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Sporeforming bacteria in beer: Occurrence, diversity, presence of hop resistance genes and fate in alcohol-free and lager beers

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ABSTRACT

The aim of this study was to assess the occurrence of sporeforming bacteria in different types of beers (n = 163) and to assess the presence of *hor* genes in the isolates. Additionally, the study aimed to evaluate the fate of five representative sporeforming bacteria harboring *horA* and *horC* genes in alcohol-free and lager beers. Two hundred and sixty (n = 260) sporeforming bacteria belonging to eight different genera were isolated from beers, i.e., *Bacillus* (n = 118), *Paenibacillus* (n = 89) and *Brevibacillus* (n = 41), *Lysinbacillus* (n = 6), *Cohnella* (n = 3), *Rummeliibacillus* (n = 1), *Alicyclobacillus* (n = 1), and *Anoxybacillus* (n = 1), respectively. A predominance of members within the *Bacillus cereus* sensu lato (n = 72; 27.1%), followed by *B. megaterium* (n = 18; 7%), *P. validus* (n = 16; 6.1%), *P. humicus* (n = 13; 5%), *P. alginolyticus* (n = 13; 5%) and *Br. brevis* (n = 13; 5%) was observed in beer samples analyzed. Only 5% (n = 14) out of 260 sporeforming bacterial isolates recovered from beers harbored one or both *horA* and *horC* genes. Only one (0.3%) isolate, i.e., *Bacillus cereus* sensu lato (identified as *B. thuringiensis* LMQA 206) presented both *horA* and *horC* genes. None of the five bacterial sporeforming strains harboring *horA* or *horC* genes inoculated was able to grow in the beers throughout the storage period studied, and no spoilage was detected. The results of this study indicated a widespread occurrence of sporeforming bacteria in several types of beers from different brands, highlighting that measures should be taken to reduce the occurrence of sporeforming bacteria considering stability and safety concerns.

1. Introduction

Beer is the oldest and most broadly consumed alcoholic beverage worldwide. Depending on the type and country, a variety of grains such as wheat, barley, oats, rice, corn, etc. might be used for beer production. The process of beer manufacture (brewing) involves the following main steps: malting, milling, mashing and wort processing (separation, boiling, clarification, cooling and aeration), fermentation, maturation, beer clarification, pasteurization and packaging (Willaert, 2007).

During the production of beer, physical and chemical changes occur that result in the inhibition of several microorganisms (Hough, Briggs, Stevens, & Young, 1982). Even though this enhances the microbiological stability of beers, wild yeasts, lactic acid bacteria (*Pediococcus* and *Lactobacillus*), acetic acid bacteria, *Zymomonas* spp., *Pectinatus* spp., *Megasphaera* spp., and several *Enterobacteriaceae*, are prevalent in the brewing environment and can cause spoilage problems either during early or later stages of processing (Sakamoto & Konings, 2003; Suzuki, Sami, Iijima, Ozaki, & Yamashita, 2006). These Gram-positive and Gram-negative microorganisms share common characteristics that allow them to grow and spoil beer. These characteristics include hop and ethanol resistance, ability to grow in the presence of carbon dioxide and under anaerobic conditions (Sakamoto & Konings, 2003; Suzuki et al., 2006; Vaughan, O'Sullivan, & Sinderen, 2005). Among these inhibitory factors, hop resistance seems to be key in driving the microbial ecology of beers. Hop resistance is associated with the presence of *horA* and *horC* genes in microorganisms able to grow in beer (Suzuki, Iijima, Ozaki, & Yamashita, 2005).

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The formation of off-flavors, production of diacetyl, hazy appearance and turbidity comprise the main changes caused in beers by the mentioned spoilage microorganisms (Sakamoto & Konings, 2003; Suzuki et al., 2006; Vaughan et al., 2005). The temperature conditions applied during beer processing (ca. 60°C/20min) should result in the inactivation of vegetative cells of bacteria. Nonetheless, their frequent association with beer spoilage could be explained by recontamination due to their widespread dissemination in the brewing environment (Sakamoto & Konings, 2003; Spitaels et al., 2015, 2014; Suzuki et al., 2006; Vaughan et al., 2005).

If on the one hand the role of wild yeasts, acid-producing bacteria, *Enterobacteriaceae* and strictly anaerobic bacteria in the microbial ecology of beer seems to be well-established (Bokulich & Bamforth, 2013), on the other hand, the occurrence and role of sporeforming bacteria in beers are not well documented. Bacterial spores are known for their chemical and physical resistances, which explain their extensive prevalence in raw materials and processed foods (Wells-Bennik et al., 2016). Regardless of this, there is limited information on the occurrence and fate of sporeforming bacteria in beers (Haakensen & Ziola, 2008; Jeon et al., 2015). Thus, it becomes clear that the knowledge of the occurrence and fate of sporeforming bacteria in beers comprise key information for the development of effective measures to ensure specific quality standards as well as the microbiological stability of this beverage during shelf-life. Then, the aim of this study was to assess the occurrence, diversity of sporeforming bacteria in different types of beers and to assess the presence of *hor* genes in the isolates. Finally, the study also aimed to determine the fate of sporeforming bacteria harboring *hor* genes during storage of three types of beers.

2. Material and methods

2.1. Beer sampling

Beer samples (n = 163) were acquired from supermarkets in Campinas-SP, Brazil. Beer samples collected were packaged in bottles of 250 and 355 mL and cans of 269 and 550 mL and belonged to 3 different classes (blond, brown and alcohol-free) of the most consumed brands (A to W) in Brazil (Table 1). All collected samples were within the shelf life period and were kept at room temperature until analysis. The pH of the beer samples was measured using a potentiometer (AK103, AKSO, São Leopoldo, Brazil) once the packages were opened under aseptic conditions. The alcoholic content of each sample was recorded from the labels.

2.2. Isolation of sporeforming bacteria

A total of 100 mL of beers and five mL of brown beers were submitted to a heat shock at 80°C/30 min to eliminate vegetative cells and allow spores to germinate further when plated in the culture medium (Stevenson & Lembke, 2015). A volume of 5 mL of brown beer samples was filtered because larger volumes led to membrane clogging. Further, after cooling down in an ice bath, the heat shocked samples were aseptically filtered through 0.45 μm cellulose nitrate membranes (Sartorius Stedim Biotech, GmbH, 37070, Goettingen, Germany). Then, membranes were placed onto Tryptone Glucose Extract Agar (TGE) plates (5 g/L casein peptone, 3 g/L meat extract, 1 g/L glucose, and 15 g/L agar), following incubation at 37°C/48 h. After incubation, plates presenting colonies were separated, and up to five colonies with different morphologies were isolated and submitted to Gram staining. Isolates were stored on TGE broth with glycerol (25% v/v) at −20 °C. All the ingredients used to prepare TGE were from Difco (Becton, Dickinson & Company, Sparks, USA).

2.3. Molecular identification of sporeforming bacteria isolated from beers

The DNA was extracted by boiling the pellets originated from the mixture of three colonies of each sporeforming bacteria isolated from beers with 300 μL of ultrapure water. The 16S ribosomal RNA gene was amplified by PCR using primers such as 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1100R (5′-AGGGTTGCGCTCGTTG-3′) (Turner et al., 1999). Each PCR mixture contained two μL of sample DNA, 1.5 U of Taq DNA Polymerase (Vivantis Technologies Sdn. Bhd., Malaysia), 0.25 mM dNTPs, 0.1 mM of each primer, 1× viBuffer A of Taq polymerase kit, and 2.5 mM of $MgCl_2$, totalizing 25 μL. PCR reaction was performed in a PTC-200 thermal cycler (Bio-Rad < Hercules, USA) with the following settings: 94 °C for 6 min, followed by 30 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and 72 °C for 3 min. PCR products (~20 μL) were purified with the kit Wizard® SV Gel and PCR Clean-up System (Promega, Madison, USA), according to manufacturer's instructions. DNA sequencing was performed in the Central Laboratory of High-Performance Technologies in Life Sciences (LaCTAD) of University of Campinas (UNICAMP, Campinas-SP, Brazil), using the sequencer 3730xL DNA Analyzer (Applied Biosystems®, Foster City, USA). Edition and alignment of the obtained sequences were performed using the BioEdit software (Ibis Biosciences, Carlsbad, USA). The treated sequences were blasted with NCBIdatabase of non-redundant nucleotides (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the best identity results were considered for identification (e-value < 10^{-5}).

2.4. Detection of horA and horC genes in sporeforming bacteria isolated from beer samples

A PCR reaction was performed in the DNA of the isolated sporeforming bacteria using the primers: forward *horA* (5′-GGGTTATTTTGACCAAACC-3′), reverse *horA* (5′-CATGATGAGCATTAAGACCA-3′) (Sami et al., 1997; Suzuki, Koyanagi, & Yamashita, 2004); and forward *horC* (5′-GGGTTATTTTGACCAAACC-3′), reverse *horC* (5′-GGGCGAACCGTGAACAAATAG-3′) (Suzuki et al., 2005). The mixture and conditions of PCR reaction were the same as described for the amplification of 16S rRNA gene. PCR products (10 μL), including positive and negative controls, underwent to an electrophoresis on a 1% agarose gel in 1× TAE buffer (pH 8.0), and the DNA amplicons were visualized by UV illumination after ethidium bromide staining. The strains were identified as positive for the genes *horA* and *horC* when they presented the amplicon size corresponding to the positive control.

2.5. Fate of Bacillus cereus sensu lato (LMQA 141, LMQA 206, and LMQA 332), B. pumilus LMQA 176, and Brevibacillus invocatus LMQA 291 in beers

2.5.1. Preparation of spore suspension

The suspensions of Espores of strains harboring *horA* and *horC* genes were prepared as previously described (Peña et al., 2014; Spinelli, Sant'Ana, Pacheco-Sanchez, & Massaguer, 2010; Spinelli, Sant'Ana, Rodrigues, & Massaguer, 2009) with some modifications. The strains of *Bacillus cereus* sensu lato (LMQA 141, LMQA 206, and LMQA 332), *B. pumilus* LMQA 176 and *Br. invocatus* LMQA 291 were inoculated in Petri dishes containing TGE agar formulated with the addition of 25% of deaerated Pilsen beer. Pilsen beer was added to the culture medium formulation as preliminary experiments indicated that the sporulation process of these strains only occurred in the presence of beer (data not shown). Petri dishes inoculated with each microorganism were incubated at 30 °C for 30 days, and after this period, the spores were harvested and centrifuged at 3000 × g

Table 1
Number of isolates (per genus) of sporeforming bacteria recovered from different beer brands analyzed.

Brands (n[a])	Number of isolates								Total
	Alicyclobacillus	*Anoxybacillus*	*Bacillus*	*Brevibacillus*	*Cohnella*	*Lysinibacillus*	*Paenibacillus*	*Rummeliibacillus*	
A (7)			9	1			2		12
B (9)			4	1			7		12
C (7)			7	2			5		14
D (22)			19	4	1	1	7		32
E (6)			1	4			1		6
F (3)			2	3			1		6
G (1)			1						1
H (6)			4	4			7		15
I (2)				1			2		3
J (2)			5	3					8
K (1)							1		1
L (1)			2	1					3
M (5)			5	3			5		13
N (15)			8	2		2	3		15
O (4)			2	1	1		3		7
P (3)			4	1			5	1	11
Q (4)			5				3		8
R (1)			1						1
S (5)			6	1			1		8
T (14)			9	6	1		10		26
U (19)	1	1	20			3	16		41
V (3)				1			2		3
W (4)			4	2			8		14
Total	1	1	118	41	3	6	89	1	260

[a] Number of samples analyzed.

at 4 °C for 20 min (Sorvall ST16, ThermoFisher Scientific Inc., Waltham, USA). The concentration of spores in the suspensions was adjusted as previously described (Peña et al., 2014; Spinelli et al., 2010, 2009), following storage at −20 °C until use.

2.5.2. Beer samples

The fate of *Bacillus cereus* sensu lato (LMQA 141, LMQA 206, and LMQA 332), *B. pumilus* LMQA 176 and *Br. invocatus* LMQA 291 was studied in Premium American Lager (5% alcoholic content and pH 4.5), Standard American Lager (5% alcoholic content and pH 4.1) and non-alcoholic Standard American Lager (0–0.5% alcoholic content and pH 3.9) beers. Lager beer was chosen for these experiments as this is the main beer style consumed in Brazil. The beer brands selected were chosen based on previous experiments that indicated no occurrence of sporeforming bacteria. The bottles of beers were purchased in supermarkets from Campinas, Brazil. After purchase, beer bottles were maintained at room temperature (21 °C) until the experiments. The beer was previously assessed for the presence of sporeforming bacteria as described in section 2.2.

2.5.3. Beer inoculation, storage conditions and growth potential assessment

Before the inoculation with sporeforming bacteria, the bottles of beers were washed with neutral detergent and water, following disinfection with 70% ethanol. Under aseptic conditions, the caps were opened with the aid of disinfected bottle opener and a total of three bottles of each beer assessed were inoculated with suspension of spores of each strain to result in a final concentration of 10^3 spores/mL of beer. Then, the bottles were immediately closed with previously washed and ethanol 70%-disinfected beer bottle caps. Before inoculation and closure, suspensions of spores were subjected to a thermal shock at 60°C/20min aiming to simulate standard condition of beer pasteurization. Then, the bottles were gently homogenized and stored at 5 °C, 25 °C and 35 °C for 30 days. The temperature of 5 °C was used to represent refrigeration conditions that beers may be exposed during commercialization, while 25 °C and 35 °C represented environmental temperatures that beers can be exposed during transportation and storage. The enumeration of spores was performed in the inoculated beer at time zero (N_0) and at the end of the storage period (N_f). Vegetative cells were only enumerated at the end of the storage period to assess whether the spores were able to germinate and outgrow. For enumeration of spores, the samples were submitted to heat shock (80°C/30min), whereas for enumeration of vegetative cells, no-heat shock was applied. A volume of 0.1 mL of heat-shocked and non-heat shocked samples were inoculated onto TGE agar, following incubation at 30 °C/48 h. Also, the pH of the samples was measured at the beginning and end of the storage period. By calculating the difference between the final (end of storage period) and initial (beginning of storage period) counts, the growth potential (δ) was obtained (Anonymous, 2003; Jesus et al., 2016). Negative values of δ ($\leq$0.5 $\log_{10}$) indicated that the beer did not support the growth of the microorganism at the storage conditions studied (Anonymous, 2003; Jesus et al., 2016). The experiments were repeated twice and all analyzes were performed in duplicate.

2.6. Statistical analysis

A chi-square test was performed to analyze differences regarding the occurrence of sporeforming bacteria among the different types of beers. The chi-square test was conducted using Statgraphics Centurion XVII version 17.1.12 (Statpoint Technologies, Inc, Warrenton, VA, USA).

3. Results and discussion

3.1. Occurrence and diversity of sporeforming bacteria in different types of beers

In this study, a total of 23 beers brands of different styles has been collected and analyzed for the occurrence of sporeforming bacteria. These microorganisms were isolated from 144 out of 163 beer samples analyzed. A total of 260 sporeforming bacteria belonging to eight different genera were isolated (Table 1). Most

isolates belonged to the *Bacillus* (n = 118; 45.5%), *Paenibacillus* (n = 89; 34.3%) and *Brevibacillus* (n = 41; 15.7%), respectively (Table 1). The other genera isolated were *Lysinbacillus* (n = 6, 2.3%), *Cohnella* (n = 3, 1.2%), *Rummeliibacillus* (n = 1, 0.4%), *Alicyclobacillus* (n = 1, 0.4%), and *Anoxybacillus* (n = 1, 0.4%), respectively. These sporeforming bacteria are widespread in the environment, including geothermal sources, soil, feces and foods and may present relevance for agriculture, biotechnology, human and animal health (Ash, Priest, & Collins, 1994; Goh et al., 2014; Grady, MacDonald, Liu, Richman, & Yuan, 2016; Kämpfer, Rosselló-Mora, Falsen, Busse, & Tindall, 2006; Oteiza, Soto, Alvarenga, Sant'Ana, & Gianuzzi, 2015; Peña et al., 2014; Shida, Takagi, Kadowaki, & Komagata, 1996; Vaishampayan et al., 2009).

The identification of isolates indicated the predominance of members within the *Bacillus cereus* sensu lato (n = 72; 27.1%), followed by *B. megaterium* (n = 18; 7%), *P. validus* (n = 16; 6.1%), *P. humicus* (n = 13; 5%), *P. alginolyticus* (n = 13; 5%) and *Br. brevis* (n = 13; 5%), respectively (Table 2). Among the 118 isolates belonging to the *Bacillus* genus, 72 belongs to *Bacillus cereus* sensu lato that is composed by *B. cereus*, *B. anthracis*, *B. mycoides*, *B. weihenstephanensis*, and *B. pseudomycoides* (Okinaka & Keim, 2016). *B. cereus* and *B. anthracis* are well recognized as human pathogens (Stenfors Arnesen, Fagerlund, & Granum, 2008), and some strains of *B. cereus* have also been associated to food spoilage (Choma et al., 2000; Choudhery & Mikolajcik, 1971). *B. thuringiensis* is another important bacteria within the *Bacillus cereus* sensu lato group, which is also abundant in the environment. Despite this, the high occurrence of *Bacillus cereus* sensu lato group can be related to the use of *B. thuringiensis* insecticides in crops, such as maize and rice (Catarino, Ceddia, Areal, & Park, 2015), highly used as beer adjuncts in Brazil. For instance, the high prevalence of *B. thuringiensis* in some vegetables has been associated to the application of *B. thuringiensis* insecticides (Frederiksen, Rosenquist, Jørgensen, & Wilcks, 2006). In spite of this, whether the occurrence of *Bacillus cereus* sensu lato in beer is related to natural occurrence of microorganisms within this group in the environment (mainly soil) or due to agricultural application of *B. thuringiensis* insecticides remains an aspect to be further investigated. Nonetheless, this raises safety concerns due to presence of pathogenic bacteria within the *Bacillus cereus* sensu lato group (Frederiksen et al., 2006; Rosenquist, Smidt, Andersen, Jensen, & Wilcks, 2005). Due to their low genetic diversity (Daffonchio et al., 2003; Priest, Barker, Baillie, Holmes, & Maiden, 2004), all these microorganisms can be considered members of only one species and that is the reason why further differentiation of the isolates within this group was out of the scope of this study. The second most frequently *Bacillus* species isolated from beer analyzed was *B. megaterium*. *B. megaterium* has been described to cause the flat sour spoilage of acid products (Silva & Gibbs, 2004), even though this is not frequently reported (Eppinger et al., 2011). Finally, *Paenibacillus* has been described to present a very diverse metabolism (Heyndrickx, 2011; Lal & Tabacchioni, 2009; McSpadden, 2004; Montes, Mercadé, Bozal, & Guinea, 2004) and along with *Bacillus*, it is widely distributed in the environment, having direct and indirect contributions to promoting crop health (McSpadden, 2004).

As seen in Table 3, among the beer types, more isolates per sample analyzed were recovered from Sweet Stout, Premium American Lager, Munich Dunkel, alcohol-free, Malzbier and Standard American Lager, respectively. Considering this, statistical analysis were performed and indicated significant differences concerning the occurrence of sporeforming bacteria recovered from the different types of beers ($\chi^2 = 17.14$; df = 4; p = 0.002). These differences might be explained by the different processing conditions and ingredients used in beer formulations. For instance, some of these beers may contain adjuncts such as rice, rye, oats, wheat and corn (Meussdoerffer & Zarnkow, 2009; Riese & Eβlinger, 2009). Adjuncts are used in order to reduce costs of production or to provide low sharpness to some beers (Standard American Lager beers, for instance) (Meussdoerffer & Zarnkow, 2009; Riese & Eβlinger, 2009). Beer adjuncts, barley and malt are known to be contaminated by bacterial spores (Buehner, Anand, & Garcia, 2014; Choma et al., 2000; Noots, Delcour, & Michiels, 1999). In other types of beers, such as Malzbier and Sweet stout, sugar syrup and caramel and lactose/chocolate are added, respectively. These ingredients may carry a specific microbiota composed of sporeforming bacteria in view of their sources and processing conditions, which may also explain the recovery of sporeforming bacteria from beers. Even though the ingredients have the potential to be a highly important source of contamination, the water used in the brewing plants could also play a significant role as source of sporeforming bacteria (Galofre, Israel, Dellunde, & Ribas, 2004; Mazoua & Chauveheid, 2005). Therefore, proper treatment of water used in the brewing environment is also key to reduce the entrance of sporeforming bacteria to the brewing environment. Finally, the establishment of sporeforming bacteria in the brewing environment should be avoided by proper cleaning and sanitization programs. This is of chief relevance as sporeforming bacteria are well-known to form biofilms (Huang, McLandsborough, & Goddard, 2016; Peña et al., 2014; Tamachkiarow & Flemming, 2003), which may result in further contamination of beers.

3.2. Detection of horA and horC genes in sporeforming bacteria isolated from beer samples

All the 260 sporeforming bacteria isolated from the different beer types were tested for the presence of *horA* and *horC* genes. These genes have a fundamental importance on microbial hop resistance (Suzuki et al., 2006) which is required for survival and growth in beer. The iso-alpha-acids and their isomerized forms are present in hop and comprise a class of compounds of major relevance for the microbial stability of beers (Caballero, Agut, Armentia, & Blanco, 2009). These acids act as protonophores, i.e., they transport protons across lipid bilayers (membranes), which result in pH changes and decrease of proton motive force (pmf). As the pmf is altered, the absorption of nutrients is reduced and the cells die (Sakamoto & Konings, 2003). For survival and growth in the presence of hop, bacteria demand specific mechanisms to maintain the internal pH of the cells and so the pmf working properly (Kashket, 1987). The *horA* and *horC* genes encode transporters that pump toxic compounds (i.e., exogenous protons) out of the cells (Suzuki et al., 2005). Even though the genes *horA* and *horC* have been found mainly in lactic acid bacteria (Sakamoto & Konings, 2003; Suzuki et al., 2005), literature support their acquisition through horizontal transfer by other bacterial genera (Suzuki et al., 2006). For instance, the gene *horA* was found in *B. cereus*, *B. licheniformis*, *P. humicus* and *Staphylococcus epidermidis* isolated from artisanal beer (Haakensen & Ziola, 2008).

Within the 260 sporeforming bacterial isolates recovered from Brazilian beers, 5% (n = 14) carried one or both mentioned genes (Table 4). Amongst the 14 positive isolates for *horA* or *horC* genes, only one (0.3%), i.e., *Bacillus cereus* sensu lato (identified as *B. thuringiensis* LMQA 206) presented both *horA* and *horC* genes. As shown in Table 4, isolates positive for *horA* and *horC* genes belonged to *Bacillus* (n = 8), *Paenibacillus* (n = 5) and *Brevibacillus* (n = 1) genera, respectively. Species identified were *Bacillus cereus* sensu lato [*B. thuringiensis* (n = 4), *B. cereus* (n = 2)], *B. pumilus* (n = 1), *B. megaterium* (n = 1), *P. polymyxa* (n = 1), *P. validus* (n = 1), *P. alginolyticus* (n = 1), *P. ehimensis* (n = 1), *P. naphtalenovorans* (n = 1) and *Br. invocatus* (n = 1) (Table 4). These species were isolated from different beer types and brands, indicating the

Table 2
Sporeforming bacteria isolated from different brands and types of beers.

Brand	Type	Species	Number of isolates
A	Standard American Lager	*Bacillus cereus*	1
		Bacillus ginsengihumi	2
		Bacillus licheniformis	1
		Bacillus thuringiensis	3
		Bacillus thuringiensis serovar kurstaki	2
		Brevibacillus brevis	1
		Paenibacillus alginolyticus	1
		Paenibacillus validus	1
B	Standard American Lager	*Bacillus pumilus*	1
		Bacillus safensis	1
		Brevibacillus fluminis	1
		Paenibacillus aestuarii	2
		Paenibacillus polymyxa	1
		Paenibacillus sp.	2
		Paenibacillus validus	1
	Premium American Lager	*Bacillus pumilus*	2
		Paenibacillus cineris	1
C	Standard American Lager	*Bacillus amyloliquefaciens*	1
		Bacillus cereus	1
		Bacillus megaterium	1
		Bacillus thuringiensis	4
		Brevibacillus brevis	1
		Brevibacillus fluminis	1
		Paenibacillus aestuarii	1
		Paenibacillus alginolyticus	3
		Paenibacillus glycanilyticus	1
D	Standard American Lager	*Bacillus cereus*	2
		Bacillus coagulans	1
		Bacillus ginsengihumi	1
		Bacillus megaterium	2
		Bacillus niacini	1
		Bacillus subtilis subsp. *Subtilis*	2
		Bacillus thuringiensis	4
		Bacillus thuringiensis serovar kurstaki	2
		Brevibacillus brevis	1
		Brevibacillus choshinensis	1
		Brevibacterium sp.	1
		Cohnella formosensis	1
		Lysinibacillus sphaericus	1
	Malzbier	*Paenibacillus cineris*	1
		Paenibacillus flavisporus	1
		Paenibacillus validus	2
	Alcohol-free	*Bacillus megaterium*	1
		Bacillus pumilus	1
		Bacillus thuringiensis	2
		Brevibacillus fluminis	1
		Paenibacillus aestuarii	1
		Paenibacillus humicus	1
		Paenibacillus polymyxa	1
E	Standard American Lager	*Bacillus arbutinivorans*	1
		Brevibacillus agri	1
		Brevibacillus brevis	1
		Brevibacillus massiliensis	1
		Brevibacillus thermoruber	1
		Paenibacillus validus	1
F	Sweet Stout	*Bacillus licheniformis*	1
		Bacillus shackletonii	1
		Brevibacillus brevis	1
		Brevibacillus sp.	2
		Paenibacillus sp.	1
G	Standard American Lager	*Bacillus circulans*	1
H	Standard American Lager	*Bacillus thuringiensis*	1
		Brevibacillus brevis	4
		Paenibacillus glycanilyticus	1
	Malzbier	*Bacillus coagulans*	1
		Bacillus thuringiensis	1
		Paenibacillus glycanilyticus	1
		Paenibacillus humicus	1
	Alcohol-free	*Bacillus ehimensis*	1
		Paenibacillus ehimensis	2
		Paenibacillus humicus	1
		Paenibacillus sp.	1
I	Standard American Lager	*Brevibacillus limnophilus*	1
	American Brown Ale	*Paenibacillus humicus*	2

Table 2 (*continued*)

Brand	Type	Species	Number of isolates
J	Standard American Lager	*Bacillus licheniformis*	2
		Bacillus megaterium	1
		Bacillus subtilis	1
		Bacillus thuringiensis	1
		Brevibacillus agri	3
K	Standard American Lager	*Paenibacillus humicus*	1
L	Standard American Lager	*Bacillus subtilis*	2
		Brevibacillus borstelensis	1
M	Premium American Lager	*Bacillus cereus*	1
		Bacillus firmus	1
		Bacillus megaterium	1
		Bacillus subtilis	2
		Brevibacillus agri	2
		Brevibacillus invocatus	1
		Paenibacillus alginolyticus	1
		Paenibacillus humicus	1
		Paenibacillus naphtalenovorans	1
		Paenibacillus sp.	1
		Paenibacillus tarimensis	1
N	Standard American Lager	*Bacillus megaterium*	1
		Bacillus thuringiensis	2
		Brevibacillus fluminis	1
		Lysinibacillus fusiformis	1
		Paenibacillus humicus	1
	Premium American Lager	*Bacillus coagulans*	1
	Malzbier	*Bacillus thuringiensis*	3
		Lysinibacillus manganicus	1
		Paenibacillus cineris	1
		Paenibacillus sp.	1
	Alcohol-free	*Bacillus thuringiensis serovar kurstaki*	1
		Brevibacillus sp.	1
O	Standard American Lager	*Bacillus aryabhattai*	1
		Bacillus oleronius	1
		Brevibacillus brevis	1
		Cohnella thermotolerans	1
		Paenibacillus alginolyticus	1
	Fruit beer	*Paenibacillus ehimensis*	1
		Paenibacillus soli	1
P	Alcohol-free	*Bacillus amyloliquefaciens*	1
		Bacillus cereus	1
		Bacillus megaterium	1
		Bacillus thuringiensis	1
		Brevibacillus parabrevis	1
		Paenibacillus aestuarii	1
		Paenibacillus alginolyticus	1
		Paenibacillus validus	3
		Rummeliibacillus stabekisii	1
Q	Standard American Lager	*Bacillus subtilis*	1
		Bacillus thuringiensis	2
	Alcohol-free	*Bacillus megaterium*	1
		Bacillus thuringiensis	1
		Paenibacillus validus	3
R	Standard American Lager	*Bacillus amyloliquefaciens*	1
S	Standard American Lager	*Bacillus amyloliquefaciens*	1
		Bacillus cereus	1
		Bacillus megaterium	1
		Bacillus pumilus	1
		Bacillus thuringiensis	1
		Brevibacillus nitrificans	1
		Paenibacillus glycanilyticus	1
	Schwarzbier	*Bacillus amyloliquefaciens*	1
T	Standard American Lager	*Bacillus bataviensis*	1
		Bacillus thuringiensis	2
		Cohnellas wuonensis	1
		Paenibacillus lautus	1
	Malzbier	*Bacillus cereus*	1
		Bacillus thuringiensis	1
		Brevibacillus fluminis	1
		Paenibacillus glycanilyticus	3
		Paenibacillus humicus	1
	Alcohol-free	*Bacillus megaterium*	1
	Munich Dunkel	*Bacillus acidiceler*	1
		Bacillus cereus	1
		Bacillus megaterium	1

(*continued on next page*)

Table 2 (*continued*)

Brand	Type	Species	Number of isolates
		Brevibacillus brevis	2
		Brevibacillus choshinensis	3
		Paenibacillus glycanilyticus	2
		Paenibacillus humicus	1
		Paenibacillus stellifer	1
		Paenibacillus validus	1
U	Standard American Lager	*Alicyclobacillus* sp	1
		Anoxybacillus flavithermus	1
		Bacillus cereus	1
		Bacillus flexus	1
		Bacillus licheniformis	1
		Bacillus megaterium	6
		Bacillus thuringiensis	9
		Bacillus thuringiensiss erovar kurstaki	1
		Lysinibacillus fusiformis	1
		Lysinibacillus sphaericus	2
		Paenibacillus aestuarii	1
		Paenibacillus alginolyticus	4
		Paenibacillus humicus	1
		Paenibacillus pasadenensis	2
		Paenibacillus polymyxa	2
		Paenibacillus validus	3
		Paenibacillus xylanolyticus	1
	Malt Liquor	*Bacillus licheniformis*	1
		Paenibacillus alginolyticus	1
		Paenibacillus contaminans	1
V	Premium American Lager	*Brevibacillus brevis*	1
		Paenibacillus alginolyticus	1
		Paenibacillus validus	1
X	Sweet Stout	*Bacillus coagulans*	1
		Bacillus licheniformis	2
		Bacillus thuringiensis serovar kurstaki	1
		Brevibacillus fluminis	2
		Paenibacillus chitinolyticus	1
		Paenibacillus cineris	1
		Paenibacillus humicus	2
		Paenibacillus peoriae	1
		Paenibacillus polymyxa	3
Total			**260**

Table 3
Number of isolates (per genus) of sporeforming bacteria recovered from different beer types analyzed.

Type of beer (n[a])	Number of isolates								Total
	Alicyclobacillus	*Anoxybacillus*	*Bacillus*	*Brevibacillus*	*Cohnella*	*Lysinibacillus*	*Paenibacillus*	*Rummeliibacillus*	
American Brown Ale (1)							2		2
Fruit beer (1)							2		2
Malt Liquor (2)			1				2		3
Malzbier (11)			7	1		1	12		21
Munich Dunkel (5)			3	5			5		13
Premium American Lager (14)			8	4			8		20
Schwarzbier (1)			1						1
Alcohol-free (15)			13	3			15	1	32
Standard American Lager (88)	1	1	79	23	3	5	34		145
Sweet Stout (7)			6	5			9		20
Total	1	1	118	41	3	6	89	1	260

[a] Number of samples analyzed.

widespread occurrence of sporeforming carrying the *horA* and *horC* genes needed for growth in the presence of iso-alpha-acids. Remarkably amongst the 14 isolates positive for *horA* and *horC* genes, four belonged to the *Bacillus cereus* sensu lato group (Table 4).

The occurrence of sporeforming bacteria harboring *horA* and *horC* genes rises several concerns to the brewery industry. Firstly, sporeforming bacteria are ubiquitous in nature and may contaminate foods and beverages through several routes (Carlin, 2011). Secondly, these microorganisms can withstand physical and chemical preservation processes and may persist in the food processing environment through several strategies (Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010), including the formation of biofilms (Peña et al., 2014). Thirdly, the *Bacillus cereus* sensu lato group comprises the well-known foodborne pathogen – *B. cereus* (Schoeni & Wong, 2005), which raises food safety concerns. Fourthly and a significant aspect is that bacteria harboring *horA* and *horC* genes can grow in beers (Haakensen & Ziola, 2008; Suzuki et al., 2006).

Table 4
Presence of *horA* and *horC* genes in sporeforming bacteria isolated from different types and brands of beers.

Brand	Type	Species	*horA*	*horC*
B	Premium American Lager	*B. pumilus*	−	+
C	Standard American Lager	*B. cereus*	+	−
	Standard American Lager	*B. thuringiensis*	−	+
D	Standard American Lager	*B. thuringiensis*	+	−
		B. cereus	−	+
	Alcohol-free	*B. thuringiensis*	+	+
		P. polymyxa	+	−
E	Premium American Lager	*P. naphtalenovorans*	+	−
		Br. invocatus	−	+
F	Standard American Lager	*B. thuringiensis*	−	+
G	Fruit beer	*P. ehimensis*	+	−
H	Alcohol-free	*B. megaterium*	+	−
I	Alcohol-free	*P. validus*	+	−
J	Malt Liquor	*P. alginolyticus*	+	−

3.3. Fate of *Bacillus cereus sensu lato* (LMQA 141, LMQA 206, and LMQA 332), *B. pumilus* LMQA 176, and *Brevibacillus invocatus* LMQA 291 in beers

The results of the fate of five bacterial sporeforming strains harboring *horA* or *horC* genes inoculated in Premium American Lager (5% alcoholic content and pH 4.5), Standard American Lager (5% alcoholic content and pH 4.1) and non-alcoholic Standard American Lager (0–0.5% alcoholic content and pH 3.9) beers following storage at 5, 25 and 35 °C are shown in Table 5. Interestingly, none of the sporeforming bacteria was able to grow in the beers throughout the storage period studied and no spoilage was detected (including no changes in pH). The decrease in the counts of spores in the beers varied with the strain tested (Table 5). The number of vegetative cells at the end of storage period was constantly lower than the counts of spores (~1–2 $\log_{10}$ CFU/mL) (data not shown), which indicates that the colonies counted were in fact spores recovered from beers after the heat shock. It is hypothesized that the lack of growth of sporeforming bacteria harboring *horA* or *horC* genes may be due to low pH values of the beers studied. The pH of inoculated beers was <4.5, which is in accordance with the low pH values of Brazilian beers (Table 6). The growth of sporeforming bacteria harboring the *horA* gene in beers containing 4 and 5% (v/v) of alcohol was reported by Haakensen & Ziola, 2008. In that study, the spoilage (turbidity) of beers was detected as early as 14 days of storage (Haakensen & Ziola, 2008). Even so, in that study, the two beers spoiled by sporeforming bacteria harboring the *horA* gene presented pH values of 4.8 and 5.2, respectively (Haakensen & Ziola, 2008). These pH values fall close to the minimum pH for growth of *B. cereus* sensu lato group. It appears that most strains are inhibited in pH values below approximately 4.8 (Lanciotti, Sinigaglia, Gardini, Vannini, & Guerzoni, 2001; Valero, Fernández, & Salmerón, 2003), whereas the production of toxins does not occur in pH values ≤ 5 (Garcia-Arribas & Kramer, 1990). Despite this, depending on other environmental parameters, the members of this group can grow in substrates with pH 4.4 (Carlin et al., 2013; Martínez, Borrajo, Franco, & Carballo, 2007). Another bacterium used to challenge the beers was *Br. invocatus*, which is known to grow in pH 6 to 8.5 (Logan et al., 2002). Other *Brevibacillus* species, however, can present different pH requirements for growth (Shida et al., 1996). *B. pumilus* commonly grows in pH > 5.7, but some strains can grow in pH as low as 4.5 (Cotter & Hill, 2003; Wilks et al., 2009). Given these data, it seems feasible to state that sporeforming bacteria can germinate and outgrow in beers when pH is appropriate.

As shown in Table 6, the pH of the beers analyzed in this study varied from 4.1 to 4.3 in most samples analyzed. Lower pH values were observed in the fruit beer (brand O) and St. Am. Lager (brand G) samples, which presented pH values of 3.1 and 3.8, respectively (Table 6). On the other hand, the samples from brand M and Q, presented pH values approaching 4.5 (Table 6). The %ABV (i.e., alcohol by volume – v/v) varied from 4.3 to 6.2 for regular beers, while for alcohol-free beer samples, %ABV was between 0 and 0.5% (Table 6). The %ABV is relevant as bacterial tolerance to ethanol has been documented (Ingram, 1990; Rigomier, Bohin, & Lubochinsky, 1980). For instance, it is known that some isolates of *B. cereus*, *Anoxybacillus*, *Thermoanaerobacter* and *Geobacillus* may tolerate up to 4 and 5% of alcohol (Fong et al., 2006; Georgieva, Skiadas, & Ahring, 2007; Lanciotti et al., 2001; Peng, Gao, & Xiao, 2008). Thus, pH appears to be the truly key factor that inhibited the growth of these microorganisms in the present study (Table 6), contrarily to the reported by Haakensen and Ziola (2008). These data indicate that pH is key to control the germination, outgrowth, and spoilage of beers by sporeforming bacteria.

4. Conclusions

This study adds novel information to the literature on the occurrence and fate of sporeforming bacteria in beers. The results indicated a widespread occurrence of sporeforming bacteria in several types of beers from different brands, highlighting that this contamination is not related to a specific kind of beer and processing plant. Considering this, measures should be taken to reduce the occurrence of sporeforming bacteria considering stability and safety concerns. However, the control sporeforming bacteria in beers will be successful only if the main sources of contamination are known. These studies will demand sampling and analysis of raw materials, water, equipment, facilities and final products combined with typing methods to track the main source(s) of these microorganisms in the brewery environment.

The testing of all 260 sporeforming bacterial strains isolated from beers showed that some (n = 14) harbored the genes associated with microbial ability to spoil beers (*horA* or *horC* genes). However, further tests indicated that none of the strains tested were able to grow and spoil alcohol-free and lager type beers with ~5% alcohol. Remarkably, the lack of growth of sporeforming bacteria harboring the *horA* or *horC* genes in the tested beers appeared to be related to the low pH (<4.5) of Brazilian beers. This finding is of key relevance for brewing industries as it sheds light on the importance of pH for the microbiological stability of these products. As microorganisms may respond differently to intrinsic and extrinsic factors (i.e., heterogeneity), each brewery should determine the adequate pH for a beer's formulation aiming to avoid the germination and outgrowth of sporeforming bacteria. In this way, the challenge tests can be valuable to assess the robustness of beer formulations towards sporeforming bacteria at specific storage conditions. This can be particularly relevant in tropical countries, in which exposure of beers for long times to temperatures >25–30 °C could provide conditions for germination and outgrowth of sporeforming bacteria harboring *hor* genes.

Throughout the years, most concerns regarding sporeforming bacteria in foods and beverages were focused on stability and safety issues (Heyndrickx, 2011; Húngaro, Caturla, Horita, Furtado, & Sant'Ana, 2016; Húngaro, Alvarenga, Peña, & Sant'Ana, 2013; Oliveira et al., 2016; Spinelli et al., 2010). Nonetheless, given their outstanding resistance (Spinelli et al., 2010), ability to form biofilms (Peña et al., 2014) and ubiquitous nature (Heyndrickx, 2011), sporeforming bacteria could also be regarded as indicators of raw materials quality and of efficiency of hygiene regimes in breweries. Then, last but not least, one of the main implications of this study is that breweries should also adjust and improve their quality control programs by considering the presence of sporeforming bacteria in

Table 5
Fate of *Bacillus cereus* sensu lato (LMQA 141, LMQA 206, and LMQA 332), *B. pumilus* LMQA 176, and *Brevibacillus invocatus* LMQA 291 in beers stored at 5, 25 and 35 °C.

Microorganism	Type of beer	Storage condition (°C)	Mean δ (log_{10} CFU)[a]
B. cereus sensu lato LMQA 141	Standard American Lager	5	−1.86
		25	−1.48
		35	−1.28
	Premiun American Lager	5	−2.39
		25	−3.39
		35	−2.09
	Alcohol-free	5	−2.09
		25	0.31
		35	−1.03
B. pumilus LMQA 176	Standard American Lager	5	−1.27
		25	−1.19
		35	−0.62
	Premiun American Lager	5	−0.80
		25	−3.41
		35	−3.41
	Alcohol-free	5	−1.15
		25	−3.41
		35	−3.41
B. cereus sensu lato LMQA 206	Standard American Lager	5	−3.42
		25	−3.42
		35	−3.42
	Premiun American Lager	5	−0.42
		25	−1.37
		35	−0.84
	Alcohol-free	5	−3.42
		25	−3.42
		35	−3.42
Br. invocatus LMQA 291	Standard American Lager	5	−3.54
		25	−3.54
		35	−3.54
	Premiun American Lager	5	−3.54
		25	−3.54
		35	−3.54
	Alcohol-free	5	−3.54
		25	−3.54
		35	−3.54
B. cereus sensu lato LMQA 332	Standard American Lager	5	−0.64
		25	−1.81
		35	−3.63
	Premiun American Lager	5	−3.63
		25	−3.63
		35	−3.63
	Alcohol-free	5	−1.93
		25	−3.63
		35	−3.63

[a] Counts of spores.

Table 6
Average pH and ABV in Brazilian beer collected in this study.

Type	pH ± SD	ABV(%)[a]
American Brown Ale	4.3 ± 0.01	4.8
Fruit beer	3.1 ± 0.01	2
Malt Liquor	4.1 ± 0.3	6.9
Malzbier	4.1 ± 0.03	4.2
Munich Dunkel	4.2 ± 0.1	4.7
Premium American Lager	4.3 ± 0.1	4.9
Schwarzbier	4.1	6.2
Alcohol-free	4.2 ± 0.2	0.25
Standard American Lager	4.2 ± 0.1	4.6
Sweet Stout	4.1 ± 0.02	5.0

[a] ABV(alcohol by volume − v/v), *i.e.*, the milliliters of pure ethanol existing in 100 mL of beer at 20 °C. ABV values were recorded from the label of the samples.

raw material, equipment and final products. This will allow breweries to better know the extension of the problem and to further establish effective measures to control the occurrence and potential spoilage of beers by sporeforming bacteria.

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References

Anonymous. (2003). Microbiological challenge testing. *Comprehensive Reviews in Food Science and Food Safety, 2*(s2), 46–50. http://dx.doi.org/10.1111/j.1541-4337.2003.tb00051.x.

Ash, C., Priest, F. G., & Collins, M. D. (1994). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus Paenibacillus. *Antonie van Leeuwenhoek, 64*(3–4), 253–260.

Bokulich, N. A., & Bamforth, C. W. (2013). The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews, 77*(2), 157–172. http://dx.doi.org/10.1128/MMBR.00060-12.

Buehner, K. P., Anand, S., & Garcia, A. (2014). Prevalence of thermoduric bacteria and

spores on 10 Midwest dairy farms. *Journal of Dairy Science, 97*(11), 6777–6784. http://dx.doi.org/10.3168/jds.2014-8342.
Caballero, I., Agut, M., Armentia, A., & Blanco, C. A. (2009). Importance of tetrahydroiso alpha-acids to the microbiological stability of beer. *Journal of AOAC International, 92*(4), 1160–1164.
Carlin, F. (2011). Origin of bacterial spores contaminating foods. *Food Microbiology, 28*(2), 177–182. http://dx.doi.org/10.1016/j.fm.2010.07.008.
Carlin, F., Albagnac, C., Rida, A., Guinebretière, M.-H., Couvert, O., & Nguyen-the, C. (2013). Variation of cardinal growth parameters and growth limits according to phylogenetic affiliation in the Bacillus cereus Group. Consequences for risk assessment. *Food Microbiology, 33*(1), 69–76. http://dx.doi.org/10.1016/j.fm.2012.08.014.
Catarino, R., Ceddia, G., Areal, F. J., & Park, J. (2015). The impact of secondary pests on Bacillus thuringiensis (Bt) crops. *Plant Biotechnology Journal, 13*(5), 601–612. http://dx.doi.org/10.1111/pbi.12363.
Choma, C., Guinebretière, M. H., Carlin, F., Schmitt, P., Velge, P., Granum, P. E., et al. (2000). Prevalence, characterization and growth of Bacillus cereus in commercial cooked chilled foods containing vegetables. *Journal of Applied Microbiology, 88*(4), 617–625. http://dx.doi.org/10.1046/j.1365-2672.2000.00998.x.
Choudhery, A. K., & Mikolajcik, E. M. (1971). Bacilli in milk. 2. Sweet curd formation. *Journal of Dairy Science, 54*(3), 321–325. http://dx.doi.org/10.3168/jds.S0022-0302(71)85836-8.
Cotter, P. D., & Hill, C. (2003). Surviving the acid test: Responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews: MMBR, 67*(3), 429–453 (table of contents).
Daffonchio, D., Cherif, A., Brusetti, L., Rizzi, A., Mora, D., Boudabous, A., et al. (2003). Nature of polymorphisms in 16S-23S rRNA gene intergenic transcribed spacer fingerprinting of Bacillus and related genera. *Applied and Environmental Microbiology, 69*(9), 5128–5137. http://dx.doi.org/10.1128/AEM.69.9.5128-5137.2003.
Eppinger, M., Bunk, B., Johns, M. A., Edirisinghe, J. N., Kutumbaka, K. K., Koenig, S. S. K., … Vary, P. S. (2011, August). Genome sequences of the biotechnologically important Bacillus megaterium strains QM B1551 and DSM319. *Journal of Bacteriology*. http://dx.doi.org/10.1128/JB.00449-11, 1752 N St., N.W., Washington, DC.
Fong, J. C. N., Svenson, C. J., Nakasugi, K., Leong, C. T. C., Bowman, J. P., Chen, B., … Rogers, P. L. (2006). Isolation and characterization of two novel ethanol-tolerant facultative-anaerobic thermophilic bacteria strains from waste compost. *Extremophiles: Life under Extreme Conditions, 10*(5), 363–372. http://dx.doi.org/10.1007/s00792-006-0507-2.
Frederiksen, K., Rosenquist, H., Jørgensen, K., & Wilcks, A. (2006, May). Occurrence of natural Bacillus thuringiensis contaminants and residues of Bacillus thuringiensis-based insecticides on fresh fruits and vegetables. *Applied and Environmental Microbiology*. http://dx.doi.org/10.1128/AEM.72.5.3435-3440.2006.
Galofre, B., Israel, S., Dellunde, J., & Ribas, F. (2004). Aerobic bacterial spores as process indicators for protozoa cysts in water treatment plants. *Water Science and Technology: A Journal of the International Association on Water Pollution Research, 50*(1), 165–172.
Garcia-Arribas, M. L., & Kramer, J. M. (1990). The effect of glucose, starch, and pH on growth, enterotoxin and haemolysin production by strains of Bacillus cereus associated with food poisoning and non-gastrointestinal infection. *International Journal of Food Microbiology, 11*(1), 21–33. http://dx.doi.org/10.1016/0168-1605(90)90037-6.
Georgieva, T. I., Skiadas, I. V., & Ahring, B. K. (2007). Effect of temperature on ethanol tolerance of a thermophilic anaerobic ethanol producer Thermoanaerobacter A10: Modeling and simulation. *Biotechnology and Bioengineering, 98*(6), 1161–1170. http://dx.doi.org/10.1002/bit.21536.
Goh, K. M., Gan, H. M., Chan, K. G., Chan, G. F., Shahar, S., Chong, C. S., … Chai, K. P. (2014). Analysis of Anoxybacillus genomes from the aspects of lifestyle adaptations, prophage diversity, and carbohydrate metabolism. *PLoS One, 9*(3). http://dx.doi.org/10.1371/journal.pone.0090549.
Grady, E. N., MacDonald, J., Liu, L., Richman, A., & Yuan, Z.-C. (2016). Current knowledge and perspectives of Paenibacillus: A review. *Microbial Cell Factories*. http://dx.doi.org/10.1186/s12934-016-0603-7. London.
Haakensen, M., & Ziola, B. (2008). Identification of novel horA-harbouring bacteria capable of spoiling beer. *Canadian Journal of Microbiology, 54*, 321–325. http://dx.doi.org/10.1139/W08-007.
Heyndrickx, M. (2011). The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. *Applied and Environmental Soil Science, 2011*, 1–11. http://dx.doi.org/10.1155/2011/561975.
Hough, J. S., Briggs, D. E., Stevens, R., & Young, T. W. (1982). Chemical and physical properties of beer. In , *Hopped wort and beer: Vol. II. Malting and brewing science* (pp. 776–838). Boston, MA: Springer US. http://dx.doi.org/10.1007/978-1-4615-1799-3_11.
Huang, K., McLandsborough, L. A., & Goddard, J. M. (2016). Adhesion and removal kinetics of Bacillus cereus biofilms on Ni-PTFE modified stainless steel. *Biofouling, 32*(5), 523–533. http://dx.doi.org/10.1080/08927014.2016.1160284.
Húngaro, H. M., Alvarenga, V. O., Peña, W. E. L., & Sant'Ana, A. de S. (2013). Hearts of palms preserves and botulism in Brazil: An overview of outbreaks, causes and risk management strategies. *Trends in Food Science & Technology, 34*(2), 80–95. http://dx.doi.org/10.1016/j.tifs.2013.07.008.
Húngaro, H. M., Caturla, M. Y. R., Horita, C. N., Furtado, M. M., & Sant'Ana, A. S. (2016). Blown pack spoilage in vacuum-packaged meat: A review on clostridia as causative agents, sources, detection methods, contributing factors and mitigation strategies. *Trends in Food Science & Technology, 52*, 123–138. http://dx.doi.org/10.1016/j.tifs.2016.04.010.
Ingram, L. O. (1990). Ethanol tolerance in bacteria. *Critical Reviews in Biotechnology, 9*(4), 305–319.
Jeon, S., Kim, N., Shim, M., Jeon, Y., Ahn, J., Lee, S., … Rhee, M. (2015). Microbiological diversity and prevalence of spoilage and pathogenic bacteria in commercial fermented alcoholic beverages (beer, fruit wine, refined rice wine, and yakju) Jeon SH1, Kim NH1, Shim MB2, Jeon YW2, Ahn JH2, Lee SH3, Hwang IG4, Rhee MS5. *Journal of Food Protection, 78*(4), 812–818. http://dx.doi.org/10.4315/0362-028X.JFP-14-431.
Jesus, A. L. T., Fernandes, M. S., Kamimura, B. A., Prado-Silva, L., Silva, R., Esmerino, E. A., … Sant'Ana, A. S. (2016). Growth potential of Listeria monocytogenes in probiotic cottage cheese formulations with reduced sodium content. *Food Research International, 81*, 180–187. http://dx.doi.org/10.1016/j.foodres.2015.12.030.
Kämpfer, P., Rosselló-Mora, R., Falsen, E., Busse, H. J., & Tindall, B. J. (2006). Cohnella thermotolerans gen. nov., sp. nov., and classification of 'Paenibacillus hongkongensis â€™ as Cohnella hongkongensis sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 56*(4), 781–786. http://dx.doi.org/10.1099/ijs.0.63985-0.
Kashket, E. (1987). Bioenergetics of lactic acid bacteria: Cytoplasmic pH and osmotolerance. *FEMS Microbiology Letters, 46*(3), 233–244. http://dx.doi.org/10.1016/0378-1097(87)90110-8.
Lal, S., & Tabacchioni, S. (2009). Ecology and biotechnological potential of Paenibacillus polymyxa: A minireview. *Indian Journal of Microbiology, 49*(1), 2–10. http://dx.doi.org/10.1007/s12088-009-0008-y.
Lanciotti, R., Sinigaglia, M., Gardini, F., Vannini, L., & Guerzoni, M. E. (2001). Growth/no growth interfaces of Bacillus cereus, Staphylococcus aureus and Salmonella enteritidis in model systems based on water activity, pH, temperature and ethanol concentration. *Food Microbiology, 18*(6), 659–668. http://dx.doi.org/10.1006/fmic.2001.0429.
Logan, N. A., Forsyth, G., Lebbe, L., Goris, J., Heyndrickx, M., Balcaen, A., … De Vos, P. (2002). Polyphasic identification of Bacillus and Brevibacillus strains from clinical, dairy and industrial specimens and proposal of Brevibacillus invocatus sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 52*(3), 953–966. http://dx.doi.org/10.1099/ijs.0.02081-0.
Martínez, S., Borrajo, R., Franco, I., & Carballo, J. (2007). Effect of environmental parameters on growth kinetics of Bacillus cereus (ATCC 7004) after mild heat treatment. *International Journal of Food Microbiology, 117*. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.08.002.
Mazoua, S., & Chauveheid, E. (2005). Aerobic spore-forming bacteria for assessing quality of drinking water produced from surface water. *Water Research, 39*(20), 5186–5198. http://dx.doi.org/10.1016/j.watres.2005.09.027.
McSpadden, B. B. (2004). Ecology of Bacillus and Paenibacillus spp. in agricultural systems. *Phytopathology, 94*(11), 1252–1258. http://dx.doi.org/10.1094/PHYTO.2004.94.11.1252.
Meussdoerffer, F., & Zarnkow, M. (2009). Starchy raw materials. In *Handbook of brewing* (pp. 43–83). Wiley-VCH Verlag GmbH & Co. KGaA. http://dx.doi.org/10.1002/9783527623488.ch2.
Montes, M. J., Mercadé, E., Bozal, N., & Guinea, J. (2004). Paenibacillus antarcticus sp. nov., a novel psychrotolerant organism from the Antarctic environment. *International Journal of Systematic and Evolutionary Microbiology, 54*(5), 1521–1526. http://dx.doi.org/10.1099/ijs.0.63078-0.
Noots, I., Delcour, J. A., & Michiels, C. W. (1999). From field barley to malt: Detection and specification of microbial activity for quality aspects. *Critical Reviews in Microbiology, 25*(2), 121–153. http://dx.doi.org/10.1080/10408419991299257.
Okinaka, R. T., & Keim, P. (2016). The Phylogeny of Bacillus cereus sensu lato. *Microbiology Spectrum, 4*(1). Retrieved from: http://www.asmscience.org/content/journal/microbiolspec/10.1128/microbiolspec.TBS-0012-2012.
Oliveira, R. B. A., Margalho, L. P., Nascimento, J. S., Costa, L. E. O., Portela, J. B., Cruz, A. G., et al. (2016). Processed cheese contamination by spore-forming bacteria: A review of sources, routes, fate during processing and control. *Trends in Food Science & Technology, 57*, 11–19. http://dx.doi.org/10.1016/j.tifs.2016.09.008.
Oteiza, J. M., Soto, S., Alvarenga, V. O., Sant'Ana, A. S., & Gianuzzi, L. (2015). Fate of Alicyclobacillus spp. in enrichment broth and in juice concentrates. *International Journal of Food Microbiology, 210*. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.05.021.
Peña, W. E. L., de Andrade, N. J., Soares, N. F. F., Alvarenga, V. O., Rodrigues Junior, S., Granato, D., … de Souza Sant'Ana, A. (2014). Modelling Bacillus cereus adhesion on stainless steel surface as affected by temperature, pH and time. *International Dairy Journal, 34*(1), 153–158. http://dx.doi.org/10.1016/j.idairyj.2013.08.006.
Peng, H., Gao, Y., & Xiao, Y. (2008). The high ethanol tolerance in a thermophilic bacterium Anoxybacillus sp. WP06. *Sheng wu gong cheng xue bao = Chinese Journal of Biotechnology, 24*(6), 1117–1120.
Priest, F. G., Barker, M., Baillie, L. W. J., Holmes, E. C., & Maiden, M. C. J. (2004, December). Population structure and evolution of the Bacillus cereus group. *Journal of Bacteriology*. http://dx.doi.org/10.1128/JB.186.23.7959-7970.2004.
Riese, J. C., & Eßlinger, H. M. (2009). World beer market. In *Handbook of brewing* (pp. 497–513). Wiley-VCH Verlag GmbH & Co. KGaA. http://dx.doi.org/10.1002/9783527623488.ch20.
Rigomier, D., Bohin, J. P., & Lubochinsky, B. (1980). Effects of ethanol and methanol on lipid metabolism in Bacillus subtilis. *Journal of General Microbiology, 121*(1), 139–149. http://dx.doi.org/10.1099/00221287-121-1-139.
Rosenquist, H., Smidt, L., Andersen, S. R., Jensen, G. B., & Wilcks, A. (2005).

Occurrence and significance of Bacillus cereus and Bacillus thuringiensis in ready-to-eat food. *FEMS Microbiology Letters, 250*(1), 129. http://dx.doi.org/10.1016/j.femsle.2005.06.054.

Sakamoto, K., & Konings, W. N. (2003). Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology, 89*(2), 105–124. http://dx.doi.org/10.1016/S0168-1605(03)00153-3.

Sami, M., Yamashita, H., Kadokura, H., Kitamoto, K., Yodi, K., & Yamasaki, M. (1997). A new and rapid method for determination of beer-spoilage ability of lactobacilli. *Journal of the American Society of Brewing Chemists, 55*(4), 137–140. Retrieved from: http://cat.inist.fr/?aModele=afficheN&cpsidt=2082475.

Schoeni, J. L., & Wong, A. C. L. (2005). Bacillus cereus food poisoning and its toxins. *Journal of Food Protection, 68*(3), 636–648.

Shaheen, R., Svensson, B., Andersson, M. A., Christiansson, A., & Salkinoja-Salonen, M. (2010). Persistence strategies of Bacillus cereus spores isolated from dairy silo tanks. *Food Microbiology, 27*(3), 347–355. http://dx.doi.org/10.1016/j.fm.2009.11.004.

Shida, O., Takagi, H., Kadowaki, K., & Komagata, K. (1996). Proposal for two new genera, Brevibacillus gen. nov. and Aneurinibacillus gen. nov. *International Journal of Systematic Bacteriology, 46*(4), 939–946. http://dx.doi.org/10.1099/00207713-46-4-939.

Silva, F., & Gibbs, P. (2004). Target selection in designing pasteurization processes for shelf-stable high-acid fruit products. *Critical Reviews in Food Science and Nutrition, 44*(5), 353–360. http://dx.doi.org/10.1080/10408690490489251.

Spinelli, A. C. N. F., Sant'Ana, A. S., Pacheco-Sanchez, C. P., & Massaguer, P. R. (2010). Influence of the hot-fill water-spray-cooling process after continuous pasteurization on the number of decimal reductions and on Alicyclobacillus acidoterrestris CRA 7152 growth in orange juice stored at 35°C. *International Journal of Food Microbiology, 137*. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.11.003.

Spinelli, A. C. N. F., Sant'Ana, A. S., Rodrigues, S., & Massaguer, P. R. (2009). Influence of different filling, cooling, and storage conditions on the growth of Alicyclobacillus acidoterrestris CRA7152 in orange juice. *Applied and Environmental Microbiology, 75*(23), 7409–7416. http://dx.doi.org/10.1128/AEM.01400-09.

Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Daniel, H. M., Van Landschoot, A., et al. (2014). The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS One, 9*(4). http://dx.doi.org/10.1371/journal.pone.0095384.

Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Van Landschoot, A., De Vuyst, L., et al. (2015). The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic beer fermentation. *Food Microbiology, 49*, 23–32. http://dx.doi.org/10.1016/j.fm.2015.01.008.

Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Reviews, 32*(4), 579. http://dx.doi.org/10.1111/j.1574-6976.2008.00112.x.

Stevenson, K. E., & Lembke, F. (2015). 23. Mesophilic aerobic endospore-forming bacilli. In Y. Salfinger, & M. L. Tortorello (Eds.), *Compendium of methods for the microbiological examination of foods* (pp. 299–304). Washington, DC: Apha Press. http://dx.doi.org/10.2105/MBEF.0222.028.

Suzuki, K., Iijima, K., Ozaki, K., & Yamashita, H. (2005). Isolation of a hop-sensitive variant of Lactobacillus lindneri and identification of genetic markers for beer spoilage ability of lactic acid bacteria. *Applied and Environmental Microbiology, 71*(9), 5089–5097. http://dx.doi.org/10.1128/AEM.71.9.5089-5097.2005.

Suzuki, K., Koyanagi, M., & Yamashita, H. (2004). Genetic characterization of non-spoilage variant isolated from beer-spoilage Lactobacillus brevis ABBC45C. *Journal of Applied Microbiology, 96*(5), 946–953. http://dx.doi.org/10.1111/j.1365-2672.2004.02244.x.

Suzuki, K., Sami, M., Iijima, K., Ozaki, K., & Yamashita, H. (2006). Characterization of horA and its flanking regions of Pediococcus damnosus ABBC478 and development of more specific and sensitive horA PCR method. *Letters in Applied Microbiology, 42*(4), 392–399. http://dx.doi.org/10.1111/j.1472-765X.2006.01860.x.

Tamachkiarow, L., & Flemming, H. C. (2003). On-line monitoring of biofilm formation in a brewery water pipeline system with a fibre optical device. *Water Science and Technology: A Journal of the International Association on Water Pollution Research, 47*(5), 19–24.

Turner, S., Pryer, K. M., Miao, V. P. W., & Palmer, J. D. (1999). Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit rRNA Sequence Analysis1. *Journal of Eukaryotic Microbiology, 46*, 327–338. http://dx.doi.org/10.1111/j.1550-7408.1999.tb04612.x.

Vaishampayan, P., Miyashita, M., Ohnishi, A., Satomi, M., Rooney, A., La Duc, M. T., & Venkateswaran, K. (2009). Description of Rummeliibacillus stabekisii gen. nov., sp. nov. and reclassification of Bacillus pycnus Nakamura et al. 2002 as Rummeliibacillus pycnus comb. nov. *International Journal of Systematic and Evolutionary Microbiology, 59*(5), 1094–1099. http://dx.doi.org/10.1099/ijs.0.006098-0.

Valero, M., Fernández, P., & Salmerón, M. (2003). Influence of pH and temperature on growth of Bacillus cereus in vegetable substrates. *International Journal of Food Microbiology, 82*(1), 71–79. http://dx.doi.org/10.1016/S0168-1605(02)00265-9.

Vaughan, A., O'Sullivan, T., & Sinderen, D. (2005). Enhancing the microbiological stability of malt and beer - a review. *Journal of the Institute of Brewing, 111*(4), 355–371. http://dx.doi.org/10.1002/j.2050-0416.2005.tb00221.x.

Wells-Bennik, M. H. J., Eijlander, R. T., den Besten, H. M. W., Berendsen, E. M., Warda, A. K., Krawczyk, A. O., … Abee, T. (2016). Bacterial spores in food: Survival, emergence, and outgrowth. *Annual Review of Food Science and Technology, 7*(1), 457–482. http://dx.doi.org/10.1146/annurev-food-041715-033144.

Wilks, J. C., Kitko, R. D., Cleeton, S. H., Lee, G. E., Ugwu, C. S., Jones, B. D., … Slonczewski, J. L. (2009). Acid and base stress and transcriptomic responses in Bacillus subtilis. *Applied and Environmental Microbiology, 75*(4), 981–990. http://dx.doi.org/10.1128/AEM.01652-08.

Willaert, R. (2007). The beer brewing process: Wort production and beer fermentation. In *Handbook of food products manufacturing* (pp. 443–506). John Wiley & Sons, Inc. http://dx.doi.org/10.1002/9780470113554.ch20.