cassava fecula (Amorim, 2011; Aplevicz & Demiate, 2007).

Starch processing from different sources is quite similar and involves washing, peeling, cell disintegration and release of the starch granules, starch separation, and drying. Fig. 5 shows cassava fecula processing. In the reception area, unloading of cassava may be carried out by tilting system or by dragging the roots. In the latter method, cassava goes through a grid, to remove part of the land. Then the cassava is washed, peeled, passed through a conveyor to be manually inspected for removal of other woody components and elimination of impurities. Grinding or milling is performed in a milling machine and consists of starch recovery from the plant cells that have not been broken (starch attached) and free starch outside the cells. Then extraction is carried out by centrifugal sifters, where the separation of the bagasse or cassava bran occurs (Jacquet, 1999). Once the extracted starch has many impurities, a purification step is needed, followed by concentration to reduce the water content. Most fecula processing plants use centrifuges for both phases. The first centrifuge concentrates the starch-milk, in which fresh water is added. The second centrifuge removes the water previously added, completing the cleaning step. In the drying process, the temperature ranges from 120 °C to 150 °C, depending on the moisture level of the concentrated starch. After drying, fecula is packaged and stored (Souza, 2011; Takahashi & Gonçalo, 2001) (see Fig. 5).

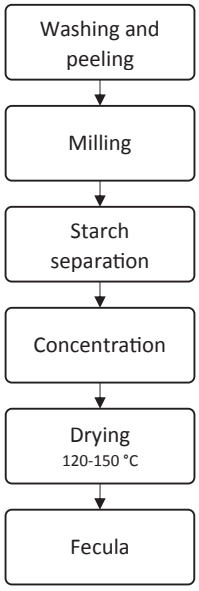

Fig. 5. Processing of cassava fecula.

5.4.1. Diversity and fate of spore forming bacteria during starch production

Due to the production method and contact of vegetables with soil, especially tubers, roots and rhizomes, starches may be contaminated by a variety of microorganisms (Pietranera & Narvaiz, 2001). Some starch applications do not require heating stages, thus allowing for the survival of microorganisms. Thermal treatment of cassava starches is a difficult task due to their low moisture content and poor thermal conductivity. Furthermore, starch containing more than 30% moisture can gelatinize at temperatures above 60 °C (Cereda, 1984). Thermophilic spore forming bacteria producing thermoresistant spores are reported as the main microbiological problem in starch. Several species of the genus *Bacillus* originating in soil can remain in the final product, including the pathogen *Bacillus cereus*. Furthermore, the presence of heat-stable amylase may remain even after destruction of vegetative cells, causing spoilage of the products manufactured with contaminated starch (Amorim, 2011; Smittle, Krysinski, & Richter, 1992).

Despite the high probability of contamination of starch by spores of spore forming bacteria and its application in the manufacturing of a huge quantity of food products, there are no studies on the diversity and fate of spore forming bacteria during starch production. Thus, further studies on microorganisms present in starch are needed, to improve the microbiological quality and efficiency of preservation methods aiming to obtain safe and shelf-stable food products.

6. Concluding remarks

The physical and chemical resistances of spores of spore forming bacteria allow them to survive various unit operations applied during food processing. Depending on the combination of unit operations applied during processing of raw materials and the responses of the spores of spore forming bacteria, qualitative and quantitative variations can be observed. These variations may further impact on the microbiological safety and stability of formulated foods.

It is known that spore forming bacteria originate mainly from the soil, but silage, composting and processing equipment are important sources too. The application of good manufacturing practices throughout raw materials processing is vital to control their occurrence and concentration. Despite this, the review of literature conducted in

this study indicates that there is still a limitation regarding the estimation (quantitative) of the effects of some unit operations employed during cocoa powder, milk powder, sugar, and starch processing on spore forming bacteria. However, data on the effects of processing and diversity of spore forming bacteria in sugar and starch are much scarcer compared with cocoa and milk powders. Thus, it becomes clear that more data on the occurrence of fouling, species present, their resistance (superdormant spores, for instance), and diversity are needed. In addition, knowledge on the effects of unit operations applied during processing of raw materials on the diversity of spore forming bacteria and their fate are required to allow the establishment of effective controls throughout their production chain. Despite this, the literature review indicates a lack of information regarding the diversity and fate of spore forming bacteria in raw materials, more specifically during sugar, and starch processing. Even though cutting-edge approaches combining quantitative data with metagenomics could improve our knowledge on the fate and diversity of spore forming bacteria in raw materials, basic and challenging aspects must still be resolved. For instance, these include improvements in approaches for a better assessment of spores' sub-populations through the development and use of proper recovery methods that could allow the recovery of very low number of spores as well as recovery of mixed populations containing, for instance, super dormant spores. This information will serve and guide further developments aiming to enhance food safety and to control food spoilage caused by spore forming bacteria.

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CAPÍTULO 2

Persistence of spore-forming bacteria including *Bacillus cereus* along processing chain of cocoa powder

Artigo formatado de acordo com as normas do periódico: "Food Microbiology"

Persistence of spore forming bacteria including Bacillus cereus along processing chains of cocoa powder

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Abstract

Spore forming bacteria can be a concern in some raw materials, like cocoa powder. Two farms and three industrial lines were sampled with the aim of investigating the impact of the process in spore forming bacteria population. Mesophilic aerobic spores, mesophilic anaerobic spores, thermophilic aerobic spores and *Bacillus cereus* populations was investigated. A total of 618 samples were collected. The persistence of *B. cereus* strains along the processing was analysed and a study about the phylogenetic groups. There is a high population spore forming bacteria at the beginning of the process, mainly mesophilic aerobic spores, but after alkalization or roasting the levels were general reduced. 59 M13 groups were identified by band similarity. Strains isolated in cocoa powder were also recovered in cocoa beans, nibs, samples after roasting, after refining and after pressing, which means that *B. cereus* can persist the process and reach final product. Phylogenetic group IV was the most frequent (72%). Strains from phylogenetic group III did not show presence of *ces* gene and just one strain from group VI show presence of *cspA* gene.

Keywords: cocoa powder, spore forming bacteria, cocoa processing, *B. cereus.*

1) Introduction

The cocoa tree *Theobroma cacao* is grown in large areas in tropical countries. Its processed beans enter production of cocoa powder, an ingredient widely used by food industry (Belscak et al., 2009; Oracz and Nebesny, 2014). As for other plant foods, contamination of cocoa bean and derived products by spore forming bacteria is almost impossible to avoid. Spores are widely present in the crop environment (Carlin et al., 2011). Processing operations of cocoa beans proceeds in open air, with frequent contact to soil, and limited inactivation on spores (Pereira and Sant'Ana, 2018). Spores of bacteria are therefore a concern for cocoa powder (Gabis et al., 1970; Lima et al., 2011a, 2011b; Witthuhn et al., 2011).

Cocoa beans processing for cocoa powder production includes several operations. Some of those modify the microbiota of the cocoa beans, increase, or decrease the levels of microbial populations. Cocoa pre-processing takes place on the farm, mainly in open air. Harvested pods are opened to expose beans. Pulp and beans are placed in wooden boxes, baskets, trays or platforms to ferment for about 6 days (Copetti et al., 2011; Wood, 1985) at the temperatures prevailing under tropical climate. Sun-drying after fermentation reduces to 6-8% (ICMSF, 2011; Nascimento et al., 2013; Thompson et al., 2013). In industrial processing, roasting consists cocoa beans dry-heating at 110 to 140°C for about 60 minutes (Beckett, 2008). The alkalization process uses alkalis, such as sodium or potassium carbonates at 1% to 6% combined to high temperatures (60°C to 120°C) for 30 to 150 minutes (Bispo, 1999; Bispo et al., 2005; Li et al., 2014; Minifie, 1999). Cocoa powder finally results of breaking and grinding of the "cocoa cake" obtained by pressing at 90°C to 110°C under a pressure of 1.5-2 to Mpa (Fang et al., 1995; Minifie, 1999; Venter et al., 2007).

In early processing stages, micro-organisms from the environment, utensils and workers contaminate cocoa seeds (Nascimento et al., 2010). However, a_w reduction, changes in pH and thermal treatments during processing creates selective pressure in favour of resistant

microorganisms, mainly spore-forming bacteria. Further germination and growth of spore-forming bacteria contaminating formulated foods through raw materials and/or ingredients can be a cause of poisonings or spoilage (Pereira and Sant'Ana, 2018). Thus, the objective of this study was to evaluate the incidence and persistence of spore-forming bacteria, along the processing chain of cocoa powder. In particular, genetic typing was applied to isolates of the foodborne pathogen *B. cereus* for tracing and hazard characterization.

2) Material and Methods

2.1) Cocoa sampling

A total of 618 samples of cocoa samples were collected along the cocoa powder processing chain, including samples collected in two cocoa farms (n = 180) and along lines from two plants processing dried cocoa beans (n = 438). Farms were located in Barreiras (Farm 1) and Gandú (Farm 2), State of Bahia, Brazil and were sampled in August 2016 and January 2017, respectively. Industrial processing lines were in plants located in Ilhéus (Line 1) and Itabuna (Line 2 and Line, 3) in the state of Bahia, Brazil and were sampled between November 2014 and June 2015. Farm samples from pre-processing were collected after the opening of the fruit, and once a day during fermentation and drying process (Fig. 1). The duration of fermentation process was 3 and 4 days in Farm 1 (Samples of fermenting cocoa beans F0-F3) and 2 (samples F0-F4), respectively. Sun-drying of fermented beans lasted for 4 days in Farm 1 (Samples D1-D4) and for 5 days in Farm 2 (D1-D5). Samples from plants were collected at different stages. As shown in Fig. 2, unitary operations were differently organized of the three processing lines producing cocoa powder. All 200 g samples from farms or industries were aseptically collected in sterile bags and delivered to UNICAMP laboratory by freeze-transportation for farm samples.

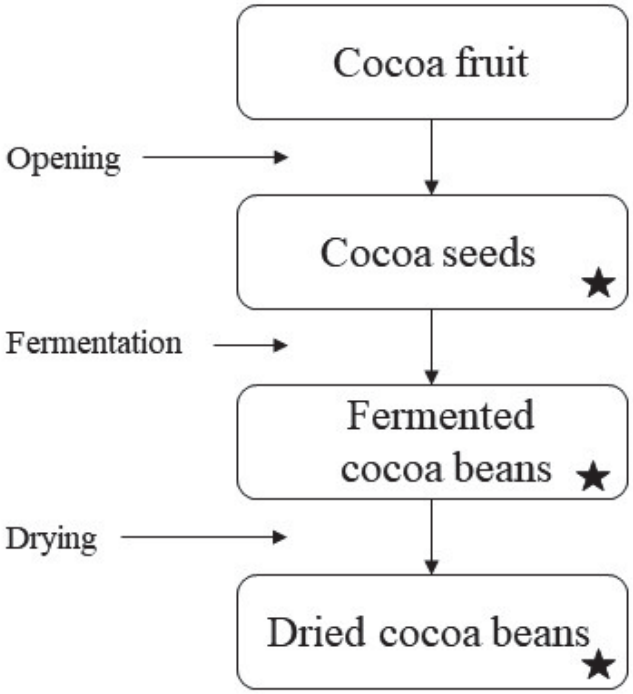
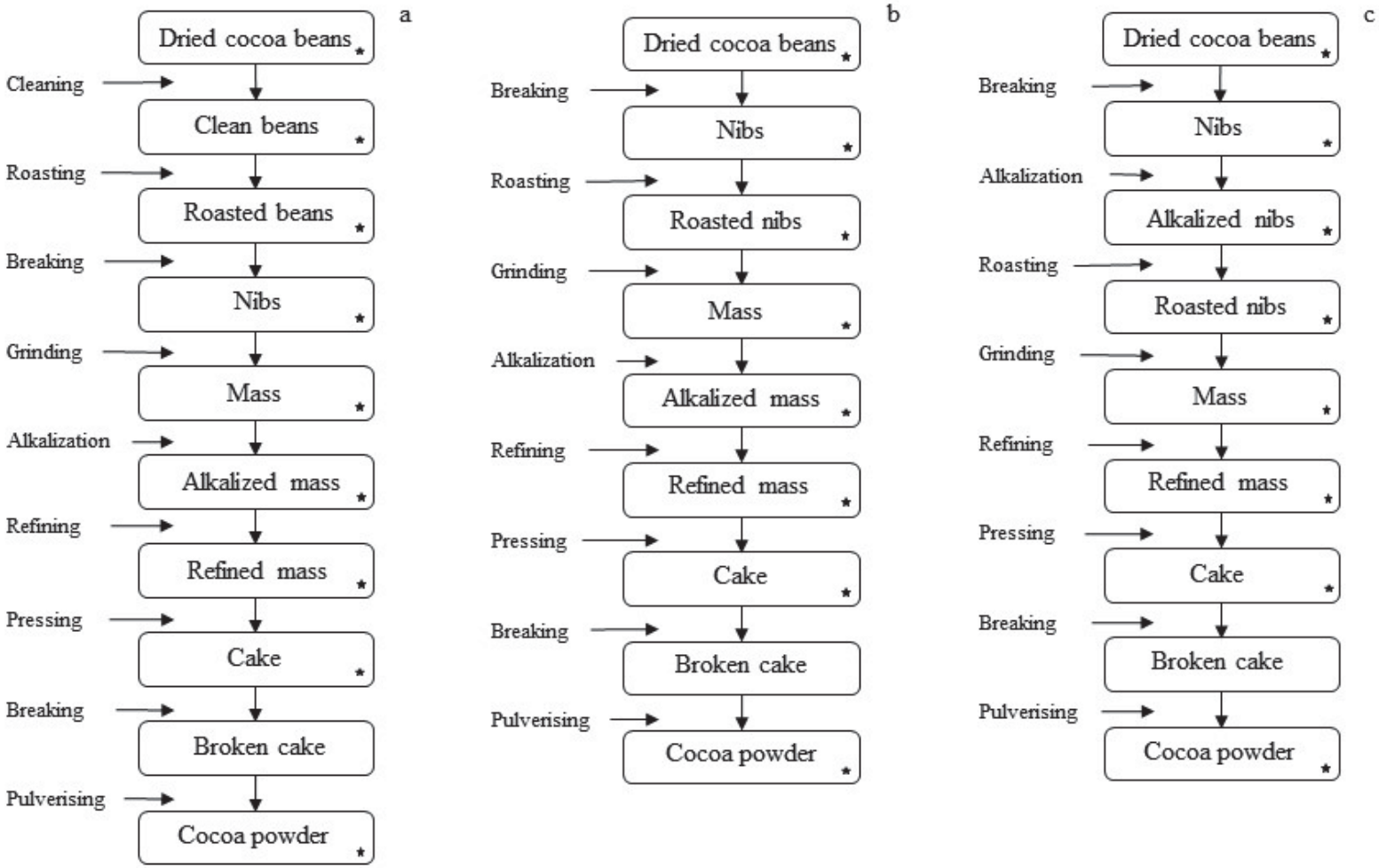


Fig. 1. Diagram of the in-farm processing operations of cocoa beans. Stars indicate sampling locations.



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116

117 Fig. 2. Cocoa industrial processing sampling. Industrial lines 1, 2 and 3 represented by a, b and c. Stars indicate sampling location.

118 ***2.2) Enumeration of microbial groups***

119 For enumeration of mesophilic aerobic bacteria ("Mesophlic aerobic spores", MAS) 10 g
120 of each sample were mixed with 90 mL of peptone water 0.1% (1 g Bacteriological Peptone
121 [Oxoid Basingstoke, UK] per liter) in a sterile bag, then homogeneized, using a Stomacher
122 (Seward, BA 7021, Worthing, UK) for 1 minute. A heat-shock at 80°C for 30 min in a water
123 bath (Quimis, Q-334M-28, Diadema, Brazil) was applied. Then the samples were serially
124 diluted and 1000 µl volumes were plated onto Tryptone Glucose Extract agar (TGE). TGE
125 contains 5g/L of Tryptone, 3g/L of Beef Extract (both from Acumedia, Neogen Co., Lansing,
126 USA), 1g/L of Glucose (Dinâmica, Diadema, Brazil) and 15g/L of Bacteriological Agar [Oxoid,
127 Basingstoke, UK]. Plates were incubated at 35°C for 48 hours (Stevenson and Lembke, 2001).
128 For enumeration of spore of *Bacillus cereus* group, samples were homogenized as
129 described for enumeration of MAS, heat-shocked at 70°C for 15 minutes in water bath, and
130 serially diluted and 100 µl volumes were plated onto Mannitol Egg Yolk Polymyxin agar
131 (MYP, Oxoid, Basingstoke, UK). Plates were incubated at 30°C for 24-48 hours (Bennett et al.,
132 2001). Strains were isolated from plates used for *B. cereus* group enumeration. Colony purity
133 was confirmed by streaing on MYP agar.
134 For enumeration of spores of thermophilic aerobic bacteria ("thermophilic aerobic
135 spores", TAS), 10 g of each sample were mixed in a sterile bag with 90 mL of sterile distilled
136 water and homogenized for 1 minute using a Stomacher. A heat-shock at 110°C for 10 minutes
137 in an oil bath (Polystat, 12105-20, Chicago, USA) was applied, followed by serial decimal
138 dilutions and plating of 1000 µl on Dextrose Tryptone agar (DTA). DTA contains 10g/L
139 Tryptone, 5g/L of dextrose [Dinâmica, Diadema, Brazil], 15g/L of Bacteriological Agar
140 [Oxoid, Basingstoke, UK], and 4 mL/L of 1% purpura of Bromocresol solution in NaOH
141 [Dinâmica, Diadema, Brazil]). The plates were incubated at 55°C for 48-72 hours (Olson and
142 Sorrells, 2001).

Spores of mesophilic anaerobic bacteria ("Mesophilic anaerobic spores" MAnS) were enumerated by Most Probable Number (MPN). Twenty grams of each sample were mixed in a sterile bag with 100mL of peptone water at 0.1% and homogenized using a Stomacher during 1 minute. Then 20 mL were distributed in 6 tubes containing 19 mL of PE-2 medium and 8 natural dried peas. PE-2 contains 20g/L of Peptone, 3g/L of Yeast Extract (Acumedia, Neogen Co., Lansing, USA), 2mL/L of a 2% Bromocresol purple of solution in ethanol (Dinâmica, Diadema, Brazil). The tubes were heat-shocked at 100°C for 20 minutes and incubated at 35°C for 3-7 days (Tortorelli and Anderson, 2001). Positive tubes were those with turbidity and color change to yellow. The results of the single dilution test were calculated.

2.3) pH and a_w measurement

The pH of samples was estimated by measuring the pH of an aliquot of the first dilution used for enumeration of TAS determined with previously calibrated pHmeter (model K39-2014B, KASVI, China) (Adolfo Lutz Institute, 2008). The a_w of samples was determined with an AquaLab 195 equipment (model CX-2, Decagon Devices, Washington, USA).

2.4) Bacterial strain

B. cereus strains isolated at different steps of processing (n=112) and additional *B. cereus* strains from white chocolate (strains 432 and 436), chocolate fortified with vitamins (strains 511 and 512) and milk (B94) (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) (TS1) were subcultured on MYP agar for purity, and then stored at - 80°C in nutrient broth with 20% (w/w) glycerol.

168 ***2.5) DNA extraction***

169 DNA extraction was performed as described previously (Guinebretière and Nguyen-The,
170 2003). The isolates were plated onto Luria Bertani agar (LBA) (10 g/L of peptone, 5 g/L of
171 yeast extract, 5 g/L sodium chloride and 12 g/L of Bacteriological Agar) and grown overnight
172 at 30°C. A 10 µL volume of bacterial culture was placed in a microtube containing 525 µL of
173 TES solution (0.2 mol.L^{-1} Tris buffer at pH= 8.0, 20 mmol.L^{-1} EDTA, 0.2 mol.L^{-1} NaCl). Then
174 50 µL of 20% Sodium Dodecyl Sulfate (SDS) and 25 µL of 10 µg/ µl proteinase K were added,
175 followed by incubation at 55°C for 1 hour. DNA was extracted with one volume of phenol,
176 followed by one volume of chloroform. The aqueous phase was precipitated with cold 100%
177 ethanol and centrifuged at 13000 rpm for 20 minutes. The supernatant was discarded, the pellet
178 was washed with cold 70% ethanol and the pellet was dried after ethanol elimination. The pellet
179 was dissolved in sterile Milli-Q water and incubated at 65ºC for 15 minutes after addition of
180 2.5 µL of RNAase. The solution was stored at – 20°C until use.
181
182 ***2.6) M13-PCR***

183 The PCR mixture contains 20 ng/µL of DNA template, 1.25 mM dNTPs mix (Eurogentec,
184 Seraing, Belgium), 6 mM $MgCl_2$ (Eurogentec), 8 µM primer, 10% (vol/vol) dimethyl sulfoxide,
185 0.1 U/µL DNA polymerase (Eurogentec) and 10x red diamond buffer (Eurogentec). The primer
186 PM13 5'-GAGGGTGGCGGCTCT-3' was used. The thermal cycling was carried out in PCR
187 9700 thermocycler (Perkin-Elmer). The following cycling conditions were used: 3 minutes at
188 94°C, 35 cycles of 1 minute at 94°C, 1 minute at 40°C and 8 minutes at 65°C, followed by 16
189 minutes at 68°C. Products from the M13-PCR were separated on 1.5% agarose gel with
190 molecular markers. The gels were stained with ethidium bromide and digitalized by a gel
191 imager. The method used was described before (Guinebretière and Nguyen-The, 2003). The
192 image of 112 strains were analysed and grouped by similar patterns.

193 ***2.7) Genotyping using panC***

194 The *panC* sequence of 59 selected *B. cereus* strains representing M13 groups and of the
195 five collection strains was determined as described previously (Candelon et al., 2004) with
196 modifications in thermal cycling (4 minutes at 94°C, 30 cycles of 15 seconds at 94°C, 30
197 seconds at 55°C and 30 seconds at 72°C, followed by 7 minutes at 72°C). Sequences of *panC*
198 were assigned to seven (I-VII) phylogenetic groups as previously described
199 (https://www.tools.symprevius.org/bcereus) (Guinebretière et al., 2010). A phylogenetic tree
200 was built with Phylogeny (www.phylogenic.fr). The *panC* sequences from 8 representative
201 strains of *B. cereus* belonging to different phylogenetic groups were included in the
202 phylogenetic analysis.

203

204 ***2.8) ces and cspA gene***

205 According to the observed characteristics of the strains from groups III and VI in previous
206 studies (Guinebretière et al., 2008), *ces* and *cspA* were PCR amplified from strains belonging
207 to these groups, respectively. PCR amplification of *ces* gene was performed as described
208 previously (Ehling-Schulz et al., 2004). PCR amplification of *cspA* gene was performed
209 following the conditions described previously (Francis et al., 1998) with minor modifications
210 (Guinebretière et al., 2008). Positive and negative control strains were used in this experiment.

211

212 ***2.9) Statistical analysis***

213 The result of plate count was log transformed and the average were calculated. Significant
214 statistical differences ($P < 0.05$) in the average levels were identified using analysis of variance
215 (ANOVA) using Assistat version 7.7 (Campina Grande, Brazil) (Silva and Azevêdo, 2002).

216

217

3) Results

3.1) Changes in spore counts along farm and industry processing of cocoa

Processing steps (i.e. a few day fermentation followed by sun drying) are quite similar in both farms 1 and 2. Initial contaminations with MAS in Farms 1 and 2 were also quite similar (about 1.5 log CFU/g). However, changes in populations during the fermentation and drying processes exhibited different patterns in the two farms. The increase during a 3-day fermentation was lower than one log cfu/g in farm 1 and was about 1.5 log cfu/g in farm 2, and higher than 4 log cfu/g in farm 2 during a 4-day fermentation. Transition between fermentation and sun-drying resulted in a reduction by approx. 1 log, and then counts remained relatively constant. There is water loss during drying, this practically means that the number of spore forming bacteria may decrease during drying operations. Finally, the differences in contamination with mesophilic spore forming bacteria of dried beans were in a factor 1:1000 (2 log cfu/g in beans from farm 1 vs 6 log cfu/g in beans from farm 2). Populations of spores of *Bacillus cereus* and of thermophilic mesophilic aerobic bacteria were, as the ones of spores of mesophilic aerobic bacteria at levels higher in Farm 2 than in Farm 1. Sharp increases in *B. cereus* group spore counts of TAS counts were observed, only in Farm2 and during fermentation. The population of MAnS remains relatively constant during fermentation and drying in farm 1 (166 MPN spores/g) and farm 2 (270 MPN spores/g).

The populations of aerobic spore forming bacteria in cocoa beans entering industrial processing were comprised between 4 and 6 log CFU/g (Fig. 4). Populations of *B. cereus* group were highly variable among samples and were about 3.5 log CFU/g in some at the beginning, but after alkalization or roasting the levels were general significantly reduced ($P < 0.05$) in some cases. After alkalization the populations of MAS and *B. cereus* group were significantly reduced ($P < 0.05$) (3.8 log cfu/g and 3 log cfu/g respectively) in industrial line 01. After roasting, the population of *B. cereus* group decreases more than 6 log cfu/g in industrial line 02

243 ($P < 0.05$), and for MAS population the reduction was of 4 log cfu/g in industrial line 03 ($P <$
244 0.05). The population of MAnS varied from > 270 MPN spores/g to bellow the limit of detection
245 in Industrial Line 1, from > 270 to 28 MPN spores/g in Industrial Line 2 and 3. The
246 contamination of spore forming bacteria can reach cocoa powder. In Industrial Line 3, roasting
247 process appeared to be most effective in inactivating MAS and TAS spores. In this industrial
248 line, roasting process occurs after the alkalization process.

249 During the pre-processing and industrial processing, pH and a_w of samples varied
250 between 4.2 and 7.1 and 0.96 and 0.35, respectively. During fermentation pH increase from 4.2
251 to 6.9. After alkalization process pH increases to 8.7, and decrease to 7.1 in subsequent steps.
252 During drying a_w decreases from 0.96 to 0.61. During industrial processing a_w remains almost
253 constant, and shows a decrease after pulverising (from 0.55 to 0.35) in Industrial Line 1.

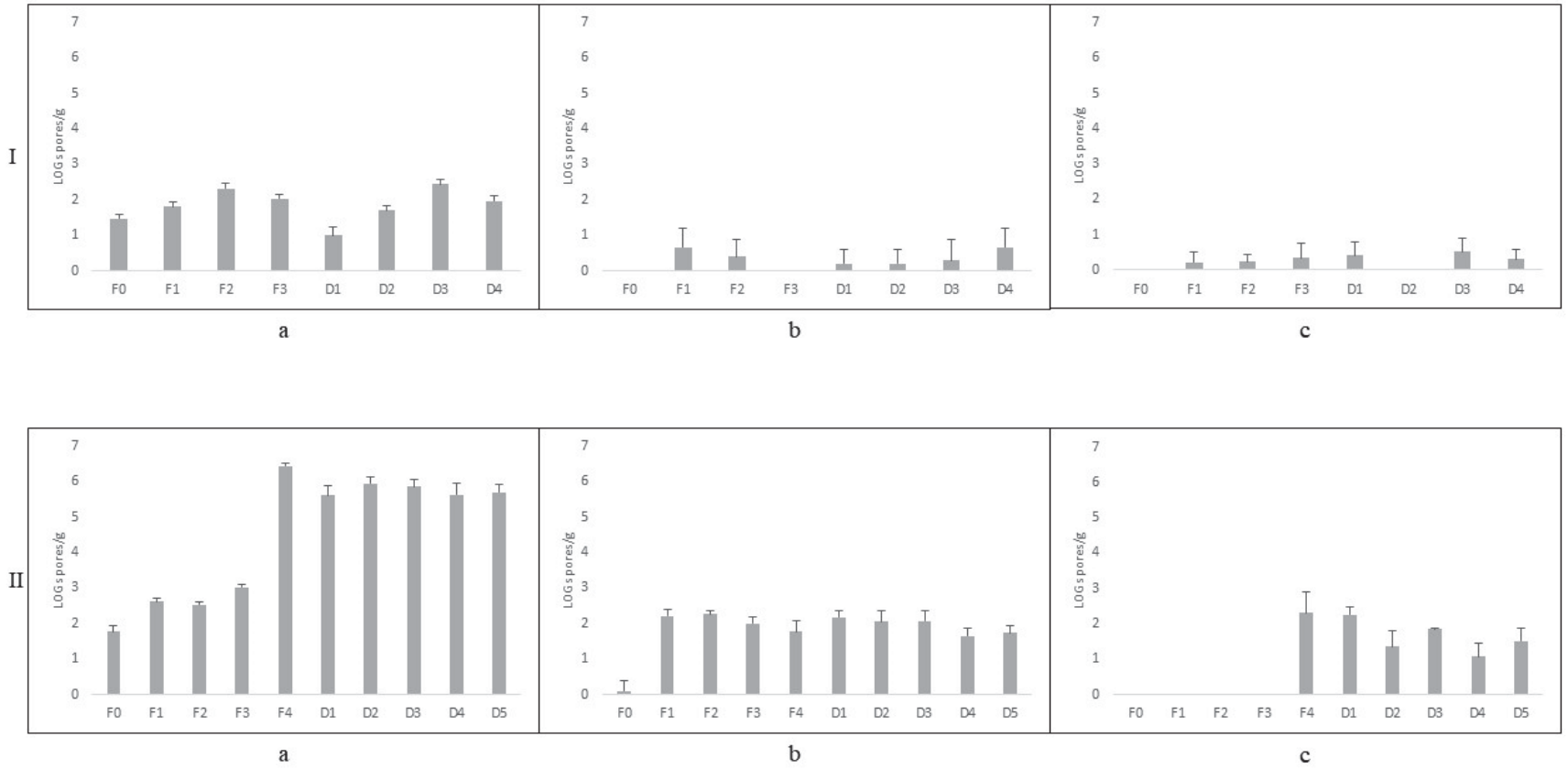

Fig. 3. Changes in spore counts during the cocoa beans pre-processing at two Brazilian farms: Farm 1 (I) and Farm 2 (II). The bars correspond to the estimated average levels (log spores/g) of a: MAS; b: *B. cereus* group; c: TAS. The absence of bars indicates levels below the detection limit of the method (1 log spore/g). F0-F4: fermentation days. D0-D5: drying days.

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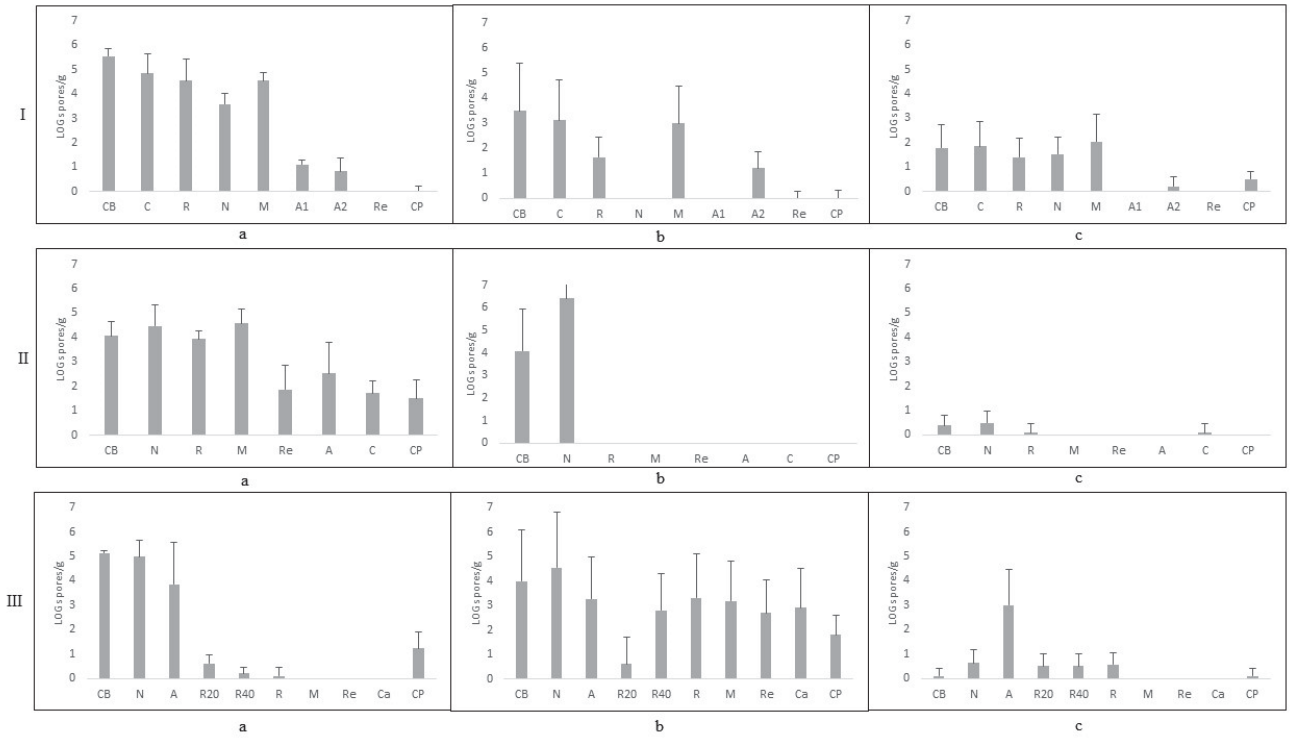

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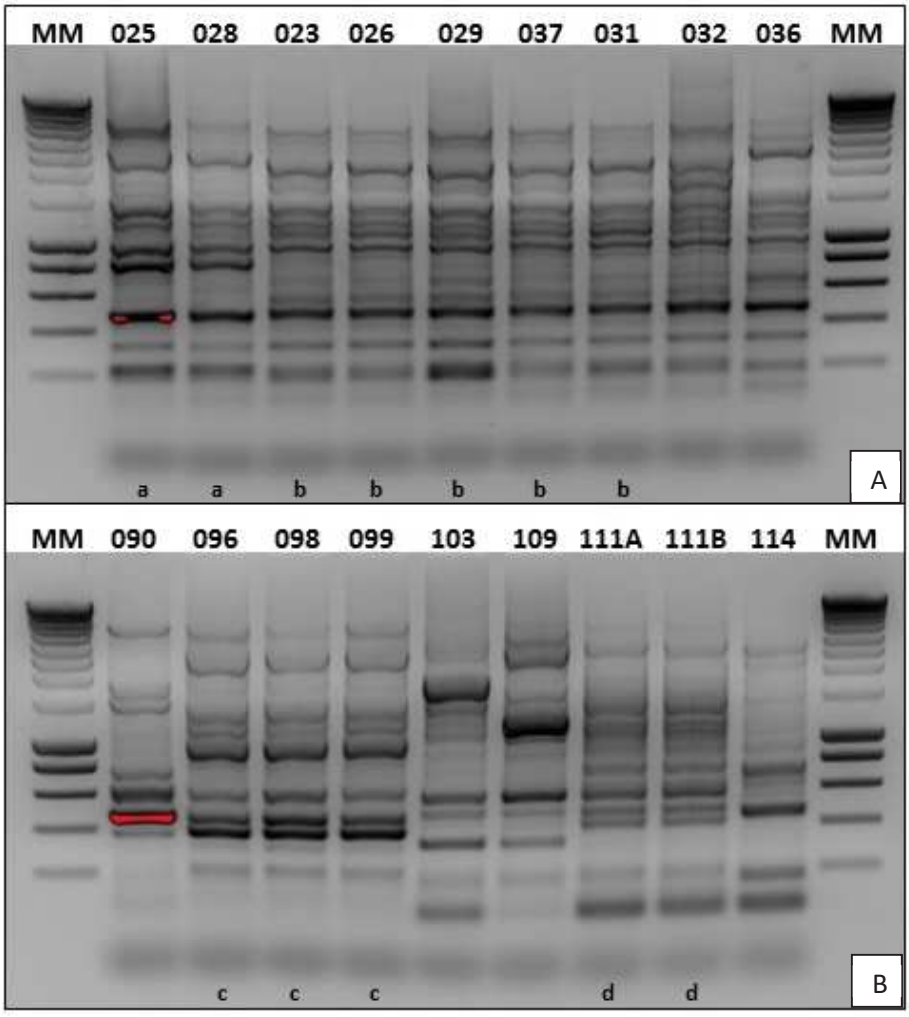
260 Fig. 4. Changes in spore counts during manufacturing of cocoa powder from cocoa beans on three processing chains at two Brazilian cocoa
261 companies: Industrial Line 1 (I); Industrial Line 2 (II); Industrial Line 3 (III). The bars correspond to the estimated average levels (log spores/g) of
262 a: MAS; b: *B. cereus* group; c: TAS. The absence of bars indicates levels below the detection limit of the method (1 log spore/g). CB: cocoa beans;
263 C: after cleaning; N: nibs; M: mass; R: after roasting; R_{20}: after 20 minutes of roasting; R_{40}: after 40 minutes of roasting; A: after alkalization; A1:
264 after alkalization 1 (110°C/60min); A2: after alkalization 2 (105°C/60min); Re: after refining; Ca: after pressing; CP: cocoa powder.

265 ***3.2) Genetic diversity of B. cereus isolates***

266 Examples of fingerprints for *B. cereus* strains generated using M13-PCR are
267 demonstrated in Fig. 5. Fingerprint were obtained for all strains. A total of 59 groups (M13
268 groups) were found by visual comparison of similarity in DNA-amplified fragments on
269 electrophoresis gels (Table 1 and 2). The visual comparison on electrophoresis gels was
270 performed separately for each farm and industry. Strains belonging of M13-groups 38, 42, 50
271 and 51 were recovered from cocoa powder and from another step of the process (Table 3).



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273 Fig. 5. Example of M13-PCR patterns for *B. cereus* strains. Strains from Line 02 (A) and
274 Farm 01 (B). Similarity pattern a: 025 (from cocoa beans), and 028 (from nibs); b: 023, 026 and
275 031 (from cocoa beans), 029 (from nibs), and 037 (from cocoa powder); c: 096, 098 and 099
276 (2° day of fermentation); d: 111A and 111B (4° day of fermentation).

277 Table 1. Distribution of *B. cereus* strains from in-farm processing according to M13 group, origin and genotypic characterization.

M13 group	N° of Strains in group	Strain origin									Representative strains	Phylogenetic group	*ces* gene	*cspA* gene
			Fermentation days				Drying days							
			1°	2°	3°	4°	1°	2°	3°	4°	5°				
1	3	F1						3				n105	III	negative	
2	2						2					n101	IV		
3	3			3								n096	IV		
4	2									2		n111A	III	negative	
5	3		2						1			n092	IV		
6	1			1								n097	IV		
7	1								1			n110	IV		
8	1									1		n112	IV		
9	1		1									n090	V		
10	1						1					n103	III	negative	
11	1								1			n109	III	negative	
12	1									1		n114	III	negative	
13	1						1					n100	III	negative	
14	3	F2	1			2						n117	IV		
15	2				1		1					n149	IV		
16	2					1	1					n170	IV		
17	2				1		1					n150	IV		
18	2							1	1			n192	IV		
19	3		3									n120	II		
20	2							1		1		n198	II		
21	6				1				2		3	n155	V		
22	3							1	1	1		n194	V		
23	1			1								n135	IV		
24	1						1					n182	IV		
25	1							1				n195	IV		
26	1									1		n223	IV		
27	1		1									n118	IV		
28	1				1							n153	IV		
29	1			1								n138	VI		positive
30	1										1	n244	IV		

278

279

Table 1. Distribution of *B. cereus* strains from in-farm processing according to M13 group, origin and genotypic characterization (cont.).

M13 group	N° of strains	Strain origin									Representative strains	Phylogenetic group	*ces* gene	*cspA* gene
		Fermentation days				Drying days								
		1°	2°	3°	4°	1°	2°	3°	4°	5°				
31	1								1		n224	IV		
32	1		1								n133	II		
33	1				1						n168	IV		
34	1		1								n140	VI		negative
Total n°	13(F1)	2(F1)	2(F1)	0(F1)	0(F1)	3(F1)	1(F1)	3(F1)	3(F1)	0(F1)				
of groups	21(F2)	3(F2)	4(F2)	4(F2)	3(F2)	4(F2)	4(F2)	3(F2)	4(F2)	2(F2)				
Total n°	21(F1)	3(F1)	4(F1)	0(F1)	0(F1)	4(F1)	3(F1)	3(F1)	4(F1)	0(F1)				
of strains	37(F2)	5(F2)	4(F2)	4(F2)	4(F2)	4(F2)	4(F2)	4(F2)	4(F2)	4(F2)				

Where F1: farm 1; F2: farm 2.

Table 2. Distribution of *B. cereus* strains from industrial processing according to M13 group, origin and genotypic characterization.

M13 group	Number of strains		Strain origin											Representative strains	Phylogenetic group	*ces* gene
			CB	C	N	R_{20}	R_{40}	R	A	M	Ca	Re	CP			
35	3	IL 1	1	1						1				n002	IV	
36	2							2						n011	III	negative
37	2			2										n005	IV	
38	2											1	1	n019	IV	
39	1		1											n001	IV	
40	1		1											n003	IV	
41	1												1	n021	IV	
42	5	IL 2	2		2								1	n023	IV	
43	2		1		1									n025	IV	
44	1				1									n032	IV	
45	1												1	n036	IV	
46	9	IL 3			1	1	1		3	2		1		n048	IV	
47	3										1	2		n075	IV	
48	2		1			1								n057	IV	
49	2		2											n040	IV	
50	2										1		1	n079	IV	
51	2						1						1	n063	IV	
52	1				1									n043	IV	
53	1		1											n041	IV	
54	1				1									n044	III	negative
55	1					1								n055	IV	
56	1					1								n056	IV	
57	1									1				n070	IV	
58	1												1	n083	IV	
59	1												1	n087	IV	

284 Table 2. Distribution of *B. cereus* strains from industrial processing according to M13 group, origin and genotypic characterization (cont.).

M13 group	Number of strains	Strain origin										Representative strains	Phylogenetic group	*ces* gene
		CB	C	N	R_{20}	R_{40}	R	A	M	Ca	Re	CP			
Total n° of groups	7(IL1)	3(IL1)	2(IL1)	0(IL1)	0(IL1)	0(IL1)	1(IL1)	0(IL1)	1(IL1)	0(IL1)	1(IL1)	2(IL1)			
	4(IL2)	2(IL2)	0(IL2)	3(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	2(IL2)			
	14(IL3)	3(IL3)	0(IL3)	3(IL3)	4(IL3)	2(IL3)	0(IL3)	1(IL3)	2(IL3)	2(IL3)	2(IL3)	4(IL3)			
Total n° of strains	12(IL1)	3(IL1)	3(IL1)	0(IL1)	0(IL1)	0(IL1)	2(IL1)	0(IL1)	1(IL1)	0(IL1)	1(IL1)	2(IL1)			
	9(IL2)	3(IL2)	0(IL2)	4(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	0(IL2)	2(IL2)			
	28(IL3)	4(IL3)	0(IL3)	3(IL3)	4(IL3)	2(IL3)	0(IL3)	3(IL3)	3(IL3)	2(IL3)	3(IL3)	4(IL3)			
		Oswaldo Cruz Foundation strain											432	III	
		Oswaldo Cruz Foundation strain											436	III	
		Oswaldo Cruz Foundation strain											511	IV	
		Oswaldo Cruz Foundation strain											512	IV	
		Oswaldo Cruz Foundation strain											B94	IV	

285 CB: cocoa beans; C: after cleaning; N: nibs; M: mass; R: after roasting; R_{20}: after 20 minutes of roasting; R_{40}: after 40 minutes of roasting;
286 A: after alkalization; Re: after refining; Ca: after pressing; CP: cocoa powder. IL, industry lines.

Table 3. Strains of *B. cereus* persistent during cocoa powder production chain

Industrial line	Group	Strain code	Origin
1	38	019	After refining
		020	Cocoa powder
2	42	023	Cocoa beans
		026	Cocoa beans
		029	Nibs
		031	Nibs
		037	Cocoa powder
3	50	079	After pressing
		082	Cocoa powder
	51	063	After roasting
		085	Cocoa powder

Assignment of *B. cereus* strains to phylogenetic groups strains belonged was determined by comparing *pan*C sequences to those deposited in database available at https://www.tools.symprevius.org/bcereus. The most frequent phylogenetic group among all strains was grupo IV, with a prevalence of 73%, followed by group III (14%), groups II and V (both with 5%) and group VI (3%). In farm 01, 57% of strains belong to group IV, and 43% belong to group III. In farm 02, the frequency of groups IV, V, VI and II was 54%, 24.3%, 5.4% and 16.3%, respectively. In industrial line 01, 83% of strains belong to group IV, and 17% of strains belong to group III. In industrial line 02 100% of strains belong to group IV. In industrial line III, 96% and 4% of strains belong to group IV and III, respectively. No strain from phylogenetic group III were positive for *ces* gene. Only one strain from (n138) phylogenetic group VI was tested positive for presence of *cspA*.

Fig. 6 presents a phylogenetic tree based on *pan*C sequences of *B. cereus* strains. High percentage of similarities with reference strains belonging to phylogenetic groups II, III, IV, V and VI was observed for the majority of strains. Strains n100 and n114 from group III shows higher percentage of similarities with reference strain from phylogenetic group II.

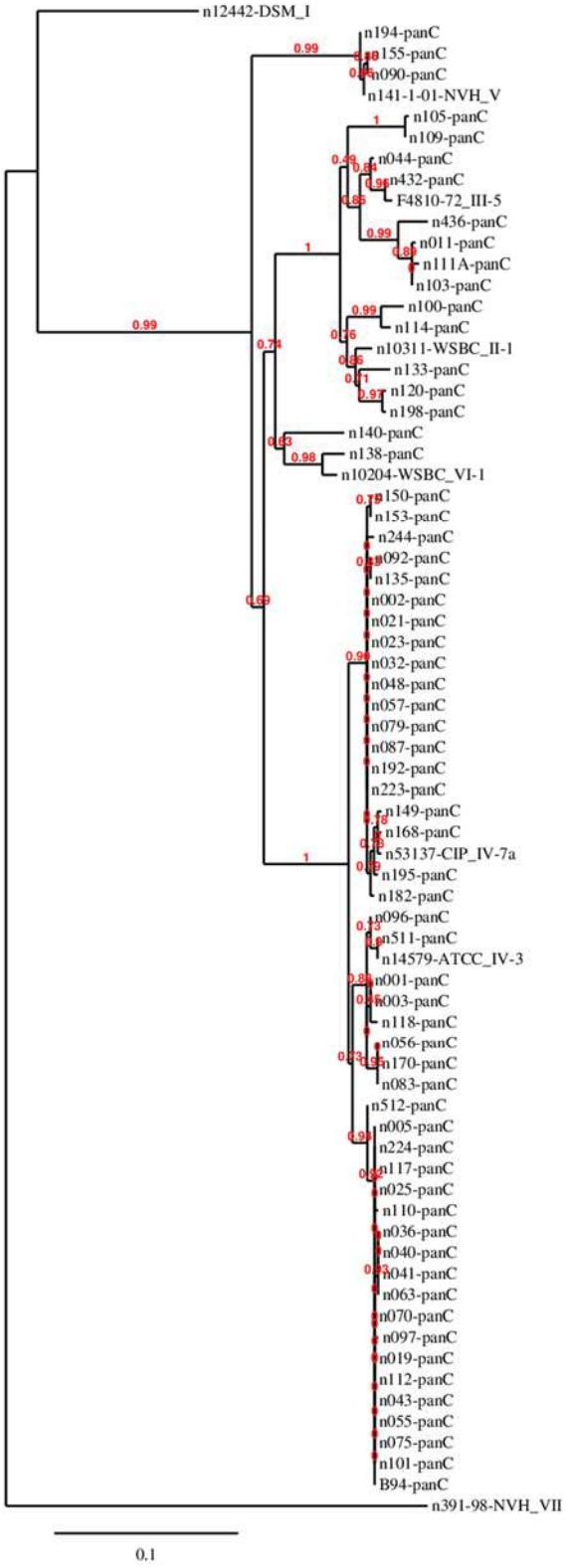

Fig. 6. Phylogenetic tree of strains based on *pan*C sequences. Sequences from reference strains belonging to different phylogenetic groups were also used.

4) Discussion

During the processing in the farms a large contamination by a variety of microorganisms occurs. The contact with soil, workers hand, re-used equipment are great sources of contamination. Besides the microbiota responsible for the natural fermentation of cocoa, there is also contamination by other types of microorganisms, such as spore forming bacteria. Some studies (Ardhana and Fleet, 2003; Nielsen et al., 2007; Ostovar and Keeney, 1973; Ouattara et al., 2011, 2008; Schwan et al., 1986) report the incidence of spore forming bacteria during cocoa fermentation. Populations of spores during cocoa fermentation normally did not present significant increase of decrease, remaining almost constant, between 5-8 log cfu/g (Ouattara et al., 2008). MAS population of farm 2 is low (1.8-3 log cfu/g) in the first days of fermentation. The seeds are considered sterile after opening the fruit, so the contamination occurs after this and increases after few days of fermentation, when there is aeration of the seeds.

In industrial processing, steps such as roasting and alkalization can reduce microbial levels by high temperature applied. During alkalization temperatures of 60-120°C are applied and alkaline agent (1-6%) is added (Bispo, 1999). During roasting temperature (110-140°C) and time (10-60 minutes) can vary (Beckett, 2008). A reduction between 2-4 $\log_{10}$ in total counts after roasting at 150°C/40 min was reported (Barrile et al., 1971). Total spore and thermoresistant spore levels were significantly reduced after alkalizing of nibs (Lima et al., 2012) in an industrial line from Netherlands. In this same study, a reduction of spores after roasting was observed too. Studies (Pia et al., 2019; unpublished data) simulated roasting and alkalization in laboratory and report that depending on the conditions applied in the industries, spore reduction may not be efficient. In this way, spore forming bacteria can persist along the processing chain and reach the final product at low levels.

During cocoa powder processing the a_w decreased to 0.35 in the final product. Low a_w may increases the thermal resistance of spores, which may explain the spore survival during

processing, as it is found in cocoa powder (Gaillard et al., 1998). The most effective inactivation of roasting process in the MAS and TAS spores in Industrial Line 3 may have occurred because after alkalization the pH increased to 8.7. In this pH range the thermal resistance of spores decreases, and this appears to be due to removal of soluble coat proteins damaging the lysis system, which is necessary for germination (Duncan et al., 1972).

M13-PCR technique provided discrimination to determinate *B. cereus* group contamination along cocoa powder processing chain. By this technique was possible to see that *B. cereus* contamination in cocoa powder can comes from cocoa beans and the processing steps. The phylogenetic group IV was the most frequent, composing 72% of strains. Group IV comprises mesophilic strains with range of growth at 10-45°C (Guinebretière et al., 2008). This temperature range corresponds to the temperature range of a tropical country, such as Brazil. Strains from Group III were reported (Guinebretière et al., 2010) carrying *ces* gene responsible for the synthesis of cereulide toxin, which leads to emetic syndrome. However, no strains in this study present the presence of this toxin gene. Strains from Group VI were reported carrying *cspA* gene characteristic of psychotrophic strains (Francis et al., 1998; Guinebretière et al., 2008). Just one strain presents the presence of this gene.

In this study, we concluded that spore forming bacteria contaminate cocoa seeds during processing in farms and may persist in the industrial process. Although spore forming bacteria levels in cocoa powder are relatively low, these spores can germinate in products produced with this raw material, leading to problems for the industry. An alternative to improve this concern would be to apply cleaning measures during the fermentation and drying stages, with the aim to reduce the level of contamination.

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487 ***SUPPLEMENTARY MATERIAL***

488 Table S1. Strains used in this study.

Strain code	Origin[a]	
n001	Cocoa beans	IL1
n002	Cocoa beans	IL1
n003	Cocoa beans	IL1
n005	After cleaning	IL1
n007	After cleaning	IL1
n009	After cleaning	IL1
n011	After roasting	IL1
n012	After roasting	IL1
n018	Cocoa mass	IL1
n019	After refining	IL1
n020	Cocoa powder	IL1
n021	Cocoa powder	IL1
n023	Cocoa beans	IL2
n025	Cocoa beans	IL2
n026	Cocoa beans	IL2
n028	Nibs	IL2
n029	Nibs	IL2
n031	Nibs	IL2
n032	Nibs	IL2
n036	Cocoa powder	IL2
n037	Cocoa powder	IL2
n038	Cocoa beans	IL3
n039	Cocoa beans	IL3
n040	Cocoa beans	IL3
n041	Cocoa beans	IL3
n042	Nibs	IL3
n043	Nibs	IL3
n044	Nibs	IL3
n048	After alkalization	IL3
n049	After alkalization	IL3
n050	After alkalization	IL3
n054	After 20 minutes of roasting	IL3
n055	After 20 minutes of roasting	IL3
n056	After 20 minutes of roasting	IL3
n057	After 20 minutes of roasting	IL3
n063	After 40 minutes of roasting	IL3
n065	After 40 minutes of roasting	IL3
n066	Cocoa mass	IL3
n068	Cocoa mass	IL3
n070	Cocoa mass	IL3
n073	After refining	IL3
n075	After refining	IL3
n077	After refining	IL3
n078	After pressing	IL3
n079	After pressing	IL3

n082	Cocoa powder	IL3
n083	Cocoa powder	IL3
n085	Cocoa powder	IL3
n087	Cocoa powder	IL3
n090	After 1 day of fermentation	F1
n092	After 1 day of fermentation	F1
n094	After 1 day of fermentation	F1
n096	After 2 days of fermentation	F1
n097	After 2 days of fermentation	F1
n098	After 2 days of fermentation	F1
n099	After 2 days of fermentation	F1
n100	After 1 day of drying	F1
n101	After 1 day of drying	F1
n102	After 1 day of drying	F1
n103	After 1 day of drying	F1
n105	After 2 days of drying	F1
n106	After 2 days of drying	F1
n107	After 2 days of drying	F1
n108A	After 3 days of drying	F1
n109	After 3 days of drying	F1
n110	After 3 days of drying	F1
n111A	After 4 days of drying	F1
n111B	After 4 days of drying	F1
n112	After 4 days of drying	F1
n114	After 4 days of drying	F1
n117	Time 0	F2
n118	After 1 day of fermentation	F2
n120	After 1 day of fermentation	F2
n122	After 1 day of fermentation	F2
n124	After 1 day of fermentation	F2
n133	After 2 days of fermentation	F2
n135	After 2 days of fermentation	F2
n138	After 2 days of fermentation	F2
n140	After 2 days of fermentation	F2
n149	After 3 days of fermentation	F2
n150	After 3 days of fermentation	F2
n153	After 3 days of fermentation	F2
n155	After 3 days of fermentation	F2
n164	After 4 days of fermentation	F2
n167	After 4 days of fermentation	F2
n168	After 4 days of fermentation	F2
n170	After 4 days of fermentation	F2
n177	After 1 day of drying	F2
n179	After 1 day of drying	F2
n181	After 1 day of drying	F2
n182	After 1 day of drying	F2
n192	After 2 days of drying	F2
n194	After 2 days of drying	F2
n195	After 2 days of drying	F2
n198	After 2 days of drying	F2

n208	After 3 days of drying	F2
n214	After 3 days of drying	F2
n215	After 3 days of drying	F2
n216	After 3 days of drying	F2
n223	After 4 days of drying	F2
n224	After 4 days of drying	F2
n226	After 4 days of drying	F2
n227	After 4 days of drying	F2
n239	After 5 days of drying	F2
n244	After 5 days of drying	F2
n245	After 5 days of drying	F2
n247	After 5 days of drying	F2
432	White chocolate	CCGB - FIOCRUZ[b]
436	White chocolate	CCGB - FIOCRUZ[b]
511	Chocolate fortified with vitamins	CCGB - FIOCRUZ[b]
512	Chocolate fortified with vitamins	CCGB - FIOCRUZ[b]
540	Boisson au chocolat	CCGB - FIOCRUZ[b]
B94	Milk	CCGB - FIOCRUZ[b]

[a]Industrial Line 1 : IL1; Industrial Line 2 : IL2; Industrial Line 3 : IL3 ; Farm 1 : F1 ; Farm 2: F2.

[b]Culture collection of *Bacillus* and correlated genera, FIOCRUZ - Oswaldo Cruz Foundation, Brazil

CAPÍTULO 3

Fate of *Bacillus cereus* and *Geobacillus stearothermophilus* spores during cocoa fermentation

Artigo formatado de acordo com as normas do periódico: "Food Microbiology"

Fate of Bacillus cereus and Geobacillus stearothermophilus spores during cocoa fermentation

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Abstract

Spore forming bacteria such as *B. cereus* and *G. stearothermophilus* play an important role as pathogen and spoilage organisms of processed foods, respectively. The aim of this study was to investigate the fate of *B. cereus* and *G. stearothermophilus* spores during fermentation of cocoa beans. Spores suspensions of five strains were used for each microorganism. The fermentation process was performed using cocoa pods (*Theobroma cacao* L.) in a pilot scale (2 kg beans/box) for 7 days. In a daily basis, a different fermentation box was inoculated with 3 log CFU/g of spores. During fermentation, samples were collected for microbiological, water activity and pH determinations. Consumption of carbohydrates, and ethanol, organic acids and volatile compounds production were also determined. The counts of *B. cereus* and *G. stearothermophilus* remained stable during the whole period of fermentation, except in the last two days of the process, in which increase (in general, approximately 2.51 log CFU/g) in populations were observed. This might be related to the increase of pH from 4.17 to 7.42 during fermentation, while water activity remained high (0.970). The most concentrated acid observed was succinic acid on the 4th day of fermentation and 88 volatile compounds were detected throughout the fermentation. There was no significant decrease or inactivation of the spore population during fermentation, but spores present at the end can survive industrial processing.

Keywords: cocoa, fermentation, spore forming bacteria, *B. cereus*, *G. stearothermophilus*

1) Introduction

Theobroma cacao is a tree originated from South America and currently grown in almost every tropical area of the globe. Cocoa seeds (beans) may be used as raw material to manufacture several products with a wide variety of applications in food industries, such as cocoa powder, cocoa butter and chocolate.

The cocoa processing chain can be divided in two main sections, *i.e.*, pre-processing and industrial processes. Among postharvest steps, the fermentation of cocoa beans is known as highly relevant for generation of compounds that result in health benefits to humans. Additionally, the fermentation of cocoa is of paramount importance for the development of flavor compounds (Kongor et al., 2016) and the microbial succession plays a role in this process (Moreira et al., 2013; Pereira et al., 2013). Cocoa is considered sterile until the opening of the fruit. After breaking the shell, the seeds are contaminated with a wide variety of microorganisms from the environment and from the skin. Some of these microorganisms are responsible for the natural fermentation of cocoa (Ardhana and Fleet, 2003; Garcia-Armisen et al., 2010; Jespersen et al., 2005; Papalexandratou et al., 2013; Schwan and Wheals, 2004). Fermentation takes place on farms after harvest right after the fruit is broken. The fruits are broken with the aid of knives, in which the seeds are removed by hand and placed in wooden boxes or baskets to ferment during 6 - 7 days. In the first step of fermentation, as the cocoa pulp is rich in sugars, the yeasts predominate and produce ethanol and pectinolytic enzymes. The growth of lactic acid bacteria is favored and lactic acid is produced. Acetic bacteria also grow in the substrate, resulting in the increase of temperature in the wooden boxes. Subsequently, spore forming bacteria predominate raising the pH, and finally filamentous fungi develop on the surface (Schwan and Wheals, 2004). At this late stage of fermentation, the pulp is liquefied, seeds lose their germination power and biochemical reactions responsible for the development of characteristic flavor precursors of cocoa products occur (Afoakwa et al., 2008; Papalexandratou et al., 2011;

Schwan and Wheals, 2004). Afterwards, the seeds are dried and prepared for industrial processing, which will favor because of harsh conditions the survival of more resistant microorganisms, such as spore forming bacteria (Barrile et al., 1971; Lima et al., 2011). Given their resistance, several studies report that the cocoa powder is a major source of spore forming bacteria, such as *Bacillus* spp. and *Geobacillus* spp. (Gabis et al., 1970; Lima et al., 2012, 2011; Lücking et al., 2013; Witthuhn et al., 2011).

The concern over the presence of spore forming bacteria in cocoa products used as raw materials in food industries relies on the fact that these microorganisms might negatively affect the quality and safety of processed foods (Pereira and Sant'Ana, 2018). Among spore forming bacteria, *Bacillus cereus* and *Geobacillus stearothermophilus* deserve special attention as reports indicate their isolation during fermentation of cocoa beans (Ardhana and Fleet, 2003; Nielsen et al., 2007; Ostovar and Keeney, 1973; Ouattara et al., 2008; Schwan and Wheals, 2004) and from cocoa powder samples (Gabis et al., 1970; Lima et al., 2012, 2011). Whereas *B. cereus* is an aerobic spore forming bacterium known to cause emetic and diarrheal syndromes (Beecher and Wong, 1997; Granum, 1994; Lund and Granum, 1997), *G. stearothermophilus* is highly thermal resistant bacterium known to cause flat sour spoilage of low-acid products (Feeherry et al., 1987; Mazas et al., 1997; Periago et al., 1998). Consequently, the significance of *B. cereus* and *G. stearothermophilus* for the safety and microbiological stability of products formulated with cocoa powder, particularly those subjected to heat treatments, such as chocolate milk is noticeable.

It is known that during the fermentation of cocoa beans, there are shifts in temperature, water activity and pH (acidity), which certainly affect the microbial ecology of the fermented cocoa beans. Furthermore, microbiological contamination can take place at different periods during the fermentation step. Depending on the moment at which contamination occurs, it may be more critical to the microbiological safety and/or to the quality of the final product

(Nascimento et al., 2010). Despite this, to the best of author's knowledge, there is no report on the fate of *B. cereus* and *G. stearothermophilus* spores as influenced by fermentation step of cocoa beans and dependent upon the moment the contamination takes place. Therefore, the current study was undertaken to assess the fate of *B. cereus* and *G. stearothermophilus* spores during cocoa fermentation as influenced by the moment the contamination occurs.

2) Material and Methods

2.1) Strains of B. cereus and G. stearothermophilus

B. cereus (n=5) and of *G. stearothermophilus* (n=5) were used in this study. The strains of *B. cereus* were isolated from cocoa-based products: CCGB FIOCRUZ ("Culture collection of Bacillus and correlated genera" - Oswaldo Cruz Foundation, Brazil) 432 and CCGB FIOCRUZ 436 from white chocolate; CCGB FIOCRUZ 511 and CCGB FIOCRUZ 512 from chocolate fortified with vitamins; CCGB FIOCRUZ 540 from mixture for chocolate drink. The strains of *G. stearothermophilus* belonged to different culture collections: CCGB FIOCRUZ 792, CCGB FIOCRUZ 793, CCGB FIOCRUZ 794, IAL (Adolfo Lutz Institute, 2008) 2128 and ATCC® (American Type Culture Collection) 795.

2.2) Preparation of B. cereus and G. stearothermophilus spore suspensions

Each strain of *B. cereus* and *G. stearothermophilus* was grown overnight separately in Nutrient Broth (Kasvi, Curitiba, Brazil) supplemented with 5 mg/L of manganese sulfate (NB-Mn), incubated at 30ºC and 55°C for *B. cereus* and *G. stearothermophilus*, respectively. 2 mL of broth was inoculated in Roux bottles (37 per strain) containing Nutrient Agar (Kasvi, Curitiba, Brazil) added of 5 mg/L of manganese sulfate (NA-Mn) (Peña et al., 2014; Pflug, 1999). Then, the Roux bottles inoculated with *B. cereus* and *G. stearothermophilus* were further incubated at 30ºC and 55ºC, respectively. The sporulation process was followed on a daily

126 basis. Once > 90% of the slides were covered by spores, confirmed with optical microscope
127 under a 1000 X magnification through the staining with malachite green (Spinelli et al., 2010),
128 the surface of NA-Mn was washed with sterile distilled water and rubbed with a glass rod. The
129 suspensions were centrifuged [4000 × g during 20 minutes at 4°C] and washed three times with
130 sterile distilled water (Oteiza et al., 2014; Peña et al., 2014; Spinelli et al., 2010). The pellets of
131 each strain were resuspended in sterile distilled water aiming to adjust the final concentration
132 of spores to 10^{10} spores/mL, following storage at -20ºC until use.
133
134 ***2.3) Preparation and inoculation of cocoa seeds***
135 Cocoa fruits were purchased from a central supply located in Campinas, state of São
136 Paulo, Brazil. The fruits were transported to the lab and manually opened. Portions of 2 kg of
137 cocoa seeds were obtained and placed inside polystyrene boxes covered with leaves of banana
138 trees. The boxes contained holes at the bottom to allow aeration and draining of liquids
139 (Nascimento et al., 2013).
140
141 ***2.4) Inoculation of cocoa seeds and simulation of cocoa fermentation process***
142 *B. cereus* and *G. stearothermophilus* spore counts in cocoa seeds used for experiments
143 were bellow of detection limit. Thus it was considered that the whole spore population present
144 throughout the experiment came from the inoculation performed at the laboratory.
145 Before the inoculation, a cocktail with equal concentrations of each strain (10^7 spores/mL)
146 and each specie was prepared. Then, the cocoa seeds placed within polystyrene boxes were
147 inoculated with a volume of the cocktail to obtain a concentration of 10^3 spores per g. This
148 inoculation was done through the assistance of a duly clean sprayer (previously disinfected with
149 100 mg/L chlorine solution for 15 min), that was used to spread over a total of 2 kg of cocoa

seeds in a sterile tray. Inoculated seeds were stirred for 3 min with a sterile spatula for homogenization. The non-inoculated boxes (C1 and C2) were used as control.

Seeds were fermented for 7 days at temperature of 25-35ºC and humidity of 60-80%. From fermentation days 3 to 7 cocoa seeds were daily aerated by transfer onto a sterile tray, stirring with a sterile spatula for approx. 02 min before replacement in the fermentation box. Experimental techniques and conditions of cocoa seed preparation and fermentation (opening technique, fermentation duration, temperature, humidity and aeration) were in accordance with practices of Brazilian cocoa farms (Beg et al., 2017; Schwan and Wheals, 2004; Suazo et al., 2014).

B. cereus and *G. stearothermophilus* spores were daily inoculated in one different box. A total of 7 boxes were inoculated for each microorganism. The boxes were identified as B0-6 for boxes inoculated with *B. cereus* spores, and G0-6 for boxes inoculated with *G. stearothermophilus* spores. From the beginning (t0) to the end (t7) of the fermentation samples from the surface, middle and bottom of the fermentation boxes were daily collected for microbiological analyses, chemical analyses (contents in organic acids, ethanol, and volatile compounds), and measurement of water activity using an AquaLab model CX-2 equipment (Decagon Devices, Washington, USA) and pH using Kasvi model K39-2014B pH-meter (Curitiba, Brazil). To pH determination 10 g of sample were homogenized in 90 ml of sterile distilled water (Adolfo Lutz Institute, 2008). The temperature of each box was ascertained on a daily basis with the assistance of a DataLogger (Hydra série II, Fluke, Everett, USA), using a flexible copper ⁄ constantan thermocouple (TC; TTT-36 type; Omega, CT, USA). The simulation of fermentation process was replicated twice.

2.5) Microbiological analysis

2.5.1) Enumeration of B. cereus and G. stearothermophilus spores in cocoa seeds during fermentation

To enumerate *B. cereus* spores, 90 mL of peptone water 0.1% (1 g Bacteriological Peptone [Oxoid, Basingstoke, UK] per liter) were added to 10 g of each sample in a sterile bag, and homogenized for 1 min with a Stomacher (Seward, BA7021, Worthing, UK). Homogenized samples were heat-shocked at 70ºC for 15 min in a water bath (Quimis, Q-334M-28, Diadema, Brazil), then serially diluted and plated onto Mannitol Egg Yolk Polymyxin agar (MYP, Oxoid - Basingstoke, UK). The plates were incubated at 30ºC for 24 to 48 hours (Bennett et al., 2001). To enumerate *G. stearothermophilus* spores, 90 mL of sterile distilled water was added to 10 g of each sample in a sterile bag, followed by homogenization by agitation during 1 minute at Stomacher (Seward, BA7021, Worthing, UK). It was applied a thermal shock of 110ºC for 10 minutes in oil bath (Polystat, 12105-20, Chicago, USA), following the serial dilutions and plating in Dextrose Tryptone agar (DTA). The plates were incubated at 55ºC for 48 to 72 hours (Olson and Sorrells, 2001). DTA contains Tryiptone (10g/L, Acumedia, Neogen Co., Lansing, USA), dextrose (5g/L, Dinâmica, Diadema, Brazil), Bacteriological Agar (15g/L, Oxoid, Basingstoke, UK) and solution of 1% purpura of Bromocresol in NaOH (4 mL/L, Dinâmica, Diadema, Brazil). Results of analysis were expressed as *B. cereus* or *G. stearothermophilus* spores/g.

2.5.2) Enumeration of yeasts, lactic acid and acetic acid bacteria during cocoa bean fermentation

To enumerate fermenting microorganisms, 10 g of each sample was homogenized in 90 mL of peptone water 0,1% g in a sterile bag, agitated for 1 minute at Stomacher (Seward, BA7021, Worthing, UK). Subsequently, serial decimal dilutions were carried out, followed by

200 plating in appropriate means according to the microorganism to be enumerated. Potato Dextrose
201 Agar (PDA, Acumedia, Neogen Co., Lansing, USA) supplemented with g 10% of tartaric acid
202 were used for yeasts enumeration; plates were incubated at 25ºC for 3 – 5 days (Mislivec et al.,
203 1992). Man-Rogosa-Sharpe agar (MRS, Oxoid, Basingstoke, UK) supplemented with 50 mg/L
204 of natamicine, was used for lactic acid bacteria enumeration; plates were incubated under
205 anaerobiosis at 37ºC for 3 days (Camu, N., Winter, T., Verbrugghe, K., Cleenwerck, I.,
206 Vandamme, P., Takrama, J.S., Vancanneyt, M., De Vuyst, 2007). Acetic bacteria were
207 enumerated in PDA containing 10% of tartaric acid and 50 mg/L of natamicine with incubation
208 on 30ºC for 3 - 5 days (Camu, N., Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P.,
209 Takrama, J.S., Vancanneyt, M., De Vuyst, 2007; Downes and Ito, 2001). Acetic bacteria
210 colonies were confirmed in ethanol agar (10g/L of extract of meat, 20 g/L of calcium carbonate,
211 20mL/L of ethanol, 20 g/L of agar, adjusted to pH 6.0) (Sievers and Swings, 2005). The results
212 of analysis were expressed in log base 10 CFU of spores per gram of cocoa beans.
213
214 ***2.6) Consumption of carbohydrates, and ethanol, organic acids and volatile compounds***
215 ***production***
216 ***2.6.1) HPLC analyses***
217 The carbohydrates, organic acid and alcohol from cocoa pulp and beans were extracted
218 as described by Rodriguez-Campos et al., 2011 and Moreira et al., 2013. Determinations were
219 made in cocoa beans and cocoa pulp inoculated with *B. cereus* spores, *G. stearothermophilus*
220 spores and control (not inoculated) during fermentation intermediate phase (4^{th} day of
221 fermentation). The analyses were carried out using a liquid chromatography system (Shimadzu,
222 model LC-10Ai, Shimadzu Corp., Japan) equipped with a dual detection system consisting of
223 a UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai). A Shimadzu ion
224 exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was operated at 30 °C for

carbohydrates and alcohols and 50 °C for acids. Perchloric acid (pH 2.1) was used as the eluent at a flow rate of 0.6 mL/min. The organic acids were detected and quantified by UV absorbance (210 nm), while the alcohols and carbohydrates were detected and quantified via RID. All samples were analyzed in triplicate, and individual compounds were identified based on the retention time of standards injected using the same conditions. The sample concentrations were determined using an external calibration method. Calibration curves were built as described by Schwan et al. (2014).

2.6.2) GC-MS analyses

The volatile compounds from cocoa samples were extracted using the HS-SPME technique, as described in Rodriguez-Campos et al. (2011), with modifications. Cocoa samples (2.0 g) from the fermentation time of each variety (Control (no contamination), *B. cereus*, *G. stearothermophilus*) were macerated using liquid nitrogen for headspace analysis. A divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm SPME fiber (Supelco Co., Bellefonte, PA, U.S.A.) was used to extract volatile constituents from the cocoa headspace. The samples were placed in a 15 mL hermetically sealed flask and heated for 15 min at 60 °C to reach the sample headspace equilibrium. The volatile compounds were extracted by the SPME fiber exposition into the headspace for 30 min at 60 °C.

The compounds were analyzed using a Shimadzu GC model QP2010 equipped with a mass spectrometry (MS) and a capillary column of silica CarboWax 20M (30. m/0.25 mm/0.25 μm). The temperature program began with 5 min at 60 °C, followed by a gradient of 60 °C to 230 °C at 10 °C/min; the temperature was then maintained at 230 °C for 15 min. The injector and detector temperatures were maintained at 230 °C. The carrier gas (He) was used at a flow rate of 1.2 mL/min. Injections were performed by fiber exposition for 2 min. Volatile compounds were identified by comparing the mass spectra. Quantitative data of the identified

compounds were obtained by integrating the peak areas of all identified compounds (Petisca et al., 2013). Principal component analysis (PCA) was conducted by SensoMaker version 1.9.

2.7) Statistical analysis

All experiments were repeated twice with each spore cocktail. It was assessed the existence of significant differences ($p \leq 0.05$) from a variance analysis (ANOVA), followed by Scott-Knott test version 7.6 (Campina Grande, Brazil) (Silva and Azevêdo, 2002).

3) Results

3.1) pH and temperature variation during fermentation

On the first three days of fermentation, the pH of beans varied slightly, rising from 4.2 to 4.8 (average), followed by a decrease on the fourth day and subsequent increase to the end of the process (Fig. 1). The temperature increased from 24.9°C to 41.1 °C (average) on the first four days of fermentation (Fig. 2 and 3). The highest recorded temperature was 44.2ºC on the fourth day of fermentation. The water activity remained high and constant (0.97).

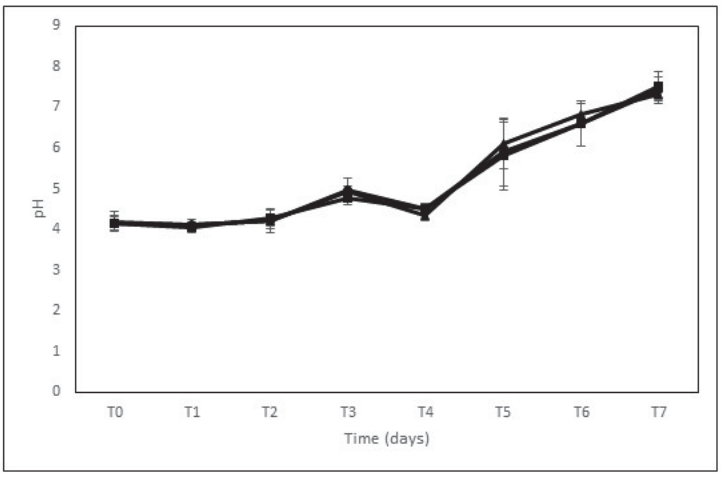
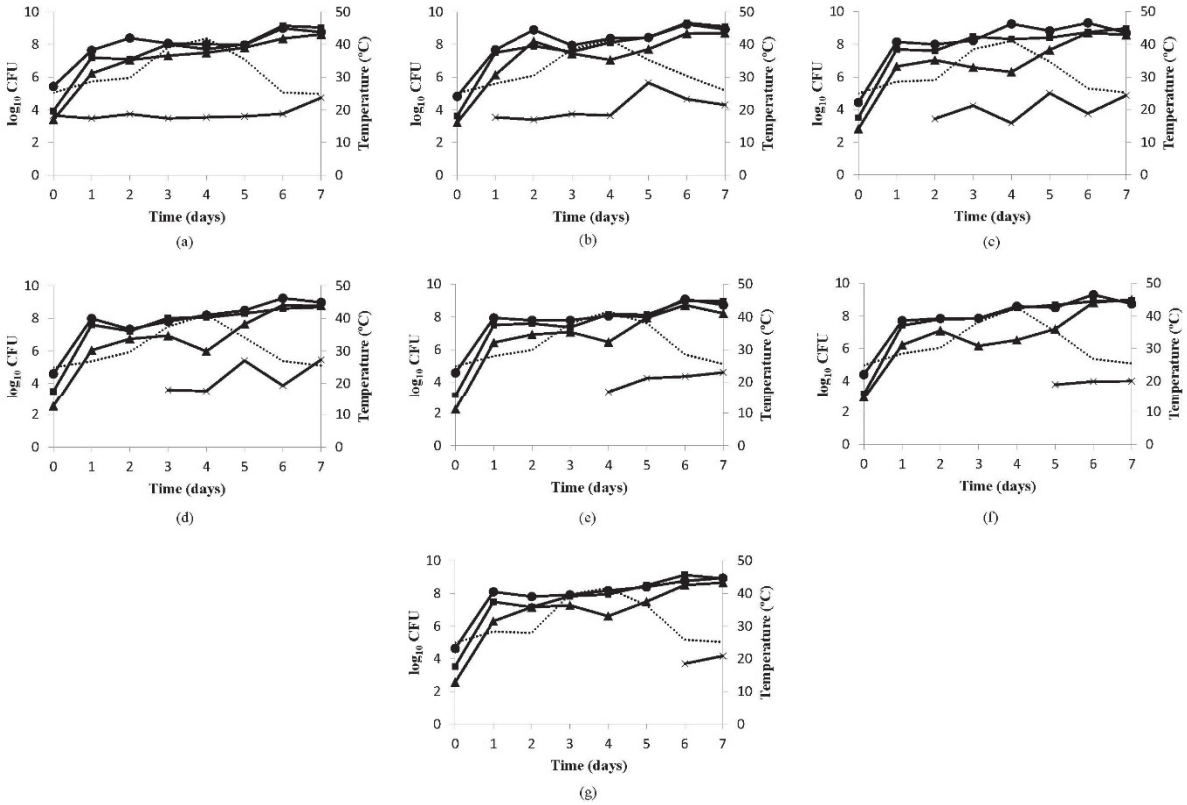


Fig. 1. pH variation during cocoa fermentation. Cocoa beans inoculated with *B. cereus* (▲) and *G. stearothermophilus* (■) spores and control (•).

268 ***3.2) Fate of B. cereus and G. stearothermophilus spores during cocoa fermentation***

269 The populations of *B. cereus* spores remain constant during fermentation (Fig. 2).
270 Counts of *G. stearothermophilus* spores remained also constant during the fermentation
271 process. In the cocoa beans inoculated with *B. cereus* spores, an increase followed by a decrease
272 in population was observed on the second and third day of fermentation. Cocoa beans from Box
273 2 (Fig. 2c) presented count of 3.44 log after inoculation and 4.24 log after 24 hours of
274 inoculation, followed by a decrease (3.18 log) and an increase (5 log) in the subsequent days.
275 Cocoa beans from Box 3 (Fig. 2d) showed a similar fate, from 3.54 log (after 24 hrs inoculation)
276 to 5.41 log (after 48 hrs), 3.88 log (after 72 hrs) and 5.47 log (after 96 hrs). The largest
277 population increase occurred on the last day, increasing 1.6 log. The *G. stearothermophilus*
278 population remained stable with little variation in their counts throughout the fermentation (Fig.
279 3). In Boxes 0 (Fig. 3a), 1 (Fig. 3b), 2 (Fig. 3c) and 6 (Fig 3g) there was an increase in population
280 between sixth and seventh days. The increase was from 3.67 to 5.85 log, 3.48 for 6 log, 3.42
281 for 4.47 log and 3.79 for 6.1 log in Boxes 0, 1, 2 and 6, respectively. *B. cereus* and *G.*
282 *stearothermophilus* spore populations did not show predominance over the other microbial
283 populations.
284

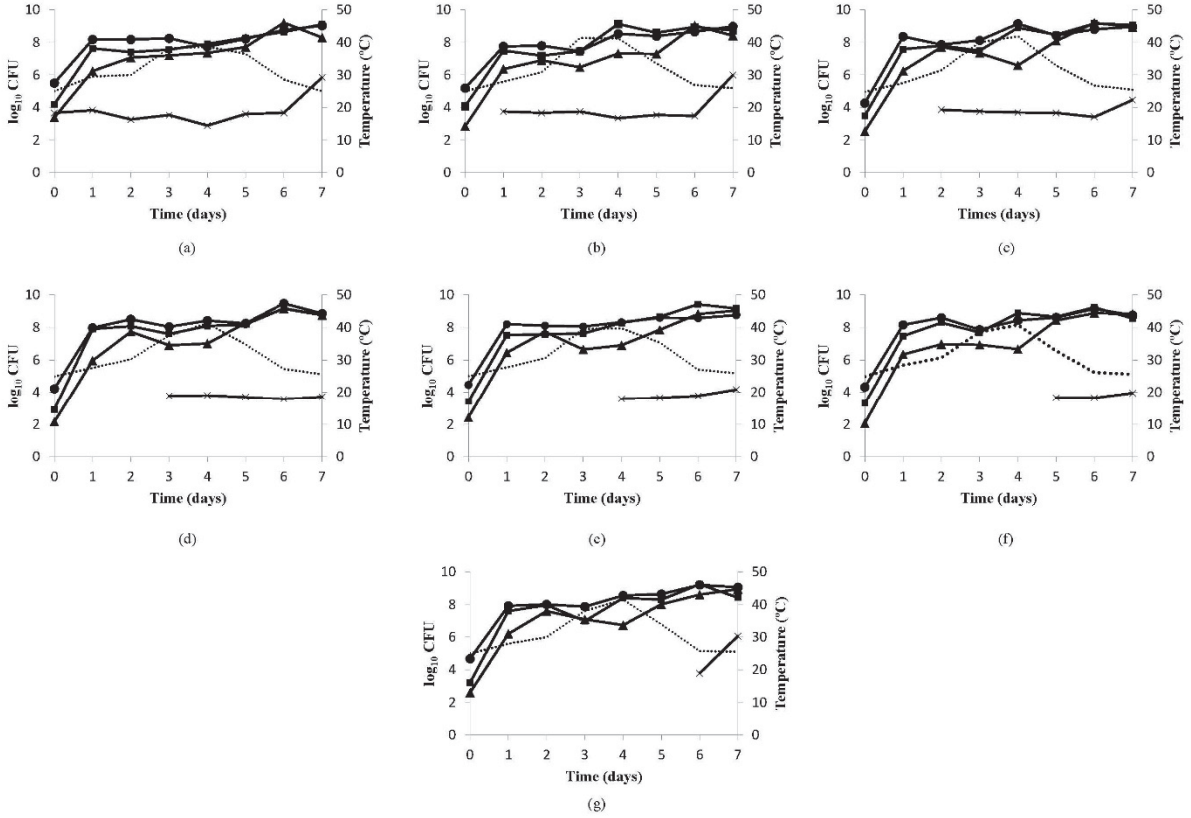


285

286 Fig. 2. Microbial count and fate of temperature during fermentation of cocoa beans inoculated
287 with *B. cereus* spores. Yeast (•), acetic acid bacteria (■), lactic acid bacteria (▲), *B. cereus* (x)
288 and temperature (….). (a) Box 0: beans inoculated at the beginning of fermentation; (b) Box 1:
289 beans inoculated on 1º day of fermentation; (c) Box 2: beans inoculated on 2º day of
290 fermentation; (d) Box 3: beans inoculated on 3º day of fermentation; (e) Box 4: beans inoculated
291 on 4º day of fermentation; (f) Box 5: beans inoculated on 5º day of fermentation; (g) Box 6:
292 beans inoculated on 6º day of fermentation.

293

294



295

296 Fig. 3. Microbial count and fate of temperature during fermentation of cocoa beans inoculated
297 with *G. stearothermophilus* spores. Yeast (•), acetic acid bacteria (■), lactic acid bacteria (▲),
298 *G. stearothermophilus* (x) and temperature (….). (a) Box 0: beans inoculated at the beginning
299 of fermentation; (b) Box 1: beans inoculated on 1º day of fermentation; (c) Box 2: beans
300 inoculated on 2º day of fermentation; (d) Box 3: beans inoculated on 3º day of fermentation;
301 (e) Box 4: beans inoculated on 4º day of fermentation; (f) Box 5: beans inoculated on 5º day of
302 fermentation; (g) Box 6: beans inoculated on 6º day of fermentation.

303

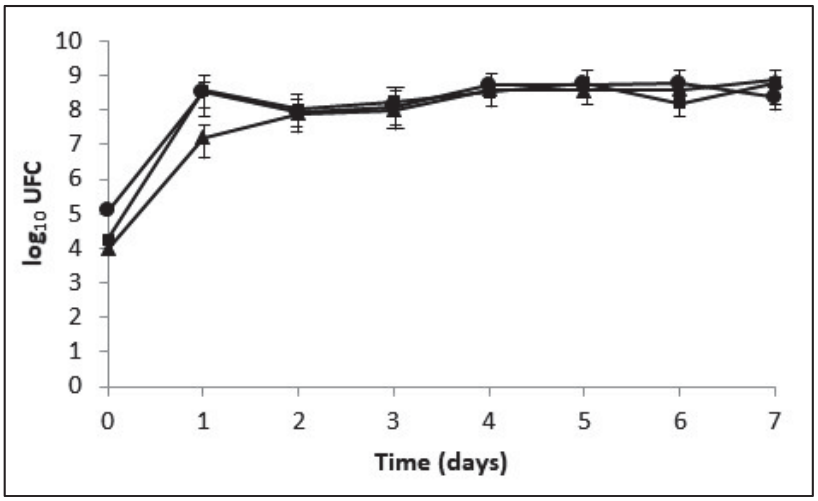


Fig. 4. Microbial count during cocoa beans fermentation without inoculation of spores. Yeast (•), acetic acid bacteria (■), lactic acid bacteria (▲).

3.3) Populations of yeasts, lactic acid and acetic acid bacteria throughout the fermentation process

It was possible to verify the yeast predominance in all the samples at the beginning of the fermentation (mean of 4.75 and 4.67 log for beans inoculated with *B. cereus* and *G. stearothermophilus*, respectively) and 24 hours after the beginning of the process (on average, 7.97 and 8.1 log for beans inoculated with *B. cereus* and *G. stearothermophilus*, respectively). In some samples, this predominance occurred up to 48h after the beginning of the fermentation process. The growth of acetic and lactic acid bacteria was similar throughout the fermentation. These bacteria showed increase in the population after 24 hours of fermentation. In the beans inoculated with *B. cereus* this increase was on average from 3.49 to 7.48 log and 2.81 to 6.28 log for acetic and lactic acid bacteria, respectively. In the beans inoculated with *G. stearothermophilus*, this increase was on average from 3.52 to 7.61 log for acetic acid bacteria and 2.57 to 6.26 log for lactic acid bacteria. At the end of fermentation, yeast, lactic acid bacteria and acetic acid bacteria showed similar counts, with a mean of 8.8 and 8.83 log for seeds inoculated with *B. cereus* and *G. stearothermophillus*, respectively. In the control experiment

323 (Fig. 4), the fate of the fermenting microorganisms was similar to the fate in the experiments
324 with inoculation of spores. The inoculation of *B. cereus* and *G. stearothermophilus* spores did
325 not affect ($P > 0.05$) the population of fermenting microorganisms.
326
327 ***3.4) Consumption of carbohydrates, and ethanol, organic acids and volatile compounds***
328 ***production***
329 The consumption of carbohydrates, the alcohols production and organics acids
330 formation during fermentation intermediate phase (4th day of fermentation) of two differents
331 microbial inoculation: *B. cereus* spores and *G. stearothermophilus* spores; and control (not
332 inoculated) in cocoa, are shown in Table 1. The sucrose was no detected in the pulp of fermented
333 cocoa. The lowest concentration of glucose (70.951 mg/L ± 2.834), fructose (193.672 mg/L ±
334 9.009), glycerol (27.702 mg/L ± 1.658) and ethanol (52.974 mg/L ± 1.774) were observed in
335 control cocoa pulp. In *B. cereus* inoculation and *G. stearothermophilus* in pulp cocoa, the sugar
336 and alcohol concentration were glucose (793.632 mg/L ± 1.667 and 658.503mg/L ± 11.958),
337 fructose (2082.118 mg/L ± 5.042 and 1636.064 mg/L ± 66.963), glycerol (117.441 mg/L ±
338 0.405 and 147.136 mg/L ± 0.893) and ethanol (247.116 mg/L ± 0.405 and 222.486mg/L ±
339 1.302), respectively. The ethanol and glycerol productions were higher than in control pulp
340 cocoa. The *B. cereus* inoculation pulp cocoa shown greater sugar utilization and ethanol
341 production, while, the *G. stearothermophilus* show greater glycerol production.
342 In control and *B. cereus* inoculation cocoas beans, the sugars concentration profiles were
343 similar and sucrose was not detected in both samples (Table 1). The control beans showed
344 glycerol (389.262 mg/L ± 0.777 and 151.103 mg/L ± 1.086) and ethanol (645.384 mg/L ± 3.475
345 and 261.170 mg/L ± 1.957) production higher than *B. cereus* inoculated beans, respectively. In
346 the case, *G. stearothermophilus* inoculated beans, there was a greater glucose (1032.340mg/L)
347 and fructose (2928.275mg/L) utilization and a lower glycerol (109.519mg/L) and ethanol

348 (241.397mg/L) production compared to other samples in cocoa beans. The sucrose was detected
349 (85.289mg/L).

350 As described in Table 1 the lactic acid was not detected in the pulp or bean of both
351 samples. The propionic acid was not detected in the beans, but in pulp was detected in control
352 (49.657 mg/L ± 0.065), *B. cereus* (75.652 mg/L ± 0.844) and *G. stearothermophilus* inoculated
353 (98.431 mg/L ± 7.546). In all samples were found concentrations of citric acid, malic acid,
354 succinic acid and acetic acid. The lowest concentration of malic acid (20.255 mg/L ± 0.434)
355 was observed in *B. cereus* inoculated cocoa pulp. There was an increase in the citric acid, malic
356 acid, succinic acid and acetic acid concentrations in the assays of cocoa beans fermentation
357 (control and inoculated). The acid of higher concentration observed in all samples was succinic
358 acid.

359 Table 1. Carbohydrates, alcohols and organic acids (mg/L)* detected in cocoa pulp and cocoa beans during fermentation intermediate phase (4th
360 day of fermentation).

	Cocoa pulp			Cocoa beans		
	Control	***B. cereus***	***G. stearothermophilus***	**Control**	***B. cereus***	***G. stearothermophilus***
Sucrose	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	85.3 ± 0.4
Glucose	71.0 ± 2.8	793.6 ± 1.7	658.5 ± 12.0	1256.1 ± 18.0	1188.7 ± 2.5	1032.3 ± 3.3
Fructose	193.7 ± 9.0	2082.2 ± 5.0	1636.1 ± 67.0	3320.4 ± 142.3	3234.1 ± 4.0	2928.3 ± 9.0
Glycerol	27.7 ± 1.7	117.4 ± 0.4	147.2 ± 1.0	389.3 ± 0.7	151.1 ± 1.1	109.5 ± 1.0
Ethanol	53.0 ± 1.8	247.1 ± 0.4	222.5 ± 1.3	645.4 ± 3.5	261.2 ± 2.0	241.4 ± 2.2
Citric Acid	555.1 ± 6.8	289.8 ± 0.8	434.3 ± 1.9	942.7 ± 4.002	464.0 ± 2.0	543.5 ± 4.3
Malic Acid	42.8 ± 0.8	20.3 ± 0.4	29.1 ± 0.2	96.9 ± 9.2	45.2 ± 0.4	81.3 ± 3.4
Succinic Acid	1004.4 ± 17.9	1214.2 ± 28.8	918.7 ± 11.8	2440.7 ± 53.2	2273.4 ± 54.1	2138.7 ± 122.8
Acetic Acid	423.7 ± 7.8	635.9 ± 3.8	568.7 ± 17.7	760.3 ± 2.4	787.6 ± 0.1	964.6 ± 3.4
Propionic Acid	49.7 ± 0.065	75.7 ± 0.844	98.4 ± 7.6	0 ± 0	0 ± 0	0 ± 0

361 *Mean ± standard deviation calculated from values of replicates.

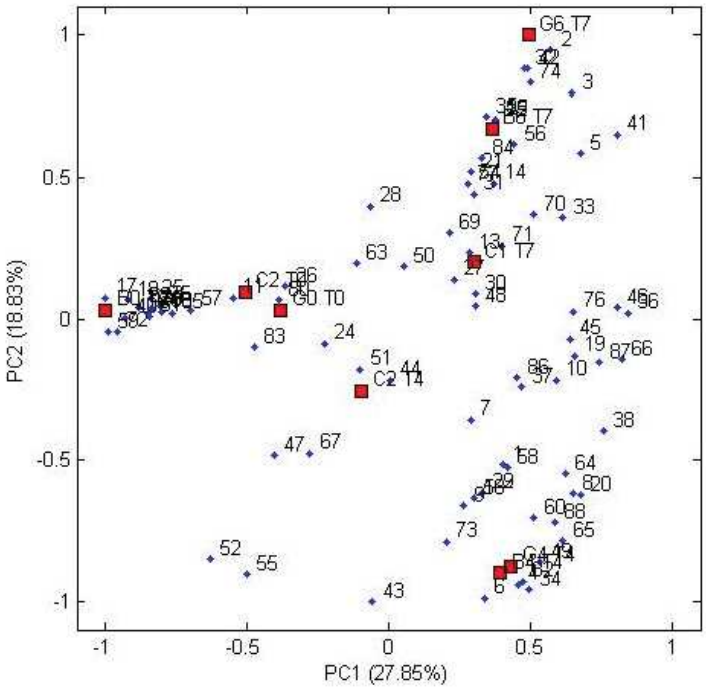
362 ***3.5) Volatile compounds produced during fermentation***

363 A total of 88 volatile compounds, in the different fermentation stages, were identified
364 in control (not inoculated) and inoculated samples with the different microbial inoculation by
365 comparing their mass spectrometry with those of mass spectrometry stored in the GC-MS
366 database. These compounds were classified as alcohols, phenols, benzenoid hydrocarbons,
367 hydrocarbons compound, aldehydes and ketones, terpenoids, heterocyclic compounds, acids,
368 sulfur compounds, fatty acid composition, esters and alkaloids as shown in Table S1. Were
369 identified about 46 volatile compounds in the initial time, while about 56 and 60 volatile
370 compounds were identified in the middle and at the end of fermentation, respectively, of both
371 samples inoculated and not inoculated cocoa. Most of the compounds observed in all
372 fermentation were alcohols and acids.

373 The compound beta-Myrcene, a hydrocarbon compound, was identified only in the end
374 of fermentation, while a Heterocyclic compounds and Undecanoic acid isopropyl ester, 10-
375 hydroxy-11-morpholin-4-yl-, ester, were identified only in the initial time of fermentation, in
376 both samples. The 1H-2-Benzopyran-1-one,3,4-dihydro-8-hydroxy-3-methyl- compound, a
377 phenol, was identified in cocoa samples inoculated only in the end of fermentation. In the
378 intermediate phase of the fermentation were identified decanoic acid, ethyl ester and 1,1-
379 ethanediol, diacetate only in cocoa samples of *B. cereus* inoculated, while the 2-acetoxy-3-
380 butanone and 4-heptanone, 2,6-dimethyl- compounds were identified only in cocoa samples of
381 *G. stearothermophilus* inoculated.

382 The results obtained for the total of volatile compounds, shows in Table S1, were
383 submitted to PCA (Fig. 5) to obtain a more simplified view of the relationship among the
384 volatile compounds analyzed. The results are show in Fig. 5. The first (PC1) and second (PC2)
385 principal components explain 27.85% and 18.83%, respectively, of the total variance (46.68%).
386 On the negative side of PC1, there are samples from fermentation initial time of control,

387 inoculated cocoa and control sample of intermediate stage of fermentation. On the positive side
388 of PC1, there are samples from final stage fermentation of control, inoculated cocoa and the
389 sample inoculated cocoa of intermediate stage of fermentation. In PC2, the samples of the final
390 stage of fermentation from inoculated cocoa were located on the positive side. The control
391 fermentation cocoa and the sample inoculated cocoa of intermediate stage fermentation for PC2
392 were located on negative side.



393

394 Fig. 5. Principal component analysis (PCA) of volatile compounds and the different
395 fermentation assays.

396

397 ## *4) Discussion*

398 The pH decrease observed on fourth day of fermentation was probably due to the large
399 production of acetic acid produced by the acetic acid bacteria (Schwan and Wheals, 2004).
400 Possibly, there was an increase in pH at the end of fermentation due to the evaporation of
401 volatile acids (Nielsen et al., 2007), which favors germination and growth of spore forming
402 bacteria (Ostovar and Keeney, 1973; Schwan et al., 1986). The final pH observed was similar

403 to that found by Nascimento et al. (2013). About the temperature Nielsen et al. (2007) observed
404 a maximum temperature of 46 ºC on a tray cocoa fermentation. Higher temperatures (51.2 ºC
405 and 50.4 ºC) was reported by Pereira et al. (2013). Cocoa beans aeration favors the development
406 of acetic acid bacteria, which increase the temperature due to exothermic reactions (Schwan
407 and Wheals, 2004).

408 As the population of spore forming bacteria remained almost constant, the fermentation
409 process did not cause any inactivation, whatever the duration of the presence of spores in the
410 fermentation process. Increases followed by decreases in population of spores observed for both
411 species occurred probably due to germination and subsequent sporulation. The conditions found
412 at the end of the fermentation favors the development of spore forming bacteria, so this explain
413 the increase in the population of spores of *B. cereus* and *G. stearothermophilus*. Factors such
414 as increased pH, aerobic conditions due to aeration and temperature rise leads to the increase
415 of spore forming bacteria (Ostovar and Keeney, 1973).

416 Other studies also observed the predominance of yeasts at the beginning of fermentation
417 (Ardhana and Fleet, 2003; Ostovar and Keeney, 1973). Due to the low pH and anaerobic
418 conditions found at the beginning of the fermentation, yeasts develop by performing alcoholic
419 fermentation, breaking of citric acid and production of organic acids, volatiles and enzymes
420 (Forsyth and Quesnel, 1963; Schwan and Wheals, 2004). After aeration of cocoa mass, an
421 increase in the population of acetic acid bacteria was observed in some boxes. This increase is
422 due to penetration of oxygen that allows a large growth and the domination of acetic acid
423 bacteria (Schwan and Wheals, 2004).

424 These compounds can be used as markers of increased activity of spore forming bacteria
425 in cocoa fermentation. Further studies are needed to verify if these compounds can change the
426 fermentative process in the sensory aspects of cocoa. Few studies have reported the role of
427 spore forming bacteria during fermentation process. Polygalacturonase and pectin lyase

production was observed in *Bacillus* spp. strains during cocoa fermentation (Ouattara et al., 2008). Species of *Bacillus* spp. may also produce lactic acid, acetic, 2,3-butanediol tetramethylpyrazine which may impair the quality of chocolate (Lopez and Quesnel, 1971; Zak et al., 1972).

Although the population of spore forming bacteria have not affected the development of fermenting microorganisms, it can alter the production of metabolites that can negatively affect the quality of the final product made with this raw material, such as chocolate. Aerobic spore forming bacteria produces some compounds which may contribute to off-flavors of fermented cocoa beans (Schwan and Wheals, 2004; Schwan et al., 1986). In addition, there was no significant decrease or inactivation of the spore population during the fermentation process. So bacterial spores present at the end of the fermentation may survive the processing of cocoa, and because of their resistance, can survive subsequent treatments such as roasting and alkalization.

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599 **SUPPLEMENTARY MATERIAL**

600 **Table S1.** Volatile compounds identified in the different fermentation stages, of two different
601 microbial inoculation (*B. cereus* and *G. stearothermophilus*) and control (not inoculated) in
602 cocoa.

Compound	Flavor	Samples[a]
1,1-Ethanediol, diacetate		B4 t4
1,2,3-Propanetriol, 1-acetate		B4 t4; G4 t4
1-Butanol, 3-methyl-, acetate		G4 t4; G6 t7; B6 t7; C1 t7
1-Butene-1,4-diol, diacetate		B6 t7; C1 t7
1-Hexanol	Fruity, green	B0 t0; C2 t0; G0 t0; G4 t4; B4 t4; C2 t4; G6 t7
1-Octanol	Fatty, Waxy	C2 t0; G0 t0; B4 t4; C2 t4; G4 t4; C1 t7; B0 t0; B6 t7
1-Pentanol	Pungent	G0 t0; B0 t0; C2 t0; G4 t4; B4 t4; C2 t4; C1 t7
1-Propanol, 3-(methylthio)-		G4 t4; C1 t7; B6 t7; G6 t7; B4 t4
1-Tetradecanol		B0 t0; G0 t0; B4 t4; G4 t4; C1 t7; B6 t7
2,3-Butanediol	Cocoa Buffer	G4 t4; C1 t7; B6 t7; G6 t7; C2 t4; B4 t4
2-Butanol, 3-methyl-, acetate		G6 t7
2-Ethyl-2-methyl-1,3-propanediol		C2 t4; G4 t4
2-Heptanol	Citrusy	G0 t0; C2 t4; B4 t4; G4 t4; C1 t7; B6 t7; G6 t7
2-Heptanol, 6-methyl-		C2 t0; B0 t0; G0 t0; G4 t4; C1 t7; B6 t7; G6 t7
2-Hexanol	Fruity, green	All samples
2-Pentanol, 3-methyl-		C2 t0; B0 t0; C2 t4; B6 t7; G6 t7
6-Methyl-2-Heptanol, acetate		G4 t4; G6 t7; C1 t7; B6 t7
alpha.-Terpineol		G4 t4; C1 t7; B6 t7; G6 t7; C2 t4; B4 t4; C2 t0; G0 t0
Benzenemethanol, .alpha.-methyl-		All samples
Benzyl alcohol	Sweet, Floral	All samples
Cyclohexanol, 3-methyl-		G6 t7; B6 t7
Ethanol, 2,2'-oxybis-		All samples
Glycerin		All samples
Phenylethyl Alcohol	Honey, Floral	All samples
Tetradecanal		G4 t4; C1 t7; B6 t7; B4 t4; C2 t4; G0 t0; B0 t0; C2 t0
Benzaldehyde	Butter	B4 t4; G4 t4; C1 t7; G6 t7
Acetophenone	Floral	All samples
Acetoin	Buttery, Creamy	G0 t0; B0 t0; C2 t0; G4 t4; B4 t4; C2 t4
4-Heptanone, 2,6-dimethyl-		G4 t4
3-Hepten-2-one, (E)-		G6 t7
3,6-Heptanedione		C2 t0; G4 t4; C1 t7; B6 t7; G6 t7; G0 t0; B0 t0
2-Pentanone, 4-hydroxy		All samples
2-Nonanone		All samples
2-Heptanone	Fruity, Floral	All samples

2-Acetoxy-3-butanone		G4 t4
Octadecanoic acid		B0 t0
n-Hexadecanoic acid		C2 t0; B0 t0; G0 t0; C2 t4; G4 t4; B6 t7
Pentadecanoic acid		C2 t0; B0 t0
Nonanoic acid	Fatty	G4 t4; G6 t7; B6 t7; C1 t7; B4 t4
Tetradecanoic acid		G4 t4; G6 t7; C2 t0; B0 t0; G0 t0; C2 t4; B6 t7
beta.-Ethoxypropionic acid		C1 t7; B6 t7; G6 t7
Dodecanoic acid	Metal	All samples
n-Decanoic acid	Rancid, Fatty	All samples
Octanoic acid	Sweaty, Fatty	B6 t7; C1 t7; G4 t4; B4 t4; C2 t4
2-Butenoic acid, 3-methyl-		G4 t4; C1 t7; B6 t7; G6 t7
Hexanoic acid	Sweaty, Pungent	All samples
Isovaleric acid	Sweaty, Rancid	All samples
Isobutyric acid	Rancid butter	B0 t0; G4 t4; C1 t7; B6 t7; G6 t7; C2 t4; B4 t4
Propanoic acid	Pungent	C2 t4; C1 t7
Acetic acid	Sour , Vinegar	C2 t0; B0 t0; G0 t0
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester		All samples
1,6-Octadien-3-ol, 3,7-dimethyl-		C2 t0; G0 t0; B4 t4; C2 t4; G4 t4; C1 t7; B6 t7; G6 t7
2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, cis-	Nutty	All samples
Acetic acid, 2-phenylethyl ester	Honey, Floral	C1 t7; B6 t7; G6 t7; G4 t4
Benzeneacetic acid, ethyl ester	Fruity, Sweet	G4 t4; C1 t7; B6 t7; G6 t7; B4 t4
Benzoic acid, pent-2-yl ester		All samples
cis-Pyranoid linalool oxide	Floral, Green	G4 t4; C1 t7; B6 t7; G6 t7; C2 t4; B4 t4; C2 t0; G0 t0
Decanoic acid, ethyl ester		B4 t4; G0 t0; G4 t4; C1 t7; B6 t7; G6 t7
Glutaric acid, di(2-propylpentyl) ester		All samples
Hexadecanoic acid, ethyl ester		G0 t0; B4 t4; G4 t4; G6 t7; B6 t7; C1 t7
Homosalate		B0 t0; B4 t4; G4 t4; C1 t7; B6 t7
Linoleic acid ethyl ester		G0 t0; B4 t4; G4 t4
trans-Linalool oxide (furanoid)	Floral, Citrus	C2 t0; B0 t0; G0 t0
Undecanoic acid isopropyl ester, 10-hydroxy-11-morpholin-4-yl-		G0 t0; B0 t0; C2 t0
Octanoic acid, ethyl ester		B4 t4; G4 t4; C1 t7; G6 t7; B6 t7
Squalene		C2 t0; B0 t0; G0 t0; C2 t4
Oleic Acid		C2 t0
Phenol, 2-methoxy-	Smoky	G4 t4; G6 t7

Morpholine, 4-octadecyl-		All samples
Phenol	Smoky	All samples
Phenol, 2,6-bis(1,1-dimethylethyl)-4-(1-methylpropyl)-		G4 t4; C1 t7; B6 t7; G6 t7; B4 t4; G0 t0; B0 t0; C2 t0
1,2-Benzisothiazole		G0 t0; C2 t4; C2 t0; B0 t0; B4 t4; G4 t4
1,6-Octadien-3-ol, 3,7-dimethyl-		C2 t0; G0 t0; C2 t4; B4 t4; G4 t4; C1 t7; B6 t7; G6 t7
Styrene		All samples
beta.-Myrcene		C1 t7; B6 t7; G6 t7
1H-2-Benzopyran-1-one, 3,4-dihydro-8-hydroxy-3-methyl-		B6 t7; G6 t7
Thiophene, 2,3-dihydro-		B4 t4; C1 t7; B6 t7
Caffeine		B4 t4; G4 t4; B6 t7

603 [a]B0 – B6: box 0 – box 6 inoculated with *B. cereus* spores; G0 – G6: box 0 – box 6 inoculated
604 with *G. stearothermophilus* spores; t0 – t7: time 0 – time 7.

CAPÍTULO 4

Inactivation kinetics of *Bacillus cereus* and *Geobacillus stearothermophilus* spores through roasting of cocoa beans and nibs

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Inactivation kinetics of *Bacillus cereus* and *Geobacillus stearothermophilus* spores through roasting of cocoa beans and nibs

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ABSTRACT

Bacillus cereus and *Geobacillus stearothermophilus* are spore-forming bacteria of concern for the quality and safety of cocoa-based foods, because of their frequent occurrence in raw materials and the capability of spores to withstand heat processing. Cocktails of spores of *B. cereus* and *G. stearothermophilus* strains were separately inoculated on cocoa beans and nibs and roasted using a laboratory equipment at 110 °C, 125 °C and 140 °C for up to 600 min to simulate an industrial roasting process. The period of time needed for the first log-reduction δ for the spores of the cocktail of *B. cereus* in cocoa nibs at 110 °C, 125 °C and 140 °C were 92.2 min, 30.5 min, and 17.2 min, respectively. In cocoa beans at 110 °C, 125 °C and 140 °C δ-values were 148.5 min, 55.9 min, and 23.3 min. The δ-values for the spores of the cocktail of *G. stearothermophilus* in cocoa nibs at 110 °C, 125 °C, and 140 °C were 105.0 min, 39.6 min and 30.4 min, respectively, and 183.6 min, 76.2 min and 49.1 min in cocoa beans at the same temperatures, respectively. The a_w of cocoa nibs and cocoa beans ranged between 0.687 and 0.753 before roasting, and between 0.355 and 0.528 after roasting, respectively. The roasting process on beans or nibs applied in the cocoa industry may have a limited effect on the inactivation of naturally present spore-forming bacteria.

1. Introduction

Spores of foodborne bacteria are resilient to heat, drying, radiation and chemical biocides such as oxidizing agents (hydrogen peroxide, chlorine-derived compounds) (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000; Wells-Bennik et al., 2016). Natural and crop environments are important habitats for spore-forming bacteria and sources of bacterial spores that contaminate raw food materials, food ingredients and environment of food processing (McKnight, Eiroa, Sant'Ana, & Massaguer, 2010; Carlin, 2011; Peña et al., 2014; Oteiza, Soto, Alvarenga, Sant'Ana, & Giannuzzi, 2014; Pereira & Sant'Ana, 2018).

Considering that soil is the main habitat of spore-forming bacteria (Carlin, 2011; Pereira & Sant'Ana, 2018), direct or indirect contact of raw materials during farm steps is critical for raw materials contamination. Because of this, several raw materials used in food industry, such as sugar (Verruma-Bernardi, Borges, Lopes, Della-Modesta, & Ceccato-Antonini, 2007; Wojtczak, Biernasiak, & Papiewska, 2012), milk powder (Alvarenga et al., 2018; Ronimus, Rueckert, & Morgan, 2006; Scott, Brooks, Rakonjac, Walker, & Flint, 2007) and cocoa (te Giffel, Beumer, Leijendekkers, & Rombouts, 1996; Lima, Kamphuis, Nout, & Zwietering, 2011; Prevost, Andre, & Remize, 2010) have been reported to be commonly contaminated with spore-forming bacteria (Pereira & Sant'Ana, 2018). Amongst these raw materials, cocoa powder has a key relevance as it is highly used in the formulation of thermally processed foods such as chocolate milk. In addition, cocoa powder has been reported as a source of spore-forming bacteria (Lima et al., 2011), linked as causative ingredient of spoilage episodes (Prevost et al., 2010) and foodborne disease outbreaks (te Giffel et al., 1996) of thermally processed foods.

Cocoa are subjected to several steps throughout processing, which are highly relevant for the quality of the final product (cocoa powder) and its further applications. In addition to being important for the development of sensory and quality characteristics of cocoa, fermentation, drying and roasting are also known to influence on the microbiota of cocoa beans and "nibs" (crushed cocoa beans). Cocoa beans are fermented for up to seven days by a complex microbial community made of the native cocoa microbiota and of the microorganisms present in the environment of processing facilities (e.g., soil, equipment, workers) (Nielsen et al., 2007; Romanens et al., 2019; Schwan & Wheals, 2004; Serra et al., 2019; Thompson, Miller, Lopez, & Camu, 2013). Moisture in fermented beans is lowered from 40-50% to 6–8% by solar drying or by forced air drying (ICMSF, 2011; Thompson et al., 2013). Dried cocoa

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beans or nibs are then roasted at temperatures between 110 °C and 140 °C for 10–60 min (Beckett, 2008). The duration of roasting depends on the harvesting period of the cocoa pods, on the moisture of cocoa beans or nibs, on the processing equipment and on the desired flavor of the final product (Schwan & Wheals, 2004). Roasting will also determine the microbiological quality of the final cocoa powder (ICMSF, 2005, pp. 467–479; Thompson et al., 2013).

The natural antimicrobial compounds of cocoa, the pH alterations during fermentation, the low water activity of dried cocoa beans and high temperature during roasting inactivate vegetative cells of microorganisms, whereas spore-forming bacteria are much less affected by processing operations. Spore-forming bacteria present in dry cocoa powder will then contaminate aqueous products that are thermally processed (Lima et al., 2012, 2011), which may result in large losses for the thermally processed beverages industries such as chocolate milk producers. Because of this, the awareness of the prevalence levels and concentration of spore-forming bacteria in cocoa powder is highly critical for the design of thermal processes applied to cocoa-based formulated foods. The accurate determination of the initial concentration of a target microorganism in raw material and its inactivation kinetics are key data to design an inactivation process (Pflug, 1999; Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017; Smelt & Brul, 2014).

Bacillus cereus and *Geobacillus stearothermophillus* stand out as the most relevant spore-forming bacteria posing food safety and microbiological stability issues, respectively, for cocoa powder formulated products subjected to thermal processing (Gabis, Langlois, & Rudnick, 1970; Lima et al., 2012, 2011). While *B. cereus* is the causative agent of emetic and diarrheal foodborne illness (Carlin & Nguyen The, 2013; Carrol et al., 2019; Lentz et al., 2018), *G. stearothermophilus* is a thermophilic bacterium the produces spores that are extremely heat resistant and is known to cause flat sour spoilage in low acid foods (Feeherry, Munsey, & Rowley, 1987; Kakagianni & Koutsoumanis, 2018; Kalogridou-Vassiliadou, 1992; Mazas, López, González, Bernardo, & Martín, 1997; Periago, Fernández, Ocio, & Martínez, 1998; Rigaux, André, Albert, & Carlin, 2014; Wells-Bennik et al., 2019).

Previous studies (Gabis et al., 1970; Lima et al., 2011, 2012) have reported on the presence of *B. cereus* and *G. stearothermophilus* in cocoa powder samples, however, the inactivation kinetics of these two microorganisms during roasting of cocoa beans and nibs has not been reported yet. Therefore, the current study aims at quantifying the impact of roasting of cocoa beans and nibs on the fate of *B. cereus* and *G. stearothermophillus* spores.

2. Material and methods

2.1. Strains and preparation of B. cereus and G. stearothermophilus spores suspensions

The study was conducted with separate five-strain cocktails of *B. cereus* and *G. stearothermophilus*. The sources, culture collection codes procedures for preparation of suspensions of spores of *B. cereus* and *G. stearothermophilus* are detailed in Pia et al. (2019). Briefly, the suspensions of spores of *B. cereus* and *G. stearothermophilus* were prepared after incubation of each separate strain on Nutrient agar (Kasvi, Curitiba, Brazil) containing manganese sulfate (5 mg/L), respectively, at 30 °C and 55 (Peña et al., 2014; Pflug, 1999). Then, harvesting took place after about 30 days (Spinelli, Sant'Ana, Pacheco-Sanchez, & Massaguer, 2010), following centrifugation and suspension washing for three times (Oteiza et al., 2014)and further resuspension in sterile distilled water of the obtained pellets (Oteiza et al., 2014; Peña et al., 2014). Heat shocks (*B. cereus:* 15 min at 70 °C; *G. stearothermophilus:* 10 min at 110 °C) were employed to determine the concentration of spores using Nutrient agar (Bennett, Tallent, & Hait, 2001, pp. 311–325; Olson & Sorrells, 2001). The suspensions of spores were stored separately under freezing and mixed using adequate volumes to reach the desired concentrations of the two bacteria in cocoa nibs and beans.

2.2. B. cereus and G. stearothermophilus spores inoculation in cocoa beans and nibs

Raw cocoa beans and nibs (fermented and dried) were donated by a Brazilian manufacturer of cocoa powder. *B. cereus* and *G. stearothermophilus* spore counts in the products used for experiments were 10^3 spores/g and 10 spores/g, respectively. This natural contamination is at least 1000-fold lower than the inoculated population and was therefore neglected. A total of 150 g of cocoa beans or nibs were placed in sterile stainless-steel trays (30 cm × 20 cm × 4 cm), two for cocoa beans and two for cocoa nibs. Then each tray containing cocoa beans or nibs was separately inoculated with the cocktails of *B. cereus* and *G. stearothermophilus* spores aiming at a final concentration of 10^6 spores per g. After homogenization with a sterile spatula, the trays containing inoculated cocoa beans and nibs were dried for 15 min in the laminar flow cabinet. Variations in spore counts of different samples of inoculated beans or nibs were lower than 0.2 $\log_{10}$ spores per g, this shows homogeneous distribution of spore inocula (data not shown). Preliminary experiments showed that water activity (a_w) of cocoa beans and nibs was not affected by this inoculation procedure (data not shown). After drying, 12 g portions were placed in open glass Petri dishes (65 mm diameter). The layer of beans and nibs on the glass Petri dishes was 7 mm and 5 mm, respectively. The procedure of preparation and inoculation of trays with spores of *B. cereus* and *G. stearothermophilus* was conducted twice on different days.

2.3. Simulation of cocoa beans and nibs roasting processes

The roasting process was simulated in a hot air sterilizer (Fanem, 315 SE, Brazil). The process was conducted at three temperatures (110 °C, 125 °C and 140 °C) with time varying from 70 to 600 min. This range of temperatures is applied by cocoa processing industries to obtain products with different sensory characteristics. During processing, the temperature was monitored with a DataLogger (Hydra série II, Fluke, Everett, USA), using a flexible copper / constantan thermocouple (TC; TTT-36 type; Omega, CT, USA) coupled to the top of the hot air sterilizer; the standard deviation of temperature measurement was lower than 2 °C. Time at the target temperature was taken as the process time.

Samples of 10 g of beans or nibs were collected at regular time intervals during roasting and were quickly placed into sterile bags and cooled on ice to approximately 30 °C. Samples were then homogenized with 90 ml sterile distilled water for enumeration of spores and for pH measurement using a pH-meter (Kasvi, K39–2014B, Curitiba, Brazil) (Adolfo Lutz Institute, 2008, p. 104). The a_w of roasted beans or nibs was measured with an AquaLab CX-2 a_w meter, (Decagon Devices, Washington, USA). The simulation of the roasting process of cocoa beans and nibs was repeated twice.

2.4. Enumeration of B. cereus and G. stearothermophilus spores after cocoa beans and nibs roasting

Ten g of each sample of cocoa beans and nibs were placed in a sterile bag, mixed with 90 mL of 0.1% peptone water (1 g Bacteriological Peptone [Oxoid, Basingstoke, UK] per liter) and homogenized for 1 min with a Stomacher® (Seward, BA7021, Worthing, UK) to enumerate surviving *B. cereus* and *G. stearothermophilus* spores. Homogenized samples inoculated with *B. cereus* were heat shocked at 70 °C for 15 min and 110 °C for 10 min for the ones inoculated with *G. stearothermophilus* using a water bath (Quimis, Q-334M-28, Diadema, Brazil) and an oil bath (Polystat, 12105-20, Chicago, USA), respectively. Subsequently, decimal dilutions were prepared in peptone water 0.1% and spread on duplicate plates of Mannitol Egg Yolk Polymyxin agar (MYP, Oxoid, Basingstoke, UK) to enumerate *B. cereus* surviving

spores. Surviving spores of *G. stearothermophilus* were enumerated using duplicate pour plates of Dextrose Tryptone agar (DTA) inoculated with 1 ml volumes of appropriate serial dilutions. DTA contains tryptone (10 g/L, Acumedia, Neogen Co., Lansing, USA), dextrose (5 g/L, Dinâmica, Diadema, Brazil), Bacteriological Agar (15 g/L, Oxoid, Basingstoke, UK) and 1% solution of Bromocresol purple in NaOH (4 mL/L, Dinâmica, Diadema, Brazil). The MYP plates were incubated at 30 °C for 24–48 h. After incubation, typical colonies (pink, round, flat, dry, surrounded by an opaque pink halo) were counted (Bennett et al., 2001, pp. 311–325). DTA plates were incubated at 55 °C for 48–72 h, and then typical colonies of *G. stearothermophilus* (round, dark and opaque center, usually surrounded by a yellow halo) were counted (Olson & Sorrells, 2001). The counts were expressed as $\log_{10}$ of spores per gram of cocoa beans or nibs.

2.5. Determination of B. cereus and G. stearothermophilus spores' inactivation kinetic parameters during roasting of cocoa beans and nibs

The numbers of spores of *B. cereus and G. stearothermophilus* that survived cocoa beans and nibs roasting processes at 110, 125 and 140 °C were plotted against time. Changes in spore counts with time were fitted with the non-linear cumulative function of the Weibull frequency distribution model (Equation (1)) (Mafart, Couvert, Gaillard, & Leguerinel, 2002):

$$\log_{10}N(t) = \log_{10}N_0 - \left(\frac{t}{\delta}\right)^p \quad (1)$$

Where $\log_{10}N(t)$ is the concentration of spores/g at time t (min); $\log_{10}N_0$ is the initial concentration of *B. cereus* or *G. stearothermophilus* as spores/g; t is the time of the process (min); δ is the time to the first decimal reduction (min), and p is a shape parameter (dimensionless). The values of δ and p were adjusted using the Microsoft® Excel® solver add-in. The time to 4-decimal reductions (t_4) of *B. cereus* and *G. stearothermophilus* spores was determined as follows (Equation (2)):

$$t_4 = \delta \times 4^{1/p} \quad (2)$$

2.6. Statistical analysis

All experiments were repeated twice with each spore cocktail. Results were subjected to ANOVA to test the effect of temperature and type of cocoa product (bean or nibs) on δ and t_4. Multiple comparisons of means were performed with the Scott-Knott test at a P-value < 0.05 using the Assistat software (Campina Grande, Brazil).

3. Results

The pH of cocoa products decreased during roasting from 5.66 to 5.72 to 5.24–5.31 for beans, and from 5.60 to 5.72 to 5.22–5.35 for nibs (Table 1). The a_w value also decreased during roasting, from 0.687 to 0.753 to 0.429–0.528 for beans, from 0.710 to 0.731 to 0.355–0.442 for nibs.

The inactivation curves of *B. cereus* and *G. stearothermophilus* on nibs and beans during roasting are shown in Fig. 1. All curves showed a slight deflection from linearity, and p-values varied between 0.92 and 1.36 (Table 2). The inactivation model satisfactorily fitted the observed data. RMSE values were lower than 0.5 in most instances. In cocoa beans, the initial *B. cereus* spore population of 6.3 $\log_{10}$ spore/g decreased to 1.7 $\log_{10}$ spore/g after 420 min at 110 °C, to 2.7 $\log_{10}$ spore/g after 140 min at 125 °C and to 2.0 $\log_{10}$ spore/g after 70 min at 140 °C. In nibs, the initial *B. cereus* spore population decreased to 2.2 $\log_{10}$ spore/g after 315 min at 110 °C, 2.1 $\log_{10}$ spore/g after 115 min at 125 °C and 2.3 $\log_{10}$ spore/g after 70 min at 140 °C. In cocoa beans, the initial *G. stearothermophilus* spore population of 6.5 $\log_{10}$ spore/g decreased to 1.7 $\log_{10}$ spore/g after 600 min at 110 °C, to 1.4 $\log_{10}$ spore/g after 280 min at 125 °C and to 1.2 $\log_{10}$ spore/g after 180 min at 140 °C. In nibs, the initial *G. stearothermophilus* spore population decreased to 2.0 $\log_{10}$ spore/g after 495 min at 110 °C, 1.5 $\log_{10}$ spore/g after 240 min at 125 °C and 1.7 $\log_{10}$ spore/g after 140 min at 140 °C. The inactivation at 140 °C was significantly ($P < 0.05$) more rapid than at 125 °C, and at 125 °C more rapid than at 110 °C, irrespective of the matrix (beans or nibs) or the species (*B. cereus* and *G. stearothermophilus*).

The δ-values (time to the first decimal reduction) and t_4-values were slightly higher in cocoa beans than in nibs at the three tested temperatures for both microorganisms (Table 1). The δ-values for *B. cereus* in cocoa beans at 110, 125 and 140 °C were (mean ± sd) 148.5 ± 1. 7 min, 55.9 ± 6.5 min, and 23.3 ± 1.4 min and t_4-values at 110, 125 and 140 °C were 412.5 ± 4.6 min, 155.3 ± 18.1 min, and 64.6 ± 3.9 min, respectively. The δ-values for *B. cereus* in cocoa nibs at 110, 125 and 140 °C were (mean ± sd) 92.2 ± 0.9 min, 30.5 ± 0.6 min, and 17.2 ± 1.9 min and t_4-values at 110, 125 and 140 °C were 331.2 ± 4.8 min, 109.6 ± 2.0 min, and 61.7 ± 6.7 min, respectively.

The δ-values for *G. stearothermophilus* in cocoa beans at 110, 125 and 140 °C were 183.6 ± 2.1 min, 76.2 ± 1.10 min, and 49.1 ± 0.25 min. The t_4-values were 572.1 ± 6.5 min, 237.5 ± 3.4 min, and 153.0 ± 0.8 min. The δ-values for *G. stearothermophilus* in cocoa nibs at 110, 125 and 140 °C were 105.0 ± 0.05 min, 39.6 ± 0.75 min, and 30.4 ± 2.04 min. The t_4-values were 477.2 ± 0.24 min, 180.0 ± 3.4 min, and 138.1 ± 9.3 min δ or t_4 values of both *B. cereus* and *G. stearothermophilus* increased by 3–5 times with a 30 °C decrease in temperature (from 140 °C to 110 °C).

4. Discussion

The control of microbiological contaminants of foods is shifting towards a risk-based approach (Kakagianni & Koutsoumanis, 2018; Membré & Boué, 2018). Risk-based approaches have been employed aiming to protect public health (Messens et al., 2018), but they can also be applied aiming to reduce or prevent losses caused by spoilage

Table 1
Changes in pH and aw of cocoa beans and nibs during roasting[a] at a range of temperature.

Cocoa product	Temperature (°C)	pH		a_w	
		Before roasting	After roasting	Before roasting	After roasting
Beans	110	5.72 ± 0.08[b]	5.29 ± 0.06	0.692 ± 0.01	0.429 ± 0.01
	125	5.69 ± 0.11	5.31 ± 0.13	0.687 ± 0.01	0.528 ± 0.01
	140	5.66 ± 0.15	5.24 (± 0.10)	0.753 (± 0.10)	0.458 (± 0.04)
Nibs	110	5.72 ± 0.04	5.22 (± 0.10)	0.710 ± 0.01	0.422 ± 0.01
	125	5.60 ± 0.02	5.35 ± 0.04	0.731 ± 0.01	0.442 ± 0.01
	140	5.67 ± 0.08	5.24 ± 0.12	0.717 ± 0.02	0.355 ± 0.10

[a] Cocoa beans and nibs were inoculated with *B. cereus* or *G. stearothermophilus* spores.
[b] Mean ± standard deviation (n = 4).

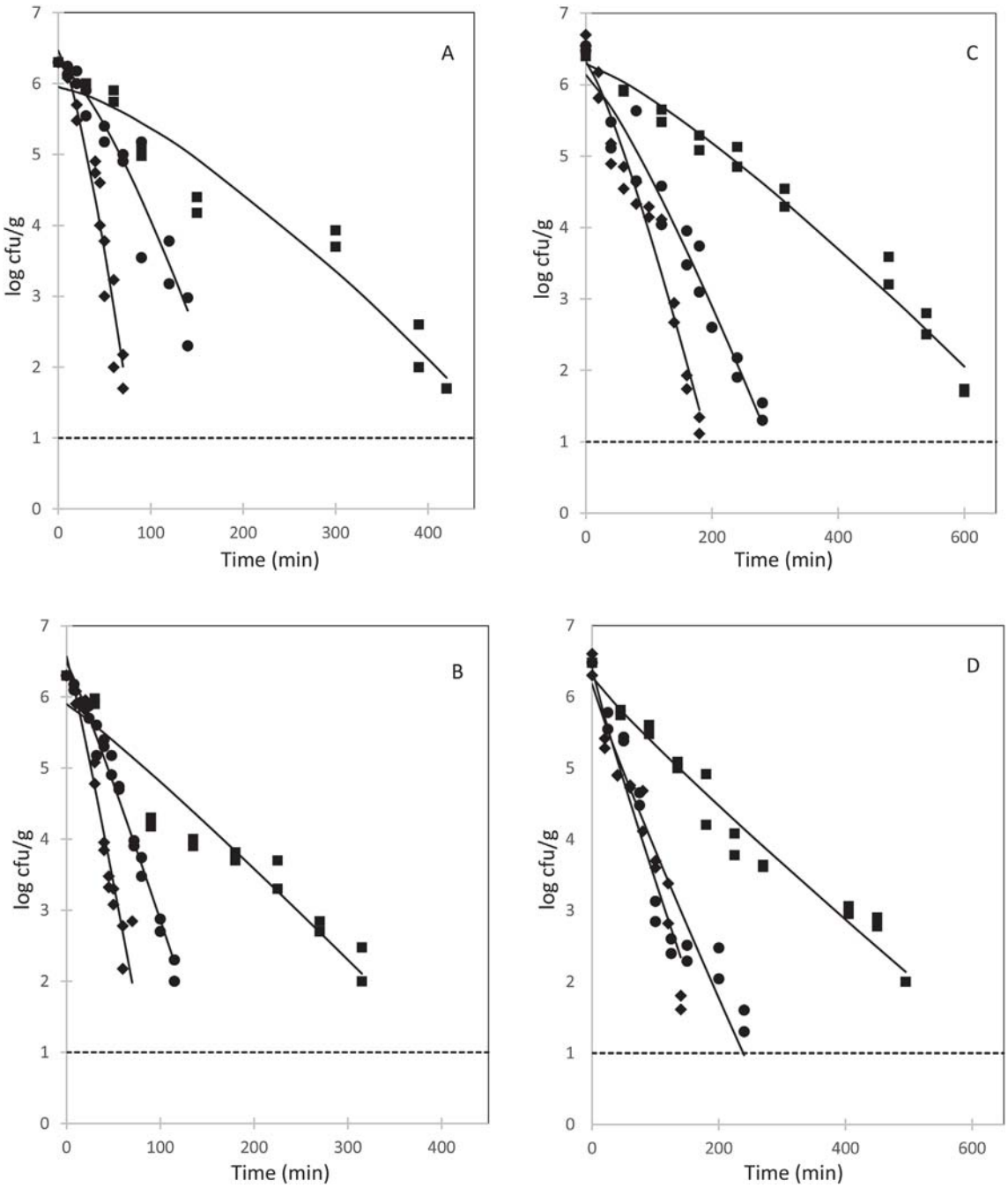

Fig. 1. Inactivation of a five-strain cocktail of *B. cereus* spores in cocoa beans (A), and in cocoa nibs (B), and of a five-strain cocktail of *G. stearothermophilus* spores in cocoa beans (C) and in cocoa nibs (D) during roasting at 110 °C (■), 125 °C (●) and 140 °C (♦). Symbols correspond to independent experimental data. The full lines correspond to the fitting of the data to the model of Mafart et al. (2002). The dotted line shows the limit of detection. See Materials and Methods for details.

microorganisms (Kakagianni & Koutsoumanis, 2018).). As a result, the interest and use of tools that allow the quantification of the effects of processing practices and unit operations towards microorganisms has grown (Alvarenga et al., 2018b; Cadavez et al., 2019; Kakagianni, Gougouli, & Koutsoumanis, 2016; Makariti et al., 2019; Pia et al., 2019). In this study, the cocoa roasting step was simulated and its impact on spores of both *B. cereus* and *G. stearothermophilus* were quantified. Several studies have been conducted to estimate the inactivation kinetics of *B. cereus* and *G. stearothermophilus* in a variety of substrates (Byrne, Dunne, & Bolton, 2006; Daryaei, Balasubramaniam, & Legan, 2013; Guérin, Dargaignaratz, Clavel, Broussolle, & Nguyen-the, 2017; Rajkovic et al., 2008; Ramaswamy, Xu, & Vatankhah, 2019; Wells-Bennik et al., 2019), however, their inactivation at low a_w conditions has been barely reported (Alvarenga et al., 2018). Determining the inactivation kinetics of these sporeforming bacteria during roasting of cocoa beans and nibs, which involves the employment of high temperatures and a_w reduction is key for proper processing design and assessment in terms of microbial destruction.

As shown in Table 1, the decrease in a_w from approx. 0.7 to approx. 0.4 during roasting is due to an additional reduction of water content and will ensure a greater stability of roasted cocoa beans or nibs over a long storage period in preventing bacterial spore germination and further growth. On the other hand, this a_w reduction during roasting will negatively affect heat transfer and further lead to a higher heat tolerance of bacterial spores (Syamaladevi et al., 2016). Roasting comprises a key step during processing for cocoa flavour development. A series of

Table 2
Time for the first decimal reduction δ, time for 4-decimal reductions (t_4) of *B. cereus* and *G. stearothermophilus* spores during roasting of cocoa beans and nibs [a,b].

SpSpecies	Temperature (°C)	Cocoa nibs				Cocoa beans			
		δ (min)[c]	Shape parameter (p)[e]	t_4 (min)[c]	RMSE[d]	δ (min)[c]	Shape parameter (p)[e]	t_4 (min)[c]	RMSE[d]
B. cereus	110	92.2 ± 0.9 aA	1.08	331 ± 4.8 aC	0.375	149 ± 1.7 aB	1.36	413 ± 4.6 aD	0.370
	125	30.5 ± 0.6 bA	1.08	111 ± 2.0 bB	0.155	56 ± 6.5 bA	1.36	155 ± 18 bB	0.227
	140	17.2 ± 1.9 cA	1.08	62 ± 6.7 cB	0.297	23 ± 1.4 cA	1.36	64.6 ± 3.9 cB	0.342
G. stearothermophilus	110	105 ± 0.1 dA	0.92	477 ± 0.2 dC	0.241	184 ± 2.1 dB	1.22	572 ± 6.5 dD	0.194
	125	39.6 ± 0.75 eA	0.92	180 ± 3.4 eC	0.527	76 ± 1.1 eB	1.22	238 ± 3.4 eD	0.329
	140	30 ± 2.0 fA	0.92	138 ± 9.3 fC	0.358	49 ± 0.3 fB	1.22	153 ± 0.8 fC	0.366

[a] For each species, different lowercase letters in the same column indicate significant difference at $P < 0.05$ of δ and t_4 as affected by temperature (Scott-Knott test).
[b] For each species, different capital letters in the same column indicate significant difference at $P < 0.05$ of δ and t_4 obtained in cocoa nibs and beans (Scott-Knott test).
[c] Mean ± mean absolute deviation, n = 2.
[d] Root mean square error.
[e] Unique shape parameter p for each species and each matrix.

precursor compounds are formed during cocoa beans fermentation and further interact during roasting (Andruszkiewicz, D'Souza, Altun, Corno, & Kuhnert, 2019; Hamdouche et al., 2019). During roasting, a_w and acidity of cocoa beans is reduced (Agus, Mohamad, & Hussain, 2018; Oracz et al., 2019), which also impacts on cocoa sensory features (Andruszkiewicz et al., 2019; Hamdouche et al., 2019; Oracz et al., 2019). As the degree of changes that take place during roasting is mainly dependent on temperature and time (Hinneh et al., 2019), all the desirable properties of cocoa beans such as colour, texture, acidity, amount of flavour compounds and fat, may be obtained by adjusting roasting conditions. However, the higher the temperature the higher the thermal-induced hydrolysis of triglycerides, which may lead to pH reduction (Tenyang et al., 2017). Other changes occurring during this step comprise the inactivation of enzymes that can lead to cocoa butter lypolysis, the development of typical chocolate aroma and colour, and dehydration which may favour stability (Pezoa, 1989; Rocha, Santana, Soares, & Bispo, 2017; Suazo, Davidov-Pardo, & Arozarena, 2014; Żyżelewicz et al., 2016). Despite the fact that *Salmonella* inactivation is should be achieved during cocoa roasting (ICMSF, 2002), the concerns with other microorganisms, such as spore-forming bacteria seem to be of minor relevance, and therefore they are not frequently considered when selecting roasting conditions. Cocoa beans are commonly roasted (Rocha et al., 2017), but in some cases, cocoa nibs are used instead, due to demand for energetic efficiency and better standardization of the obtained product (Hii, Menon, Chiang, & Sharif, 2017; Żyżelewicz et al., 2016).

As shown in Table 2, the inactivation in beans of spores of both *B. cereus* and *G. stearothermophilus* required a longer time than in nibs. This may be due to a greater contact surface of nibs than in beans providing a more efficient heat transfer. Roasting processes in industry operates between 110 °C and 140 °C and last 10 min–60 min (Beckett, 2008), which is much shorter than tested in the current study. Reduction of spores of *G. stearothermophilus* after 60 min roasting was less than 2 $\log_{10}$ and commonly close to 1 $\log_{10}$ at 110 °C and 125 °C. Reduction of spores of *B. cereus* after 60 min roasting was approximately 3 $\log_{10}$ at 140 °C and 1 $\log_{10}$ at 110 °C and 125 °C. This suggests that roasting in the industrial process at temperatures of 110 °C and 125 °C contributes to limited spore inactivation. The natural contamination of unroasted cocoa beans or nibs has been estimated at 6.3 log cfu/g for spores of *B. cereus*, at 3.0 log cfu/g for thermophilic aerobic spores (Pereira et al., unpublished data), and at 4.3–5.5 log spores per g for total spores (Lima et al., 2012). This mean that spore-forming bacteria will persist after roasting and enter at low levels further processing steps.

Extending the duration of the roasting process is difficult to achieve, as this may alter sensory characteristics, flavour and texture of cocoa (Barrile, Ostovar, & Keeney, 1971; Żyżelewicz et al., 2016; Suazo et al., 2014, Rocha et al., 2017). The partial reduction in spore counts during the roasting process was previously observed (Lima et al., 2012). These authors reported from 1 to 2 log reductions of spore-forming bacteria naturally present in alkalized cocoa nibs during roasting, even though the roasting conditions were not clearly indicated (105 °C and 140 °C). . Barrile et al. (1971) reported a 2 to 4 $\log_{10}$ reduction in total counts during roasting at 150 °C for 40 min; only spore-forming bacteria were isolated from roasted bean samples, and *G. stearothermophilus* and *B. coagulans* were the predominant species.

The particular pattern of inactivation of spores of *B. cereus* and *G. stearothermophilus* observed in the present work is certainly due to the low a_w of cocoa beans and nibs all along the roasting process. The low a_w of cocoa beans and nibs has a profound effect on the thermostability of the spores. Firstly, heat-resistance parameters were relatively poorly affected by the increase in temperature. The δ and t_4 values decreased by 3–5 times with a 30 °C increase in temperature. In an aqueous environment, the decrease would have been by 20–40 times considering z-values (the increase of temperature, in °C, needed, so a tenfold reduction in decimal reduction times is achieved) in the range 7 °C–13 °C (den Besten, Wells-Bennik, & Zwietering, 2018; Nicholson et al., 2000). A relatively low influence of temperature changes on heat-resistance parameters of spore-forming bacteria has previously been reported (Ababouch & Busta, 1987; Alderton & Snell, 1969). Secondly, heat-resistance parameters in cocoa beans and nibs observed for both species tremendously exceed the values reported in an aqueous environment (ICMSF, 1996; Luu-Thi, Khadka, & Michiels, 2014; Rigaux, Denis, Albert, & Carlin, 2013). den Besten et al. (2018) also reported that the survival of spores of *B. cereus* was higher in oily products than in wet foods. For instance, decimal reduction time of *G. stearothermophilus* at 125 °C does not exceed a few minutes and a few seconds at 140 °C (Rigaux et al., 2013; den Besten et al., 2018). Thirdly, differences in resistance between *B. cereus* and *G. stearothermophilus* during a roasting process are only marginal, while their difference in resistance to wet heat is huge (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013; Sadiq et al., 2016). More generally, differences in resistance to dry heat between species of spore-forming bacteria, in particular between thermophilic, mesophilic and psychrotrophic species are greatly reduced, when compared to the differences in resistance to wet heat (Murrell & Scott, 1966).

In conclusion, the roasting process may not promote a relevant reduction in populations of spore-forming bacteria despite relatively long process times at high temperatures. The use of high temperatures (140 °C) during roasting and of cocoa beans and nibs with low population of spore-forming bacteria can improve the efficiency of the roasting process in terms of microbial inactivation, which should also be a concern for cocoa processors. Populations of spore-forming bacteria not inactivated by roasting may remain at further processing steps

and potentially affect microbiological quality and safety of thermally processed cocoa-based products.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lwt.2019.05.063.

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CONSIDERAÇÕES FINAIS

DISCUSSÃO

No Brasil, há poucos estudos a respeito de bactérias esporuladas no processamento do cacau brasileiro. Desta forma, foi realizado um estudo grande sobre diferentes grupos de bactérias esporuladas (mesófilos aeróbios, mesófilos anaeróbios, termófilos aeróbios e *B. cereus*), tanto no pre-processamento realizado nas fazendas, quanto no processamento industrial.

A contaminação das sementes que ocorre nas fazendas é essencial pois parte dela atua na fermentação natural do cacau (Nascimento, et al., 2010). Porém, as condições em que as sementes são manipuladas pelos trabalhadores levam a contaminação por micro-organismos que podem prejudicar a qualidade e segurança dos produtos.

Foi observada que populações podem sobreviver ao processamento, principalmente populações de bactérias esporuladas mesófilas aeróbias e de *B. cereus*. É importante destacar que muitas espécies no gênero *Bacillus* apresentam poder de deterioração (Ehling-schulz & Messelhäusser, 2013; Lucking et al., 2013). Embora que as cepas de *B. cereus* pertencentes ao grupo filogenético III, conhecido por carregar o gene *ces* responsável pela sintetização da toxina cereulide, não tenha apresentado a presença deste gene, a sua presença no cacau em pó é preocupante devido ao poder que estas podem ter de provocar também a síndrome diarréica (Fogele et al., 2018). A maioria das cepas de *B. cereus* estudadas pertencem ao grupo filogenético IV, caracterizado por cepas mesófilas, o que reflete o fato de serem cepas isoladas do cacau brasileiro, um país tropical.

O impacto da fermentação natural do cacau sobre cepas de *B. cereus* e *G. stearothermophilus* foi estudado. A fermentação não forneceu condições de crescimento para estas bactérias, tampouco de redução. Porém, devido a sua resistência, esporos presentes no final do processo de fermentação podem sobreviver a etapas do processamento industrial, como a torrefação e alcalinização. A população de bactérias esporuladas não afetou o desenvolvimento dos micro-organismos fermentadores. Porém bactérias esporuladas podem alterar a produção de metabólitos que podem afetar negaticamente a qualidade do produto final produzido com tal matéria prima, como o chocolate. Bactérias aeróbias esporuladas podem produzir compostos que contribuem para a formação de off-flavors na amêndoa fermentada (Schwan and Wheaks, 2004).

O impacto da torrefação também foi estudado, aplicando diferentes temperaturas em nibs e amêndoas. O processo de torra pode promover uma redução da população de esporos, porém em altas temperaturas e em um processo relativamente longo, o que nem sempre é ideal para a qualidade sensorial do produto final. O uso de uma temperatura mais alta no processo de

torrefação (140°C) pode ser eficaz na inativação de bactérias esporuladas durante o tempo real do processo, mas é importante que a matéria prima chegue na indústria com baixo nível de contaminação. A população de esporos sobreviventes a torrefação pode permanecer nas etapas de processamento adicionais e afetar a qualidade microbiológica e a segurança dos produtos à base de cacau.

A presença de micro-organismos esporulados em matérias primas como o cacau em pó, não deve ser ignorada, pois estes apresentam um risco a estabilidade dos produtos formulados. Estes micro-organismos ao serem introduzidos no processo, porem formar biofilme e futuramente contaminar o alimento. O conhecimento sobre a diversidade de bactérias esporuladas ao longo da cadeia de processamento do cacau em pó e no produto final é critico para estabelecer medidas de controle efeticas para garantir a segurança e estabilidade microbiana (Byrer et al., 2000).

CONCLUSÃO GERAL

O estudo sobre a população de bactérias esporuladas presente no pre processamento proveniente das fazendas e do processamento industrial mostrou que há uma grande contaminação das sementes durante as etapas de fermentação e secagem, principalmente devido as condições higiênicas precárias aplicadas. Tal contaminação é levada para a indústria, e pode persistir ao longo do processamento chegando ao cacau em pó.

Etapas do processamento industrial onde se aplicam altas temperaturas, como a torrefação, pode permitir a sobrevivência de esporos de *B. cereus* e *G. stearothermophilus.* Desta forma, medidas de controle para se garantir uma matéria prima de melhor qualidade se torna necessário.

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ANEXOS

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