Comparative analysis of the role of *Bacillus* species as food fermenting and food spoilage organisms

by

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Abstract

Bacillus species are fermenters in some food fermentation but spoilage organisms in other food products. For instance, bacilli and fungi are the predominant organisms in daqu fermentation. The antifungal lipopeptides produced by bacilli, including surfactins, fengycins and iturins, may impact fungal growth during daqu fermentation, thereby influencing the microbial community. The interaction between *Bacillus* lipopeptides and fungi in *daqu* is unclear. Therefore, a simulated model of *daqu* was created to investigate the role of antifungal lipopeptides *in situ*. The results showed that iturin A displayed the strongest antifungal activities in vitro. *B. velezensis* FUA2155 exhibit higher antifungal activity than two strains of *B. amyloliquefaciens* in the *daqu* model, due to the production of iturin A. Additionally, hydrolytic enzymes produced by *Bacillus* spp. can cause ropy bread spoilage, which leads to economic losses in bakeries. *Bacillus* spp. are wheat grain endophytes and form heat resistant endospores, consequently, process hygiene and heating during baking are insufficient to prevent ropy spoilage. Therefore, to reduce the negative impact of *Bacillus* and increase the shelf life of bread, sourdough was investigated as a biopreservative. The results indicated more than 2 d delay of spoilage with an addition of 12 % sourdough fermented by lactic acid bacteria. Bread with reutericyclin exhibited a bactericidal mode of action against strains of *Bacillus*.

In food industries, the high thermal loads that are required for inactivation of *Bacillus* spores can result in significant loss of food quality. Pressure-assisted thermal sterilization is a promising approach for spore inactivation without the need for high thermal stress. Nevertheless, the pressure resistance of *Bacillus* spores varies greatly. The *spoVA*^{2mob} operon increases heat resistance of spores and multiple copies of the operon further increases the heat resistance. However, the role of *spoVA*^{2mob} operon in pressure resistance is not well explored. In this study, the pressure

resistance of 17 strains of *Bacillus* were correlated with the copy number of the *spoVA*^{2mob} operon. To study the role of *spoVA*^{2mob} operon in pressure resistance and spore germination triggered by different germinants, an isogenic mutant strain of *Bacillus subtilis* 168 with insertion of *spoVA*^{2mob} operon was created. The *spoVA*^{2mob} operon increased pressure resistance and decreased pressure induced spore germination significantly.

Overall, this research provides insights into the interaction of *Bacillus* and other microorganisms, such as fungi and lactic acid bacteria in food fermentations, to better understand the role of *Bacillus* as fermenting or food spoilage organisms.

Preface

This thesis is an original work by Zhen Li, which is written according to the guidelines provided by Faculty of Graduate Studies and Research of University of Alberta.

Chapter 2 of this thesis is a literature review, and a version of manuscript will be submitted as Zhen Li, Mengzhuo Zheng, Jinshui Zheng, Michael G. Gänzle "*Bacillus* species in food fermentations: an under-appreciated group of organisms for safe use in food fermentations" to *Current Opinion in Food Science*. I was responsible for literature review and manuscript preparation. Dr. Michael Gänzle provided suggestions and contributed to concept formation and manuscript revision. Dr. Jinshui Zheng and Mengzhuo Zheng contributed to bioinformatics analysis and data visualization of the heatmap with query sequences provided by me.

Chapter 3 is an experimental work prepared for submission as Zhen Li, Kleinberg X. Fernandez, John C. Vederas, Michael G. Gänzle "Composition and activity of antifungal lipopeptides produced by *Bacillus* spp. in *daqu* fermentation" to *Applied and Environmental Microbiology*. I contributed to the study design, conducted the experiments and prepared the manuscript. Kleinberg Fernandez performed the LCMS analysis. Dr. John Vederas provided suggestions. Dr. Michael Gänzle contributed to the hypothesis development and editing of manuscript.

A version of Chapter 4 of this thesis has been published as Zhen Li, Felix Schottroff, David J. Simpson, and Michael G. Gänzle. "The copy number of the *spoVA*^{2mob} operon determines pressure resistance of *Bacillus* endospores." *Applied and Environmental Microbiology* 85, no. 19 (2019): e01596-19. I was responsible for conducting the experiments and writing the manuscript. Dr. Felix Schottroff contributed to the cooperation of the experiment of DPA analysis. Dr. David Simpson

contributed to the experiment design and manuscript revision. Dr. Michael Gänzle contributed to the hypothesis development, experimental design, and manuscript revision.

Chapter 5 has been published as Zhen Li, Francieli Begnini Siepmann, Luis E. Rojas Tovar, Xiaoyan Chen, and Michael G. Gänzle. "Effect of copy number of the *spoVA*^{2mob} operon, sourdough and reutericyclin on ropy bread spoilage caused by *Bacillus* spp." *Food Microbiology* 91 (2020): 103507. My contribution to this study includes data analysis, data visualization and writing of manuscript. Dr. Francieli Begnini Siepmann and Luis E Rojas Tovar contributed to the execution of the experiments and manuscript revision. Dr. Xiaoyan Chen created the mutant strain of *Limosilactobacillus reuteri*. Dr. Michael Gänzle provided suggestions to the experimental design and manuscript editing.

Chapter 6 is an experimental work prepared for submission. I contributed to the study design, conducted the experiments, and prepared the manuscript. Dr. Michael Gänzle contributed to the hypothesis development, experimental design and manuscript revision.

Dedication

This thesis is dedicated to my beloved parents, Mr. Li Chunbo and Mrs. Chen Guanhua, and my sister, Mrs. Li Ya for their endless love, encouragement, and support.

To my husband, Mr. Li Jiyuan, thank you for your unconditional companionship, love and praise.

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List of Abbreviations

AGFK	L-asparagine, D-glucose, D-fructose, potassium ions
ANOVA	Analysis of variance
a_W	Water activity
BLAST	Basic local alignment search tool
CFU	Colony forming units
CLE	Cortex lytic enzyme
CPEC	Circular polymerase extension cloning
CRISPR	Clustered regularly interspaced short palindromic repeats
ddPCR	Droplet digital polymerase chain reaction
DPA	Dipicolinic acid
GR	Germinant receptor
Kan	Kanamycin
LAB	Lactic acid bacteria
LB	Luria-bertani
Log	Logarithmic
MIC	Minimum inhibitory concentration
mMRS	Modified de Man, Rogosa, and Sharpe
MPa	Mega pascal
NCBI	National Center for Biotechnology Information
OD	Optical density
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
sgRNA	Single guide RNA
spp.	Species

Chapter 1 General Introduction and Thesis Objectives

1.1 Introduction

Bacillus are part of the microbiota of some food fermentations, such as *daqu* fermentation (Zheng et al., 2011; Shrestha et al., 2013). In addition, *Bacillus* spp. cause spoilage of food such as bread and dairy products (Valerio et al., 2012; Lücking et al., 2013; Glasset et al., 2016), and produce toxins that can cause foodborne illness. However, the understanding of genetic determinants for this difference remains limited.

Bacillus spp. are regarded as members of fermentation microbiota, possibly due to their hydrolytic activity as well as tolerance to low water activity and high temperature. For instance, *Bacillus* spp. are predominant organisms generally present in *daqu* fermentation (Zheng et al., 2014). *Daqu* is a spontaneous solid state cereal fermentation that is used as saccharification culture in Chinese liquor and some steamed bread (Zheng et al., 2013; Yan et al., 2019). In *daqu* fermentation, hydrolytic enzymes including amylolytic enzymes are mainly produced by *Bacillus* and fungi (Li et al., 2015b; Liu et al., 2018). Amylases produced during *daqu* fermentation are the major contributors to starch liquefaction and saccharification in the subsequent mash fermentation (Li et al., 2015a). In addition, proteolytic enzymes produced by *Bacillus* and fungi generate amino acids as precursors for volatile flavor compounds (Liu et al., 2018). *Bacillus* also produce lipopeptides with a broad spectrum of antimicrobial activity (Cochrane and Vederas, 2016; Zhang et al., 2022). However, whether the *Bacillus* lipopeptides impact community assembly in *daqu* fermentations is not well studied.

Some *Bacillus* spp. also cause food spoilage. For example, *B. amyloliquefaciens* and closely related species possess amylolytic and proteolytic enzymes that are involved in ropy bread

spoilage. Ropy bread spoilage is characterized by an unpleasant fruity odor followed by discolored, enzymatic degradation resulting in soft, sticky and stringy bread crumb (Valerio et al., 2015). *Bacillus* spores have been widely isolated from bakery environments and from raw materials, such as wheat flour, yeast and bread improvers (Bailey and von Holy, 1993; Pepe et al., 2003). Contamination of wheat flour with *Bacillus* spores relates to the stable occurrence of these organisms as part of commensal microbiota of plants including wheat (Chen et al., 2007; Fan et al., 2011), and to the presence of *Bacillus* as member of seed-borne endophytic microbial community (Robinson et al., 2016; Shahzad et al., 2016). *Bacillus* spores remain viable throughout storage and processing of cereal grains and flours (Needham et al., 2005; Fangio et al., 2010), which makes the contamination not accidental but unavoidable. Due to the ability of spores to survive during baking, the concern of heat resistant spores cannot be fully addressed by sanitary prevention of contamination and heat treatment during baking process.

Bacillus endospores are resistant to heat, UV irradiation and chemicals, and thus survive in insufficiently processed foods (Setlow, 2003). In addition, heat-resistant spores may survive thermal processes that are currently used to achieve commercial sterility (Holdsworth and Simpson, 2016). In food industries, inactivating *Bacillus* spores with high temperature heat treatment during food processing is critical. However, the high thermal loads required for inactivation of *Bacillus* spores can result in significant food quality losses (Nerandzic and Donskey, 2010; Setlow, 2014; Zhang and Mathys, 2019). The alternate germination-inactivation strategies, such as high pressure thermal sterilization is a promising approach for spore inactivation without the need for high thermal stress (Farkas, 2016; Delbrück et al., 2021a). It aims to germinate spores to mitigate their resistance to inactivation processes. Nevertheless, *Bacillus* endospores exhibit a strong variation in resistance to pressure (Margosch et al., 2004b). Yet, the mechanisms

of spore inactivation by pressure are not fully understood and warrant further investigation. The *spoVA*^{2mob} operon is a mobile genetic element present in *Bacillus* spp. Heat resistance is correlated with the copy number of the operon in *Bacillus* spp. (Berendsen et al., 2016a). However, the relationship between the copy number of the *spoVA*^{2mob} operon and pressure resistance of *Bacillus* spores has not been studied. Therefore, comparison of the high pressure resistance of *Bacillus* spores with different copy number of *spoVA*^{2mob} operon is needed. Additionally, creation of isogenic mutant strain of *B. subtilis* 168 is a good option to help explore the role of *spoVA*^{2mob} operon in pressure resistance and different germinants induced germination of *Bacillus* spores.

In summary, the role of *Bacillus* as food fermenting organisms and spoilage or toxinogenic organisms has been widely studied; however, the understanding of genetic determinants remains limited. Therefore, studying the phenotypic and genotypic characteristics of *Bacillus* spp. related to spoilage, and identifying *Bacillus* spp. that could be safely used in food fermentations are important (Sewalt et al., 2016). Experimentation described in this thesis therefore aimed to test the following hypotheses:

1.2 Hypotheses

1) Antifungal lipopeptides produced by *Bacillus* spp. inhibit the growth of fungi in *daqu* fermentation.
2) Sourdough and specifically reutericyclin produced by *Limosilactobacillus reuteri* inhibit the ropy spoilage of bread caused by *Bacillus* spp.
3) The *spoVA*^{2mob} operon in *Bacillus* endospores confers pressure resistance and the strains with more copies of *spoVA*^{2mob} operon have higher pressure resistance.
4) The *spoVA*^{2mob} operon decreases the heat-, pressure-, and nutrients-induced germination of *Bacillus* spores.

1.3 Objectives

1) Describe the role of *Bacillus* in food fermentations and identify metabolic traits that impact product quality and safety (Chapter 2).
2) Compare the antifungal activity of three strains of *Bacillus* in *daqu* fermentation *in situ* (Chapter 3).
3) Explore the effect of sourdough, and specifically reutericyclin produced by *Limosilactobacillus reuteri* on inhibition of the ropy spoilage of bread caused by *Bacillus* spp. (Chapter 4).
4) Determine the relationship between copy number of *spoVA*^{2mob} operon in *Bacillus* genomes and pressure resistance of *Bacillus* spores (Chapter 5).
5) Create the isogenic mutant strain of *Bacillus subtilis* 168 with the insertion of *spoVA*^{2mob} operon to further determine its role in high pressure resistance and germination of spores (Chapter 6).

Chapter 2. Literature Review: *Bacillus* Species in Food Fermentations – An Under-Appreciated Group of Organisms for Safe Use in Food Fermentations

2.1 Background

Food fermentations recruit the activity of microorganisms for conversion of food components to improve shelf-life and safety as well as sensory and nutritional properties of food. The presence of live microorganisms in fermented foods is increasingly recognized to improve gastrointestinal health (Marco et al., 2017; Pasolli et al., 2020; Wastyk et al., 2021; Gänzle, 2022). The assembly of communities of fermentation microbes is determined by the fermentation conditions, i.e., the ingredients, temperature, pH, and moisture content, and back-slopping or starter cultures (M. Gänzle, 2019). Most fermentations include yeasts and/or lactic acid bacteria as major fermentation microbes but mycelial molds, acetic acid bacteria, staphylococci, propionibacteria, *Enterobacteriaceae* and bacilli also play dominant roles in some fermented foods (Gänzle, 2019). However, the contribution of *Bacillus* species to food quality is not as well documented as the contribution of other groups of microbes.

Bacteria belonging to the genus *Bacillus* are Gram positive, endospore-forming, rod-shaped, and obligate aerobes or facultative anaerobes. The relevance of *Bacillus* in agriculture, in food production and fermentation, and in animals is indicated in Figure 2.1. *Bacillus* are frequently isolated from natural habitats including soil, water and growing plants, and are almost ubiquitously present in foods (Nicholson, 2002). In addition, bacilli are among the most robust bacteria on earth, an important characteristic of the genus is the ability to sporulate and withstand adverse conditions. The high resistance of endospores is believed to be a key factor determining the ecology of these bacteria (Mandic-Mulec et al., 2016). *Bacillus* endospores are more resistant than vegetative cells

to severe environment, such as heat, freezing, desiccation, and chemical assaults (Setlow, 2003; Mandic-Mulec et al., 2016). The resistance of spores to high temperature and high pressure is linked to the presence and copy number of the *spoVA*^{2mob} operon (Berendsen et al., 2016a; Li et al., 2019).

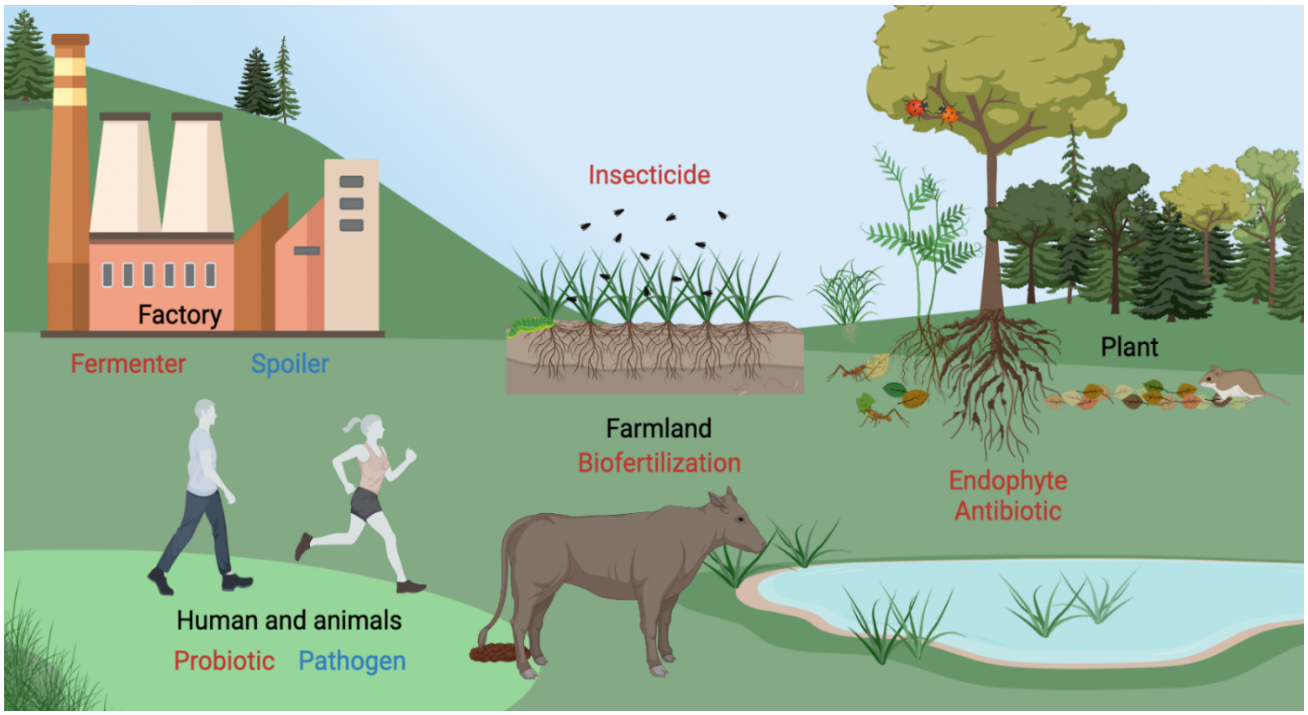


Figure 2.1 A simplified view of the lifestyle of *Bacillus* in different environmental niches. Mutualistic interactions of *Bacillus* with plants or humans are highlighted in red letters; pathogenic interactions are in blue letters. Soil is the largest reservoir of *Bacillus*. *Bacillus* species form stable symbiotic associations with plants and occur in the rhizosphere or as endophytes (Robinson et al., 2016; Shahzad et al., 2016). The plants provide nutrients to bacilli; in turn, *s*trains of *Bacillus* help the plant to defend against harmful microbes by production of antimicrobial peptides (Szymańska et al., 2016). *B. thuringiensis* additionally produces an insecticidal spore protein, which is used commercially as biopesticide (Bravo et al., 2011). *Bacillus* spp. are generally present in plant foods and may cause food spoilage (André et al., 2017) or improves food quality as a food fermenting organism (Parkouda et al., 2009; Gänzle, 2022). In vertebrate hosts, pathogenic *Bacillus* species of the *B. cereus* group cause emetic or diarrheal illness cause illness or death (EFSA Panel on Biological Hazards (BIOHAZ), 2016), whereas some non-pathogenic *Bacillus* spp. are used as probiotics to promote host health (Elshaghabee et al., 2017).

Bacillus spp. form stable symbiotic associations with plants and occur in the rhizosphere or as endophytes (Robinson et al., 2016; Shahzad et al., 2016). The plants serve as sheltered ecological niches and provide nutrients to *Bacillus*; in turn, *s*trains of *Bacillus* produce antimicrobial peptides and help the plant to defend against harmful microbes (Szymańska et al., 2016). Therefore, the presence of *Bacillus* endospores in plants including cereals, pulses and cassava relates to the stable occurrence of these organisms as endophytes (Tilak and Reddy, 2006; Zhang et al., 2012; Jamal et al., 2019; Ferreira et al., 2021). The thermostable endospores of *Bacillus* survive the entire storage and processing of plants, even cooking steps (André et al., 2017). Accordingly, cereal grains, pulses and cassava generally harbor endospores or vegetative cells of *Bacillus* (Fangio et al., 2010) and are invariably present in the cooked or uncooked substrates for plant food fermentation (Li et al., 2020). They have been isolated mainly from alkaline fermented foods, as well as various unprocessed and processed foods. In alkaline fermented foods, secondary microorganisms such as lactic acid bacteria (LAB) have been also reported but play a lesser role than *Bacillus* species during the fermentation (Parkouda et al., 2009).

Most traditional food fermentations using *Bacillus* spp. are produced from pulses or tubers in Asia, Africa and South America (Figure 2.2). Until recently, research on food fermenting bacteria has focused on meat, dairy and cereal fermentations; thus, the role of *Bacillus* as a food fermenting organism is under-appreciated and not described in recent reviews. This review aims to provide an overview on the role of *Bacillus* in food fermentations by presenting traditional fermented foods where *Bacillus* spp. are employed as one of the major fermentation organisms, outlining their specific contribution to food quality and safety, and exploring the potential of using these organisms in non-conventional fermentation processes.

Figure 2.2 Geographic location of *Bacillus* species associated food fermentation. **A.** cereal fermented foods; **B.** fermented soy and legumes; **C.** fermented cassava or yams.

2.2 The presence of *Bacillus* in food fermentations

2.2.1 Soybean fermentation products

Soybeans contain a high amount of protein as well as phytochemicals, including isoflavones, lipids, and oligosaccharides (Barnes, 2010). Consumption of soybean and soybean derived fermented products has a long history in many Asian countries, including China, Japan, Korea, Indonesia, and India (Jang et al., 2021). The traditional fermented soybean products can be largely categorized into products fermented by *Bacillus* spp. and products fermented by fungi. *Bacillus* spp. are frequently associated with alkaline food fermentation in which soybeans are used as substrates (Parkouda et al., 2009). An overview on fermented soybeans and the organisms that occur in the fermentation is provided in Table 2.1; several examples are presented in more detail below.

Natto

Natto is a traditional Japanese fermented food produced from soybeans fermented by *natto* starter strains of *Bacillus subtilis* var. *natto*. To make *natto*, soybeans are soaked and steamed, mixed with *B. subtilis* var. *natto* and fermented at 40 °C for 24 h. The *natto* is cooled and aged in the refrigerator for up to 1 week to allow the development of stringiness (Table 2.1). The process of *natto* fermentation is divided into several steps, bacterial growth on the surfaces of steamed soybeans, secretion of proteases from bacteria degrading soy proteins, bacterial incorporation and metabolism of oligopeptides and amino acids, the production and racemization of L- and D-glutamic acids in the bacterial cytoplasm, and the synthesis and secretion of longer chains of poly-γ-glutamate (Kada et al., 2013).

Table 2.1 *Bacillus* spp. associated fermented foods. With exception of fish sauces, all fermentations are solid state fermentations.

Product	Substrate	Main microorganisms	Recipe	Food use
Soybeans or soy protein and legumes				
Natto	Soybeans	*B. subtilis* var. *natto*	Soaked and steamed soy or legumes are inoculated with spores of *B. subtilis* var. *natto* and fermented for 15-24 h (Hitosugi et al., 2015).	Japan; main course as meat substitute
Cheonggukjang	Soybeans, local pulses	*B. subtilis, B. amyloliquefaciens, Rhizopus oligosporus*	Soaked and steamed seeds are inoculated with spores of *Bacillus* and fermented for 1-3 d (Man Cho et al., 2011).	Korea; main course as meat substitute
Doenjang	Soybeans, local pulses	*B. subtilis*, *Rhizopus* spp., and *Aspergillus* spp.	*Meju* is made by soaking, steaming, crushing, and then fermenting for 2 to 3 months. The solid fraction of *meju* is used for ripening for over 2 months to make *doenjang* (Jo et al., 2011).	Korea; condiment
Gochujang	Soybeans with malt, salt, rice flour, and red pepper powder	*Bacillus* spp., including *B. subtilis*, *B. amyloliquefaciens; Aspergillus* spp.; and *Rhizopus* spp.	Ingredients (25 % red pepper powder, 22.2 % glutinous rice, 5.5 % *meju* powder, 12.8 % salt, 5 % malt, and 29 % water) are fermented for 6 months to 1 year (Kim et al., 2010).	Korea; condiment
Hawaijar	Whole soybeans	*B. subtilis, B. licheniformis, S. sciuri, Alkaligenes* spp., *Providencia rettgeri*	The soybeans are soaked, boiled, washed and wrapped with clean cotton cloth/healthy leaves, and packed tightly in a bamboo basket with a lid. The basket is kept warm for fermentation for 4-5 d (Keishing and Banu, 2013).	India up (Asia); staple
Sufu	Tofu (soybean curd)	*Bacillus* spp. or *Micrococcus* spp.	Sufu production consists of four steps: preparation of tofu; brining, inoculation with a pure starter culture to prepare pehtze; and ripening in dressing mixture (Liang et al., 2019).	China up (Asia); side dish
Dawadawa	Locust bean and local pulses	*Bacillus* spp., including *B. subtilis, B. pumilus, B. licheniformis* or *B. subtilis* var. *natto*	The locust beans are cleaned, boiled, pounded and separated the seed coat from the cotyledons. The cotyledons are re-boiled and packed into baskets or perforated pots and allowed to ferment spontaneously for about 48 h. Before fermentation, ash, maize, or millet flour is sprinkled on the cotyledons (Dakwa et al., 2005).	West and central Africa; meat substitute

Table 2.1 (continued).

Product	Substrate	Main microorganisms	Recipe	Food use
Ugba	Locust bean and local pulses	*Bacillus* spp., including *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. pumilus*; *Staphylococcus spp.,* and *Micrococcus* spp.	Boil bean seeds overnight, slice off the cotyledons, cook followed by washing them in water, soaking overnight and then fermenting the sliced cotyledons for a period of 3 to 5 days (Ahaotu et al., 2013).	Nigeria; side dish
			Tubers and roots	
Ntoba Mbodi	Cassava leaves	*Bacillus* spp., including *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. pumilis*, *B. sphaericus*, and *B. xylanilyticus*	The cassava leaves are harvested, allowed to wilt for 2-3 days, cleaned, cut into small pieces, washed with water, distributed into small portions, and wrapped in large leaves, and allowed to ferment at ambient temperature for 2-4 d (Amoa-Awua and Jakobsen, 1995; Mbozo et al., 2017).	Congo
Agbelima	Cassava	*Bacillus* spp., including *B. subtilis*, *B. licheniformis* and *B. pumilus;* lactic acid bacteria and yeasts.	Agbelima is prepared by peeling cassava roots and grating them together with a traditional inoculum which is also prepared from cassava roots. The grated mash is packed into plastic sacks and allowed to ferment whilst weights are placed on top of the sacks to dewater the mash (Mante et al., 2003; Obilie et al., 2003).	West Africa; staple
Tape	Cassava	*Bacillus* spp., including *B. subtilis, B. amyloliquefaciens,* and *B. thuringiensis*	In general, the cleaned and peeled cassava tubers are steamed, cooled, and placed in basket. Powdered ragi is sprinkled over the cassava. The cassava is covered with banana leaves and incubated at room temperature for 2-3 d (Barus et al., 2013).	Indonesia; dessert or ingredient for baking
Elubo (yams)	Yams	*L. plantarum*, *L. brevis* and *B. subtilis*	Yam slices are blanched at 60 °C for 10 min and then fermented at 30 °C for 24 h (Achi and Akubor, 2000).	West African, staple
Taruba	Cassava	*L. plantarum*, *L. brevis* and *B. amyloliquefaciens*	Cassava is crushed and pressed, toasted for 30 min and fermented at ambient temperature for 12 d (Ramos et al., 2015; Jimenez et al., 2022).	Amazon, beverage

Table 2.1 (continued).

Product	Substrate	Main microorganisms	Recipe	Food use
			Vanilla	
Vanilla	Seed pods of *Vanilla planifolia*	*Bacillus* spp., including *B. subtilis*, *B. fusiformis*, and *B. pumilus*	Mature vanilla beans are blanched by immersing in hot water for 3-5 min to destroy the cell tissue structure. Then, the blanching vanilla beans are treated under conditions of high humidity and temperature. The sweating vanilla beans are further dried by sun or air to inhibit mold growth and stored in a closed box for few months (Chen et al., 2015; Gu et al., 2015)hen et al., 2015; Gu et al., 2015).	Madagascar, Indonesia, Mexico, others; aroma
			Cereals	
Daqu	Grains (wheat, rice, sorghum and barley)	*Bacillus* spp., including *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis*, *B. licheniformis* LAB, *Enterobacteriaceae, Aspergillus* spp., *Rhizopus* spp., *Saccharomyces*, and *Saccharomycopsis*	Grains are grounded and mixed with water to ~35 %. The mixture is shaped to bricks and then fermented for 2 months and matured for 2 months (Zheng et al., 2013; Li et al., 2014).	China; starter culture for liquor and vinegar
			Fish	
Fish sauce	Fish	*Filobacillus, Bacillus, Micrococcus, Virgibacillus, Pseudomonas, Halobacillus, Halococcus*	The fishes are mixed with salts and fermented for 6 to 12 months at room temperature (Udomsil et al., 2011; Du et al., 2019).	Southeast Asia up; sauce

Cheonggukjang

Cheonggukjang is a traditional Korean soybean paste made from cooked whole soybeans fermented with *Bacillus* species, which usually prepared at around 40 °C for 2-3 days in the air or in rice straw (Table 2.1) (Man Cho et al., 2011). Fermentation with *Bacillus* breaks down soybean protein and polysaccharides into peptides and monosaccharides, resulting *cheonggukjang* with sticky gums and bioavailable nutrients. In addition, isoflavones, including genistein, daidzein, and daidzin, are converted to the corresponding aglycones during fermentation (Man Cho et al., 2011; Go et al., 2016). In *cheonggukjang* fermented with *B. subtilis* CS90, genistein increased from an undetected level to 26 mg/kg after 60 h of fermentation; likewise; daidzein increased from undetectable levels to 330 mg/kg while the daidzin content decreased from 487 to 67 mg/kg (Man Cho et al., 2011).

Doenjang

Doenjang is a fermented soybean paste widely used for many Korean foods. *Doenjang* is manufactured by fermentation of *meju*, dried soybean blocks. *Meju* is traditionally prepared by soaking, steaming, crushing, and then fermenting for 2-3 months with *B. subtilis*, *Rhizopus* spp., and *Aspergillus* spp. The fermented *meju* is separated into two parts; the supernatant liquid part is filtered to prepare soy sauce and the precipitated solid part is used for further ripening for over 2 months to make *doenjang* (Table 2.1) (Jo et al., 2011). It takes more than 6 months to manufacture *doenjang* using the traditional method.

Gochujang

Gochujang is a traditional Korean fermented paste made from *meju*, red pepper powder, and glutinous rice. The characteristic flavor of gochujang is a combination of hot taste from red pepper,

sweet taste from sugars, umami taste from amino acids, and salty taste from NaCl. *Gochujang* is produced by fermentation with *Aspergillus* spp., *Bacillus* spp., and *Rhizopus* spp. for several years in large earthen pots by mixing glutinous rice powder, salt, and red pepper powder with *meju* powder (Table 2.1) (Kim et al., 2010).

Hawaija

Hawaijar is an indigenous traditional fermented soybean product in Manipur, India, with a distinct flavour and stickiness (Tamang, 2015). It is consumed commonly in the local diet as a low-cost and staple source of high protein food (Jeyaram et al., 2008). Whole soybeans are soaked overnight, washed and boiled until the seeds are soft. After draining the excess water, the cooked soybeans are washed with hot water, wrapped with clean cotton cloth or healthy banana leaves, and packed tightly in a basket with lid. The base and sides of the basket are layered and lined with banana leaves. The basket is wrapped with cloth and kept in the sun or near stove or buried in paddy straw for fermentation for 4 to 5 days (Table 2.1). The final product has a pH of 8.0 to 8.2 and is brown in colour with a sticky slimy white appearance and a light ammonia odour (Jeyaram et al., 2008; Keishing and Banu, 2013). In *hawaijar* fermentation, no starter culture is added during its preparation. The different *Bacillus* strains that dominate the fermentation originate from raw soybeans (Zhang et al., 2012) or is acquired from other materials used during fermentation (Keishing and Banu, 2013).

Sufu

Sufu is a Chinese fermented soft cream cheese-type product made from cubes of soybean curd (tofu) by mold ripening (Feng et al., 2013). Five steps are normally involved in *sufu*-making; preparing tofu *via* salt precipitation from boiled soymilk; pre-salting; preparing pehtze with a pure starter culture; salting; and ripening for 2-3 months in a dressing mixture. During the pre-salting

procedure, the tofu adsorbs the salt until the salt content of tofu reaches about 6.5 %, which takes about 2 days. Pehtze is prepared by inoculating pure culture of *Bacillus* spp. or *Micrococcus* spp. to tofu and incubated at 30-38 °C for about 1 week. To maintain the shape of the final product, pehtze is dried at 50-60 °C for 12 h before salting. The composition of the microbiota is the most important factor in the characteristics and flavor of *sufu*. The most common types are mold-fermented *sufu*, and bacteria-fermented *sufu*, which includes *Bacillus* and/or *Micrococcus* as major members of fermentation microbiota (Table 2.1) (Liang et al., 2019).

2.2.2 Roots, tubers, and vanilla fermentation products

Starchy root and tuber crops are second only to cereals in importance as a global source of carbohydrates. In most African and Asian countries, the traditional diets of a majority of people rely largely on starchy staples such as cassava and yam, which are poor in other nutrients, particularly proteins, essential amino acids, vitamins and minerals, but contain significant amounts of calories and dietary fiber (Chandrasekara and Josheph Kumar, 2016).

Cassava

Cassava is an important food and one of the most important sources of energy throughout tropical regions of Africa, Asia and Latin America (Chandrasekara and Josheph Kumar, 2016). The root of the cassava plant contains a high amount of digestible starch but a low content of protein and free amino acids. Cassava also contains the cyanogenic glycosides linamarin and lotaustralin that release cyanide during digestion unless the β-glucosidic bonds are hydrolysed during food processing (Salvador et al., 2014). An overview on fermented tubers and the organisms that occur in the fermentation is provided in Table 2.1; several examples are presented in more detail below.

Ntoba Mbodi

Ntoba Mbodi is a popular alkaline fermented food in Congo where it constitutes a significant source of protein in the diet of the consumers (Mbozo et al., 2017). It is made by fermenting cassava leaves in the following way: the leaves are harvested, wilted for 2-3 days, cleaned, cut into small pieces, wrapped small portions in large leaves and allowed to ferment at ambient temperature for 2-4 days. Cassava leaves also contain cyanogenic glucosides that need to be eliminated by fermentation or processing (Amoa-Awua and Jakobsen, 1995; Mbozo et al., 2017). A rise in pH of up to 10 is observed during the process. The main microorganisms responsible for the fermentation are *Bacillus* spp. (Table 2.1) (Mbozo et al., 2017).

Cassava tape

Cassava tape (fermented cassava) is an Indonesian traditional food made by fermentation of cassava. It is made from steamed cassava mixed with a starter commonly known as "Ragi Tape". In general, the cleaned and peeled cassava tubers are steamed, cooled, and placed in basket. Powdered ragi is sprinkled over the cassava. The cassava is covered with banana leaves and incubated at room temperature for 2-3 d (Table 2.1). The quality of cassava tape depends on many conditions, including the quality of cassava, preparation method, and microbes (Barus et al., 2013). Several *Bacillus* species including *B. subtilis*, *B. amyloliquefaciens* and *B. thuringiensis* have been isolated from cassava tapes. It was reported that *B. subtilis* mainly determined the quality of cassava tape (Barus and Wijaya, 2011).

Elubo

Yams (*Dioscorea* spp.) are processed and fermented to the traditional West African dried yam flour "Elubo". Yam slices are blanched at for 10 min 60 °C and fermented for 24 h at 30 °C. During

the first 24 h of spontaneous fermentation, the microbial population grows as the pH falls from 6.2 to 5.4. Back-slopping at a rate of 10 % (w/v) is used to accelerate natural lactic fermentation by repeatedly using the preceding fermentation batch as an inoculum. Fermentation experiment of blanched samples with pure cultures of the isolates indicated that *Lactiplantibacillus plantarum*, *Levilactobacillus brevis* and *B. subtilis* to be the main species responsible for pH reduction and in changes in the browning levels of the reconstituted flour paste (Table 2.1) (Achi and Akubor, 2000).

Taruba

Indigenous populations in the Amazon region of South America produce fermented cassava beverages for daily consumption from cassava alone, or from cassava with addition of corn or potatoes. Cassava roots are generally washed and soaked, followed by crushing and sieving, cooking or toasting, and fermentation for one or several days. Bacilli including *B. subtilis* and *B. amyloliquefaciens* were consistently identified as members of fermentation microbiota, which also includes *Lp. plantarum*, *Lv. brevis* and pediococci (Ramos et al., 2015; Jimenez et al., 2022).

Vanilla

Vanilla flavoring obtained from cured *Vanilla planifolia* (Andrews) beans is widely used in food, beverages, and cosmetics (Kaur and Chakraborty, 2013). The characteristic vanilla flavor is formed during a curing process that yields the character impact compound vanillin and other flavor volatiles. The conventional curing processes involves four steps: blanching, sweating, drying, and conditioning. Mature fresh vanilla beans are blanched and then stored at high humidity and temperature. Sweating retains a sufficiently high moisture content for the enzyme-catalyzed reaction. The vanilla beans are dried by sun or air to inhibit mold growth and stored in a closed box for several months for formation of vanillin (Chen et al., 2015; Gu et al., 2015). Thermophilic

bacilli develop during sweating (Table 2.1) (Röling et al., 2001). *Bacillus* isolated from vanilla beans produced β-D-glucosidase, which hydrolyses glucovanillin to vanillin (Chen et al., 2015).

2.2.3 Cereal fermented product

Daqu

Daqu is a spontaneous solid-state cereal fermentation that is used as a saccharification starter to initiate the alcoholic mash fermentation for production of cereal liquors and vinegars (Figure 2.3). It provides amylolytic and proteolytic enzymes to sustain alcoholic fermentation and flavor formation in the subsequent mash fermentation (Zheng et al., 2013; Li et al., 2014). The liquor starter is prepared in an open system with different grains including wheat, rice, sorghum, that are shaped in blocks and fermented with controlled temperature and humidity (Zheng et al., 2011). The microbiota composition of *daqu* is diverse and includes *Bacillus* spp., *Enterobacteriaceae*, and lactic acid bacteria (Table 2.1) (Gänzle, 2019). Fungal organisms include *Aspergillus* spp., *Mucor* spp., *Penicillium* spp. with *Aspergillus* spp. (Wang et al., 2008; Deng et al., 2021); the most frequently isolated yeasts belong to the genus *Saccharomyces* (Wang et al., 2008). In China, *daqu* is also used as a starter culture for sourdough fermentation but bacilli were not identified as members of microbiota of the corresponding sourdough (Yan et al., 2019).

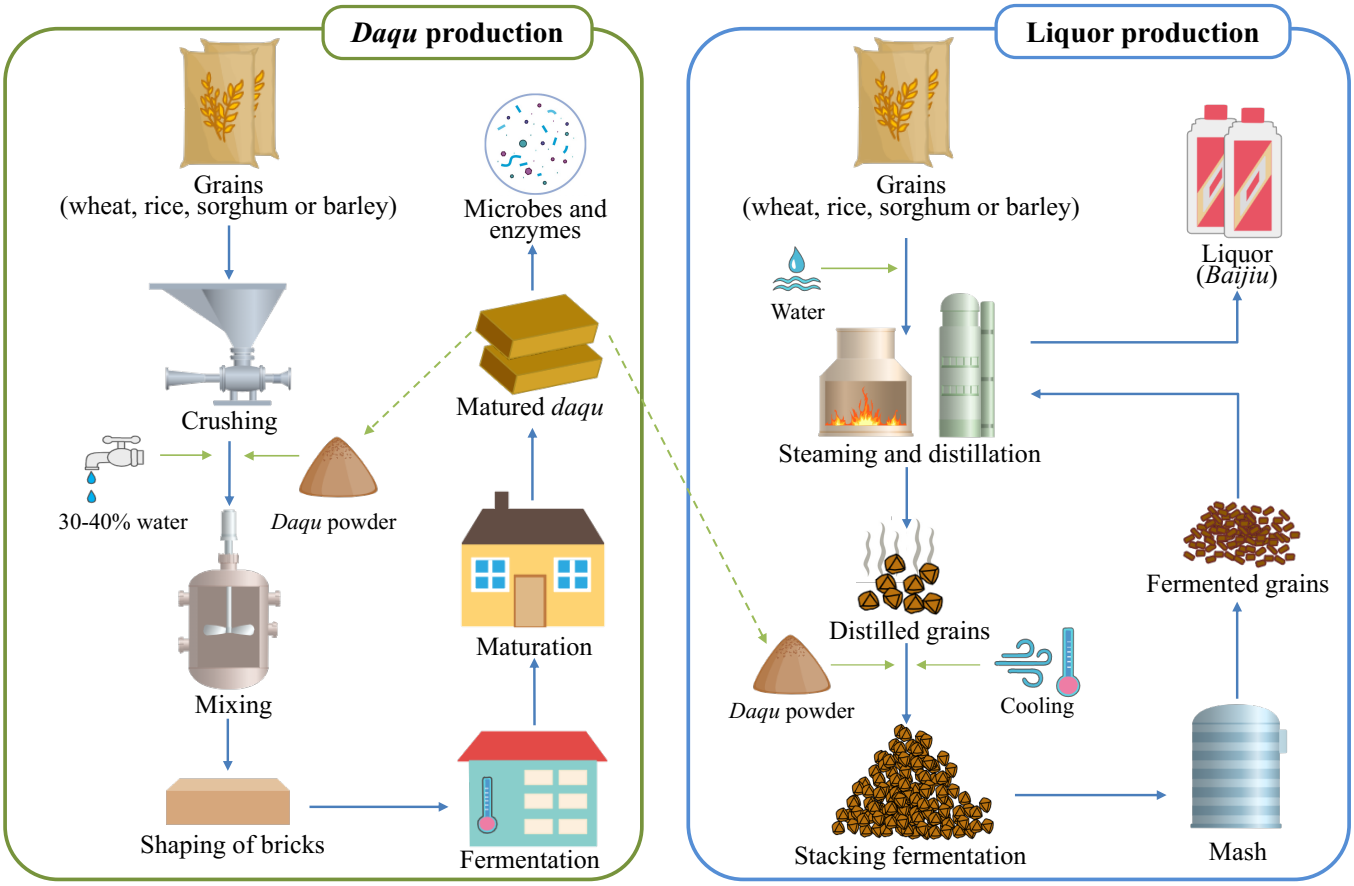


Figure 2.3 Flow chart of traditional Chinese liquor brewing process including the process of the *daqu* production (left) and the liquor production (right). The stacking fermentation is used for production of few but not all liquors (Yang et al., 2017; Wang and Li Wang, 2022).

2.2.4 Fish sauce

Fish sauce is a popular seasoning that is traditionally consumed in Southeast Asia but becomes increasingly popular in other places of the world (Redzepi and Zilber, 2018). It is produced by mixing fish, such as anchovies, with salts and fermenting for 6 to 12 months at room temperature (Toyokawa et al., 2010). Fish proteins are hydrolyzed by proteases from the fish and from halotolerant/halophile microorganisms, resulting in umami-tasting peptides and amino acids (Park

et al., 2002; Curtis, 2009). The fermentation organisms in fish sauces predominantly include halophilic or salt tolerant bacteria (Table 2.1) (Udomsil et al., 2011; Du et al., 2019).

2.3 The role of *Bacillus* in food fermentation

Bacillus spp. have a variety of desirable characteristics, including their ability to form endospores, and several strains or species have been awarded GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (Yan et al., 2013) and QPS (Qualified Presumption of Safety) by European Food Safety Authority (Koutsoumanis et al., 2022). Bacilli are also used in agriculture as pesticides or plant growth promoters, and as cell factory for production of chemicals, enzymes, and antimicrobial compounds. Figure 2.4 provides an overview on the presence of desirable (production of hydrolytic enzymes and antifungal lipopeptides) or undesirable (toxins producing) metabolic traits in type strains of the genus *Bacillus*. The role of *Bacillus* as source of industrial enzymes has been well reviewed elsewhere (Danilova and Sharipova, 2020; Su et al., 2020). In this review, the microbial enzymes produced by *Bacillus* during food fermentations are discussed.

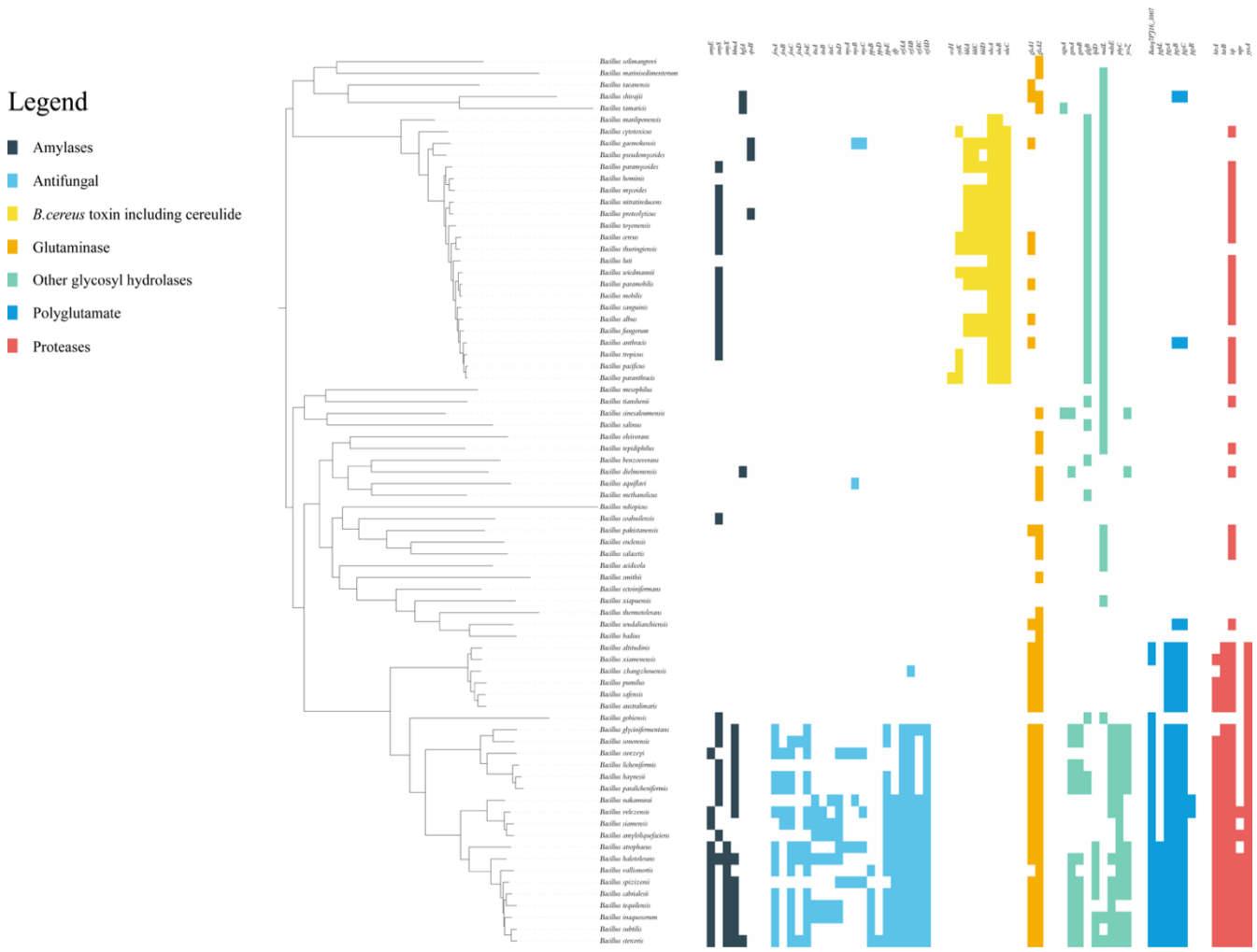


Figure 2.4 Core genome phylogenetic tree of type strains in the genus *Bacillus* (left) and genes coding for useful enzymes, or toxins, in these type strains of *Bacillus* spp. (right).

Amylolytic system: AmyE (P00691), α-amylase; AmyS (P06279), heat stable α-amylase; BbmA (O06988), β-amylase**;** SpoII (P36924), β-amylase; AmyX (C0SPA0), pullulanase or debranching enzyme.

Other glycosyl hydrolases: BglA (P22073), β-glucosidase; GanA and GanB (O07012 and O07013), exo- and endo-β-galactosidases/β-galactanases; YesZ (O31529), β-galactosidase; LplD (P39130), α-galactosidase; AguA (Q09LY5), thermostable xylanase; PhyC (O31097).

Proteolytic system: HtrA and HtrB (O34358 and Q9R9I1), envelope-associated serine proteases; YyxA (P39668), uncharacterized serine protease.

Polyglutamate synthesis and glutaminases: PghL (A0A6M3ZBI1), poly-γ-glutamate hydrolase, PgsABCR (A0A6M3ZGL0, E0U4Z3, A0A6M4JPT9, and Q45449), poly-γ-glutamate synthetase and regulatory genes; GlsA1 and GlsA2 (O31465 and O07637), glutaminases.

Antimicrobial lipopeptides: FenABCDE (A0A7G7U8D7, H9TE69, A0A7G7U8D5, A0A7T5SHH4 and A0A7G7U8D6), fengycin synthase; ItuABCD (Q93I56, Q93I55, Q93I54 and X5F8R7), iturin synthase; MycABC (Q9R9J1, Q9R9J0 and Q9R9I9), mycosubtilin synthase; PpsBDE (A0A6H2JR43, A0A6H2JR81 and O31827), plipastatin synthase (doi: 10.6026/97320630007384); SrfAA SrfAB, SrfAC and SrfAD (P27206, Q04747, Q08787 and Q08788), surfactin synthase.

Toxins: CesH (Q20CJ2), cereulide synthetase; CytK (Q63EQ2), cytotoxin K; HblACD (A0A2A8M4Y0, Q9L4L8 and Q9REG7), Hemolysin BL; NheABC (A0A8F1XQI7, A0A8I1GLU0 and A0A8F1XT31), Nhe enterotoxin.

2.3.1 Amylases

The amylolytic system of *Bacillus* species includes the glucan branching enzyme GlgB, extracellular amylases including α-amylases, β-amylases, pullulanases and glucoamylases, and intracellular oligosaccharide hydrolases. Amylases are produced by most strains of the *B. cereus* and *B. subtilis* groups; type species of the latter clade typically encode for multiple amylases. The *Bacillus* amylases play an essential role in fermentation of starchy crops (Zheng et al., 2011). Roots and tubers, such as yams and cassavas have a low content of fermentable carbohydrates and low amylase activity (Srichuwong et al., 2005). Therefore, extracellular amylases produced by *Bacillus* spp. are particularly important for the hydrolysis of starch in cassava and yams fermentation (Senthilkumar et al., 2012).

Thermostable amylases of bacilli are also a key element of *daqu* fermentations (Wang et al., 2017). Strains of the *B. subtilis* group that are present in *daqu* fermentations have a high starch degrading ability including thermotolerant α-amylase activities (Jin et al., 1990; Yan et al., 2013). Amylase activity in *daqu* was significantly increased by inoculation with *B. licheniformis* (Wang et al., 2017). Moreover, amylases produced by *B. licheniformis* in *daqu* fermentation yielded maltose, maltotriose, and maltodextrins as major products from starch (Li et al., 2018).

2.3.2 β-Glucosidase, phytase and α-galactosidase

β-Glucosidase (β-D-glucopyranoside glucohydrolase) removes nonreducing terminal glucosyl residues from saccharides and glycosides. It hydrolyzes β-glucosidic linkages between carbohydrate residues in aryl-amino- or alkyl-β-D-glucosides, cyanogenic glucosides, short chain oligosaccharides and disaccharides under different physiological conditions (Cairns and Esen, 2010). The gene that produce BglA is encoded by most type strains of the *B. subtilis* group (Figure 2.4).

Isoflavones are abundant in soybeans and have various health benefits related to their oestrogenic activities (Wei et al., 2008). They have bioactivity only when they are decomposed into free isoflavone aglycones. However, isoflavones in soybeans usually exist in the form of inactive glycosides and are rarely found in aglycone forms (Setlow et al., 2004). The *Bacillus*-mediated soybean fermentations are similar, and *natto* is given as an example of soybean fermentations. During the *natto* fermentation, glucoside conjugates of isoflavones are converted into aglycones by β-glucosidase (Li et al., 2021a), which are an important factor for the health benefits of *natto* (Zhou et al., 2018). The activity of β-glucosidase reached the highest level after 12 h of *natto* fermentation and the expression level of the genes encoding β-glucosidase also significantly increased. Consequently, the content of free isoflavones in *natto* was 3.2 times higher than that in soybeans, which further improving the health value of *natto* (Li et al., 2021a).

The β-Glucosidase of *Bacillus* species also plays an essential role in the hydrolysis of cyanogenic glycosides. Cassava is one of the most important sources of calories in some countries in Africa and South America but the poisonous cyanogenic glucoside linamarin must be removed prior to consumption (Alitubeera et al., 2019). Linamarin accounts for 80 % of the cyanide content of cassava and is known to cause severe disease upon continual consumption. Several strains of

bacteria were isolated from cyanide-rich cassava peel waste and tested for their potential to detoxify cassava. β-Glucosidase activity of bacilli detoxifies cassava cyanides without compromising other nutrients (Murugan et al., 2014).

Flavor of cured vanilla beans is quite complex, but vanillin is the main aromatic constituent in cured vanilla pod (Pérez Silva et al., 2011). In fresh vanilla beans, vanillin is present as glucovanillin, a β-D-glucoside of vanillin and fresh beans have no trace of vanilla flavor. Curing consists mainly to maintain vanilla pods in high temperature and humidity conditions as long as possible while limiting proliferation of microorganisms. One of the most important aspects of curing is when glucovanillin encounters β-glucosidase, releasing free vanillin (Odoux et al., 2006). *Bacillus* isolates from vanilla fermentation produce β-glucosidase, which mediates glucovanillin hydrolysis and influences vanillin formation during conventional curing (Chen et al., 2015). Its formation may be due to the action of thermo-tolerant bacteria such as *B. subtilis*, as they survive in high temperature environments (up to 65°C) during vanilla curing.

Fermentation is considered a necessary and accepted method to reduce the anti-nutritive compounds in cereals and pulses, including phytic acid and raffinose family oligosaccharides (Budhwar et al., 2020). Most of total seed phosphorus (P) in legumes and cereal grains is present as phytate, which chelates minerals, decreasing their bioavailability to humans and monogastric animals (Raboy, 2020). In cereal fermentations with LAB, microbial acidification in conjunction with cereal phytases strongly reduces the phytate content of product (Poutanen et al., 2009). *Bacillus* spp. are effective producer of phytases, which hydrolyze phytate to less-phosphorylated inositol and orthophosphate and increases the bioavailability of minerals in cereals and legumes (Figure 2.4) (Phengnuam and Suntornsuk, 2013). The *Bacillus* phytases, which are active at a wide range of pH, high thermal stability, and strict substrate specificity for the calcium–phytate

complex, have considerable potential in commercial and environmental applications. Soybean products are frequently made from heat-processes which necessitates the presence of microbial phytase activity. Soaking, heating, and fermentation during *natto* manufacture decrease phytate content, which in turn, could improve nutrient bioavailability. Recently, the *natto* prepared using low-phytate soybean have been studied to be an alternative of traditional *natto* as a cheap and nutrient-rich food, with higher bioavailability of nutrients, and could contribute to nutrient and food security in low-income countries (Qin et al., 2020).

Raffinose family oligosaccharides (RFOs) (i.e., raffinose, stachyose, verbascose) are abundant in soybean and legumes. They are complex sugars with one or more galactose residues joined by α-1,6-glycosidic bonds to a sucrose (Aulitto et al., 2021). α-Galactosidases are enzymes that react on α-galactosides and are becoming increasingly significant with respect to our diet. Due to the lack of pancreatic α-galactosidase in monogastric animals (including man), these indigestible carbohydrates, identified as anti-nutritional factors, can lead to flatulence and other gastrointestinal disorders (di Stefano et al., 2007). Additionally, the increased consumption of soybean or other legume products in human and animal food contributes to the growing importance of the use of using α-galactosidases in the degradation of RFOs to improve the digestion efficiency and nutritional value of food and feed (Álvarez-Cao et al., 2019). The ability to produce α-galactosidase has been investigated in some novel isolates from different habitats. In many applications, such as the pasteurization of soymilk, thermostable enzyme from extremophilic bacteria can resist high processing temperatures (Álvarez-Cao et al., 2019; Bhatia et al., 2020). *Bacillus* species, particularly species of the *B. subtilis* group also encode for α-galactosidases, which hydrolyse raffinose-family oligosaccharides in soybean and legumes (Bhatia et al., 2020).

2.3.3 Proteases

Strains of the *B. subtilis* group but not the other bacilli produce multiple extracellular proteinases (Figure 2.4). Because of their broad pH and temperature activity and stability range, proteases from *Bacillus* play an essential role in soybean and fish fermentations to obtain bioactive peptides and process different foods as they are tolerant to alkaline environments and high temperature.

Proteases are secreted in large amounts by *B. subtilis* var. *natto* during *natto* fermentation. They break down soybean proteins into amino acids, which serve not only as nutrients for bacterial growth, but also provide flavor, and serve as substrates producing two important characteristics of *natto*, the viscous texture and a specific smell.

Fermented fish sauces are also rich in soluble proteins, peptides, and amino acids (Park et al., 2002; Curtis, 2009). They are produced during proteolytic degradation by proteases in the muscles or digestive tracts of fish, and various microorganisms existing in the fermentation broth (Yongsawatdigul et al., 2007). Proteases produced by *Bacillus* play an essential role in the degradation process. To continuously progress proteolytic degradation of fish protein over a long ripening period, a thermotolerant and halotolerant enzyme is a more useful catalyst than a mesophilic or thermophilic enzyme. A halotolerant protease produced by *B. licheniformis* has been found in Thai fish sauce with a high enzymatic activity, even under high salt conditions (30 % NaCl) (Toyokawa et al., 2010).

2.3.4 Polyglutamate and glutaminase

Poly-γ-glutamic acid (γ-PGA) is synthesized by polymerizing D- and L-glutamate to an anionic biopolymer comprised of only glutamic acid residues. It is water soluble and biodegradable and has good thickening capacity, and excellent absorbability (Hsueh et al., 2017). γ-PGA is produced

by *Bacillus* spp. to form part of the mucilage of *natto* and *cheonggukjang* (Ashiuchi et al., 2001; Kada et al., 2008). Polyglutamate production is also observed mainly but not exclusively by strains of the *B. subtilis* group (Figure 2.4).

The stringy consistency of *natto* is mainly due to γ-PGA produced by *B. subtilis* var. *natto* which is the main microorganism used in the soybean fermentation of *natto* (Kada et al., 2008). The viscosity of *natto* caused by γ-PGA-producing *Bacillus* strain can be decreased by the addition of NaCl to the medium. The production of γ-PGA from *natto* starters were reduced in medium containing NaCl greater than 3 % (Ogawa et al., 2014). A salt tolerant *B. subtilis* strain was found in a *cheonggukjang* fermentation, which also produce γ-PGA (Ashiuchi et al., 2001).

L-glutaminase is an enzyme majorly produced by microorganisms that catalyzes L-glutamine to L-glutamic acid. The glutamic acid is an important "Umami" substance that plays a role in both sensory and nutritional properties of food (Wakayama et al., 2005). L-glutaminases are relevant enzymes for a variety of applications. In food processing industry, the enzyme has been thought to be a useful additive to improve the quality of food in terms of both flavor and nutrition (Wakayama et al., 2005). In addition, L-glutaminase is used as a flavor stimulator by increasing the glutamic acid level in many fermented foods such as soy sauce and others (Orabi et al., 2019). Therefore, finding more viable strains with higher yields as well as economically viable bioprocesses for large-scale production are both required by its commercial value. Furthermore, the enzyme should possess distinctive characters such as thermostability, resistance to severe environments, and the capacity to function at a wide range of pH values. *Bacillus* species play important roles as the dominant bacteria in industrial production of L-glutaminase (Lyngwi and Joshi, 2014). Numerous *Bacillus* spp. have been shown to produce L-glutaminase with tolerance

to high temperature and high salt conditions. (Sathish and Prakasham, 2010; Ye et al., 2013; Lyngwi and Joshi, 2014; Orabi et al., 2019).

2.3.5 Lipopeptides

Bacillus species produce a wide range of lipopeptides with broad spectrum of antimicrobial activities (Khan et al., 2018). The lipopeptides produced by *Bacillus* spp. can be broadly grouped into three families depending on their amino acid sequence: surfactins, fengycins and iturins (Gordillo and Maldonado, 2012; Meena and Kanwar, 2015). The non-ribosomal peptide synthases that synthesize these lipopeptides are present almost exclusively in strains of the *B. subtilis* group.

The surfactins are powerful biosurfactants, which have antibacterial activity but, with some exceptions, no marked antifungal activity (Ongena and Jacques, 2008). Iturins display a strong antifungal action against a wide variety of yeast and fungi but only limited antibacterial and no antiviral activities. For example, iturin from *B. pumilus* HY1 inhibited *A. flavus* and *A. parasiticus* with an MIC of 50 mg/L (Cho et al., 2009). This fungitoxicity of iturins has been attributed to their membrane permeabilization properties (Gordillo and Maldonado, 2012). Fengycins also have strong fungitoxic activities, specifically against filamentous fungi (Ongena and Jacques, 2008). Presence of the lipopeptides produced by *Bacillus* was shown in many fermented products, such as *cheonggukjang*, *daqu*, and some other solid-state fermentations (Slivinski et al., 2012; Lee et al., 2016a; Chen et al., 2020; Owusu-Kwarteng et al., 2020) but studies on *in situ* antimicrobial activity of bacilli are scarce. *In vitro* studies indicate that the ability of *B. subtilis* to inhibit aflatoxin-producing fungi was substantially greater than the inhibitory effect of lactic acid bacteria (Kim, 2007).

2.3.6 Health

Probiotics

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agricultural Organization of the United Nations and World Health Organization, 2001). Over the years, probiotics have gained popularity and have found application in several general health and clinical scenarios (Elshaghabee et al., 2017). The role of LAB as probiotics for the maintenance of human and animal health is widely recognized and well documented (Hill et al., 2014; Merenstein et al., 2020). Species of the *Lactobacillus, Lacticaseibacillus, Ligilactobacillus, Lactiplantibacillus, Limosilactobacillus* and *Bifidobacterium* are the most prevalent LAB found in probiotics; however, they are not heat resistant. Endospores of *Bacillus* species have been proposed as heat resistant alternatives (Konuray and Erginkaya, 2018). In addition to the commonly explored LAB strains, the genus *Bacillus* also carry probiotic attributes. Although the health benefits of strains of *Bacillus* as probiotics are not as well documented as those of lactobacilli, randomized clinical trials support reducing levels of serum blood cholesterol, antimutagenic effects, inhibiting gastrointestinal pathogens, and antioxidant activity (Nithya and Halami, 2013; Shobharani and Halami, 2014; Yogesh and Halami, 2015).

The distinction between fermented foods and probiotics was clarified by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Marco et al., 2021). It is not acceptable to use the term "fermented food" and "probiotic" interchangeably. The term "probiotic" should only be used when well-defined and characterized live microorganisms have proved to provide a health benefit. The health benefit must outweigh any nutritional advantage provided by the food matrix. Evidence of a strain-specific benefit from a well-controlled intervention study, as

well as proven safety and confirmation of sufficient numbers of that strain in the final product to confer the claimed benefit, are required to label a product as a probiotic fermented food with an additional stipulated health benefit. Fermented food containing probiotic strains can be labeled as a "probiotic fermented food", if the evidence of a strain-specific benefit from a well-controlled intervention study, as well as proven safety and confirmation of sufficient numbers of that strain in the final product to confer the claimed benefit, are provided (Marco et al., 2021). Therefore, to use *Bacillus* strains as probiotic, the randomized clinical trial must be completed. Additionally, the efficacy and health benefits of *Bacillus* spp. as probiotics have been screened in several *in vitro* and *in vivo* animal models and a few have also been validated in human clinical trials. They were well summarized in other review (Elshaghabee et al., 2017).

However, in comparison to LAB, even though *Bacillus* spp. offer these benefits, the *Bacillus* spp. as probiotics have not gained high popularity since the debate over probiotic *vs.* pathogen tag of *Bacillus* in the research and production terrains is confusing consumers (Elshaghabee et al., 2017). Hence, it is important to clearly understand the phenotypic and genotypic characteristics of selective beneficial *Bacillus* spp. and their substantiation with those having safe status, to reach a consensus over the same (Bader et al., 2012).

Toxins

Bacillus species are also of concern for food safety. Toxins are produced by certain strains of the *B. cereus* group (Figure 2.4). The *B. cereus* group includes eight species, *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus* and the recently described species *B. toyonensis* (EFSA Panel on Biological Hazards (BIOHAZ), 2016). Toxins produced by these organisms include the non-haemolytic enterotoxin (*nhe)*; haemolysin BL (*hbl*); cytotoxin K (*cytK*), cereulide, which is produced by a non-ribosomal peptide synthase,

and the anthrax toxin. Production of these toxins is observed only in the *B. cereus* clade (Figure 2.4). The non-haemolytic enterotoxin (*nhe)* and the haemolysin BL (*hbl*), as well as the single protein cytotoxin K (*cytK*) have been linked to the diarrheal type of *B. cereus* food poisoning, which is characterized by abdominal pain and watery diarrhea (Schoeni and Lee Wong, 2005; Ehling-Schulz et al., 2015). A heavy diarrhea of a day or two will happen if large numbers of cells are ingested. These enterotoxins are also frequently found in other *B. cereus* group members, including *B. thuringiensis*. Nearly all *B. cereus* strains harbor the *nhe* genes, while *hbl* and *cytK* are detected in about 30-70 % of isolates (EFSA Panel on Biological Hazards (BIOHAZ), 2016).

The emetic toxin cereulide produced by *B. cereus* and *B. weihenstephanens* is a major cause of food borne intoxications leading to diarrheal and emetic syndrome. Cereulide is commonly produced in food resulting in rapid onset of vomiting (15 min to 6 h) after ingestion of the contaminated food. So far, the cereulide toxin synthesis (*ces*) gene has only been identified in few clades of the *B. cereus* group (EFSA Panel on Biological Hazards (BIOHAZ), 2016).

B. anthracis is the causative agent of anthrax, an acute fatal disease in animals and in humans. Toxins specific for *B. anthracis* are among the most dangerous toxins because they are associated with deadly illness in humans and animals (Elshaghabee et al., 2017; Griffiths and Schraft, 2017). The *B. anthracis* generated bipartite exotoxins include protective antigen-lethal factor (PA-LF) and PA-edema factor (PA-EF) (EFSA Panel on Biological Hazards (BIOHAZ), 2016).

The *B. cereus* group was not evaluated for the QPS list by EFSA because the majority of strains in this category produce toxins. In contrast, foodborne intoxication caused by *Bacillus* species other than *B. cereus* is uncommon. The production of enterotoxins and poisonous peptides is a qualification in the QPS technique that eliminates *Bacillus* strains linked to safety problems. *Bacillus* species having a substantial body of knowledge and a history of apparent safe use in the

feed or food chain were thus included to the QPS list, with the qualification of "absence of toxigenic potential" (Koutsoumanis et al., 2020, 2022).

2.4 Conclusions

Bacillus species are an under-appreciated group of bacteria that are prevalent in numerous food fermentations, particularly in Asia and Africa. Strains of the *Bacillus subtilis* group produce extracellular hydrolytic enzymes, potent antifungal lipopeptides and extracellular polymeric compounds. These properties that are not or only rarely found in other dominant groups of food fermenting bacteria, including lactic acid bacteria, acetic acid bacteria, or propionic acid bacteria. With the better understanding and further study of *Bacillus*, the fundamentally positive characteristics of the *Bacillus* species will make them the preferred hosts for the many novel and improved food fermentation products.

Chapter 3 Composition and Activity of Antifungal Lipopeptides Produced by *Bacillus* spp. in *Daqu* Fermentation

3.1 Introduction

Chinese liquor (*Baijiu*) is a distilled liquor and one of the most popular alcoholic beverages in China (Liu and Tong, 2017). Chinese liquor is made with sorghum, wheat, rice, barley, or corn (Zheng and Han, 2016; Chetrariu and Dabija, 2021). Different from alcoholic cereal fermentations in Africa and Europe, where malt is used as a source of enzymes, hydrolytic enzymes are provided by *daqu,* which is based on spontaneous fermentation (Jin et al., 2017). *Baijiu* also differs from Japanese sake, which uses back-slopped *koji* with domesticated strains of *Aspergillus oryzae* as the main or sole fermentation organism (Gänzle, 2019). The fermentation process of Chinese liquor consists of two stages: production of the saccharification starter *daqu* and the mash fermentation for ethanol production (Huang et al., 2017; He et al., 2019). *Daqu* can be divided into three categories: low-, medium-, and high-temperature *daqu*, which are used to produce Chinese liquor with light flavor, sauce flavor or strong flavor, respectively (Sakandar et al., 2020). The medium-temperature *daqu* is the most widely used starter in the production of traditional Chinese *baijiu* (Xiao et al., 2017).

The composition of microorganisms in *daqu* mainly consists of bacteria, mycelial fungi, and yeasts (Chen et al., 2021). Bacterial species include *Bacillus* spp., *Enterobacteriaceae* and lactic acid bacteria. Strains of *Bacillus* were found to be the dominant species throughout the fermentation (Xu et al., 2022). Fungal strains consist of *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., with *Aspergillus* spp. being the dominant species (Wang et al., 2008; Deng et al., 2021). The most frequently isolated yeasts belong to the genus *Saccharomyces* (Wang et al., 2008).

Daqu is produced using unsterilized raw materials in an open environment, and the fermentation organisms are derived from the raw materials or the environment. *Bacillus* spp. form stable associations with plants, are present in the rhizosphere or as endophytes (Robinson et al., 2016; Shahzad et al., 2016), and thus are invariably present in or on cereal grains (Li et al., 2020). The shape of the *daqu* blocks provides a large surface area to support the growth of the aerobic bacilli, and the low moisture content slows the growth of *Enterobacteriaceae* (Xie et al., 2020). This allows *Bacillus* spp. to become the dominant organism during the fermentation process (Yan et al., 2013). Endospores formed by *Bacillus* remain active at low moisture content and high temperature conditions, supporting their presence as the most frequently isolated bacteria from *daqu* (Wang et al., 2008).

During the *daqu* fermentation, hydrolytic enzymes including amylolytic enzymes are produced by bacteria and fungi (Li et al., 2015b; Liu et al., 2018). Of the bacterial fermentation organisms, *Bacillus* spp. are the major group that produces extracellular amylolytic enzymes (Setlow, 2006; Valerio et al., 2012). Several genes encoding amylases have been identified in the genomes of *Bacillus* spp. (Li et al., 2020). Amylases produced during *daqu* fermentation are the major contributors to starch liquefaction and saccharification in the subsequent mash fermentation (Li et al., 2015a). In addition, proteolytic enzymes produced by fungi and bacilli generate amino acids as precursors for volatile flavor compounds (Liu et al., 2018).

Bacillus spp. also produce a wide range of lipopeptides with a broad spectrum of antimicrobial bioactivity (Cochrane and Vederas, 2016; Zhang et al., 2022). These lipopeptides are produced by polyketide synthases (PKSs) and nonribosomal peptide-synthetases (NRPS) (Moyne et al., 2004). Lipopeptides produced by *Bacillus* spp. can be broadly grouped into three families: surfactins, fengycins and iturins (Jasim et al., 2016). These antifungal lipopeptides inhibit or kill fungi either

by inhibiting mycelial growth, or causing the hyphae or spores to become broken or swollen (Roongsawang et al., 2010; Li et al., 2021b). Genes coding the synthesis of antimicrobial lipopeptides, i.e., *bioA*, *bmyB*, *ituC*, *fenD*, *srfAA*, *srfAB*, *yngG*, and *yndJ*, were identified in the genomes of bacilli isolated from *daqu* fermentations (Wu et al., 2021). In addition, several lipopeptides were identified in both *daqu* fermentations and *baijiu* (Zhang et al., 2014; Chen et al., 2020). For example, surfactin accumulated to a concentration of more than 7 mg/kg in the *daqu* stage of Moutai fermentation and was diluted to 1.5 mg/kg by the addition of cooked cereals at the mash stage. The concentration of the non-volatile peptide in the distilled end product was less than 1 µg/L (Chen et al., 2020). It remains unknown, however, whether the production of antifungal lipopeptides in *daqu* impacts community assembly in *daqu* and mash fermentations. It was therefore the aim of this study to investigate the role of *Bacillus* lipopeptides in the interaction between *Bacillus* spp. and fungi in *daqu* fermentation.

3.2 Materials and methods

3.2.1 Strains used in this study and preparation of the inocula

The origin and growth conditions of strains used in this study are listed in Table 3.1. Luria-Bertani (LB) broth (Difco™ Agar, BD Biosciences, Franklin Lakes, USA) was inoculated with a single colony of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, *B. velezensis* FUA2155, or *Kosakonia cowanii* FUA10121 and incubated overnight at 37 °C at 200 rpm agitation. A culture of *Weissella cibaria* FUA3456 was prepared in a similar manner but the strain was grown in modified de Man, Rogosa, and Sharpe (mMRS) broth (Müller et al., 2001) at 30 °C for 2 d without agitation.

Table 3.1 List of origin and purpose of strains used in this study.

Microorganism	Strains and origin	Incubation	Purpose in this study	Reference
B. amyloliquefaciens	Fad We; ropy bread	37 ℃, LB	Simplified and complex *daqu* fermentation	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad 82; ropy bread	37 ℃, LB	Simplified and complex *daqu* fermentation	(Röcken and Spicher, 1993)
B. velezensis	[a] FUA2155; *daqu*	37 ℃, LB	Simplified and complex *daqu* fermentation	(Wang et al., 2018)
K. cowanii	FUA10121; *daqu*	37 ℃, LB	Complex *daqu* fermentation	(Wang et al., 2018)
W. cibaria	FUA3456; sourdough	30 ℃, mMRS	Complex *daqu* fermentation	
S. cerevisiae	FUA4002; sourdough	30 ℃, MEA	Complex *daqu* fermentation and MIC test	
S. fibuligera	FUA4036; *daqu* starter	30 ℃, MEA	Complex *daqu* fermentation and MIC test	
P. kudriavzevii	FUA4039; *daqu* starter	30 ℃, MEA	Complex *daqu* fermentation and MIC test	
A. niger	FUA5001	25 ℃, MEA	Complex *daqu* fermentation and MIC test	
M. racemosus	FUA5009	25 ℃, MEA	Complex *daqu* fermentation and MIC test	
P. roqueforti	FUA5012	25 ℃, MEA	Complex *daqu* fermentation and MIC test	
A. clavatus	FUA5004	25 ℃, MEA	MIC test	
A. clavatus	FUA5005	25 ℃, MEA	MIC test	

[a] FUA number, Food microbiology culture collection at the University of Alberta.

Spore suspensions of the following fungal strains, *Aspergillus niger* FUA5001, *A. clavatus* FUA5004, *A. clavatus* FUA5005, *Mucor racemosus* FUA5009, and *Penicillium roqueforti* FUA5012 were prepared as described (Zhang et al., 2010). In brief, the strains were cultivated on malt extract agar (MEA) plates at 25 °C for 7 d. Conidia were harvested from agar plates by adding 10 mL of sterile distilled water and harvesting of fungal biomass with an L-shaped cell spreader (Fisher Scientific, Ottawa, Canada). The spore suspensions were filtered to eliminate mycelial cells and spores were harvested by centrifugation. The spores were quantified with a hemocytometer (Fein-Optik, Jena, Germany).

Inocula of *Saccharomyces cerevisiae* FUA4002, *Saccharomycopsis fibuligera* FUA4036, and *Pichia kudriavzevii* FUA4039 were prepared by inoculating malt extract broth (MEB) with single colonies, followed by incubation for 2 d at 30 °C. The cell counts were confirmed with a hemocytometer.

3.2.2 *In silico* prediction of lipopeptides produced by *Bacillus* spp.

The genome sequences of *Bacillus* strains used in this study were published (Li et al., 2019). Biosynthetic gene clusters in the genome and their corresponding secondary metabolites were identified using the bacterial version of the antiSMASH (Blin et al., 2021). Identification of gene clusters encoding different lipopeptides in the genomes of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155 were verified by BLAST on NCBI. All gene clusters identified by antiSMASH were used as query sequence for BLASTn against the NCBI nucleotide database.

3.2.3 Extraction and purification of the antifungal peptides from LB cultures

To study the antifungal peptides produced by *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155, the peptides were extracted from 150 mL of the stationary phase cultures in LB broth. The cultures were incubated at 37 °C, 200 rpm for 3 d and cells were removed by centrifugation at 12,000 × *g* for 20 min. The pH of the supernatants was adjusted to 2 with 6M HCl, followed by incubation at 4 °C overnight. Solid crude lipopeptides were collected by centrifugation at 12,000 × *g* for 20 min, and the precipitate was extracted with methanol. The organic layer was evaporated *in vacuo* at 50 °C (Yang et al., 2015). The extracted peptides were dissolved in 1 mL methanol and filtered through 0.45 µm filters to remove solids.

3.2.4 Minimum inhibitory concentration assay

The minimum inhibitory concentration (MIC) of surfactin, fengycin and iturin A was determined in a critical dilution assay. Iturin A and fengycin (Sigma-Aldrich, Oakville, Canada) and surfactin (MedChemExpress, Monmouth Junction, USA) were dissolved in DMSO to a concentration of 5 g/L as stock solutions and stored at -80 °C until further use. The mycelial fungi *A. niger* FUA5001, *A. clavatus* FUA5004, *A. clavatus* FUA5005, *M. racemosus* FUA5009 and *P. roqueforti* FUA5012, as well as the yeasts, *S. cerevisiae* FUA4002, *S. fibuligera* FUA4036 and *P. kudriavzevii* FUA4039 were used as indicator strains. Inocula were prepared as described above. The growth was observed visually, and the MIC was recorded 1 day after visible growth. The MIC values were the means of three independent experiments using three different preparations of conidiospores. For each experiment, 90 µL of the lipopeptides iturin, surfactin, fengycin were mixed with MEB (for fungi) or mMRS broth (for yeasts) in a 96-well microtiter plate, followed by serial 2-fold dilutions with the respective growth medium. Each well was

inoculated with 10 μL of spores or vegetative cells with a cell count of 10^6 CFU/mL. The controls contained inocula, but distilled water was used instead of the lipopeptide solutions.

3.2.5 Preparation of simplified model of *daqu* fermentation

The production of antifungal peptides by the three strains of *Bacillus* (*B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155) was initially assessed in a simplified model of *daqu* fermentation that were inoculated with only one bacterial strain. Overnight cultures of the three *Bacillus* strains were prepared by inoculating single colonies in 100 mL LB broth and incubating at 37 °C, 200 rpm for 20 h. Cells were harvested by centrifugation and resuspended in tap water to obtain a cell count of 10^9-10^{10} CFU/mL. One mL of this inoculum, 60 g wheat flour and 30 mL sterile tap water were mixed in a sterile plastic bag, resulting in a water content of around 35 % and a final cell count of around 10^7-10^8 CFU/g. Wheat flour mixed with water without inoculation of strain of *Bacillus* served as the control. The *daqu* samples were manually pressed and shaped in Petri-dishes and incubated at controlled temperatures and relative humidity (rH) as follows: shaping stage, 30 °C and rH 95 % for 1 d; ripening stage, 37 °C, rH 95 % for 2 d; high-temperature stage, 55 °C, rH 90 % for 7 d; maturation stage, 37 °C, rH 75 % for 6 d. This temperature and relative humidity profile closely matches the medium-temperature *daqu* fermentation conditions (Zheng et al., 2011; Li et al., 2016). Samples were incubated in hermetically sealed containers and the relative humidity in these containers was controlled with the following saturated salt solutions: K_2SO_4, rH 95 %; KNO_3, rH 90 %; NaCl rH 70 %.

3.2.6 Determination of pH and total bacterial cell counts of the simplified *daqu* fermentation model, and observation of mold growth

To measure the pH and viable cell counts, 0.5 g samples were collected and diluted 10-fold with sterile 18 MΩ water. Samples were further diluted in peptone water and plated on LB agar. The

pH of the first dilution was then measured with a glass electrode. The LB agar plates were incubated at 37 °C for 24 h prior to counting the total number of colonies per plate; differential cell counts of *Bacillus* species were also recorded based on the colony morphology.

Fungal growth during the simplified *daqu* fermentation model was observed daily and recorded as follows: -, no mycelial mold growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % pf the surface covered by mycelium. The reference pictures for defining the fungal growth are indicated in Figure 3.5.

3.2.7 Quantification of expression of genes encoding the three antifungal lipopeptides under the simplified *daqu* fermentation model by reverse transcription quantitative PCR (RT-qPCR)

To extract mRNA and prepare cDNA of the 1st, 2nd, and 3rd day of the simplified *daqu* samples, aliquots of 0.5 g *daqu* were mixed with 3 mL RNAprotect Bacteria Reagent (Qiagen, Germantown, USA) and incubated for 10 min. The solids were then removed by centrifugation at 500 × *g* for 10 min. The cell in the supernatant were harvested through further centrifugation. The RNA was isolated from cell pellets using TRIzol LS reagent based on the instructions of manufacturer (ThermoFisher Scientific, Waltham, USA). Contaminant genomic DNA was digested by DNase treatment using the RQ1 RNase-Free DNase (Fisher Scientific), and cDNA libraries were generated by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen).

To quantify the expression of genes encoding surfactin, fengycin and iturin A production in *daqu* fermentation, the genes *srfAA*, *fenA and ituA* coding part of the peptides synthetase subunit of surfactin, fengycin and iturin A were chosen. Primers used in qPCR are shown in Table 3.2 and the specificity of the primers was verified by PCR using chromosomal DNA of the three strains as

template. The single-copy gene *gyrB*, which encodes for the DNA gyrase subunit B (la Duc et al., 2004), was used as the housekeeping gene for relative quantification of gene expression.

Table 3.2 List of primers used in RT-qPCR.

Target	Primer	Sequence (5'-3')	Reference
gyrB	*gyrB* Forward	ATCGTCGACAACAGTATTG	(Li et al., 2019)
	gyrB Reverse	CTTTATATCCGCTTCCGTC	(Li et al., 2019)
srfAA	*srfAA* Forward	GACAAGCGGCGTCATCAATC	This study
	srfAA Reverse	CTGCCACGCATAATTCACCG	This study
fenA	*fenA* Forward	TGCGGTTAACGGCAAACGG	This study
	fenA Reverse	TCAAGAAGCCATTCAGTTCGCG	This study
ituA	*ituA* Forward	CCGGCACGATTGATATCGC	This study
	ituA Reverse	CCGGCCTGCTTGATAAAGC	This study

Gene expression was detected by QuantiFast SYBR Green PCR Kit (Qiagen) and reverse-transcriptase qPCR (7500 Fast, Applied Biosystems, Foster City, CA). Cells growing exponentially in LB broth (OD_{600nm} 0.8) were used as reference conditions. Negative controls included DNase-treated RNA and a no-template control. Gene expression relative to cultures in LB was calculated with the $\Delta\Delta C_T$ method and $\log_2$ transformed. Significant differences in the relative gene expression were assessed with a t-test and an error probability of 5 % ($P < 0.05$). Data are presented as mean ± standard deviation of three independent fermentations.

3.2.8 Preparation of complex model of *daqu* fermentation

To better study the role of antifungal peptides produced by *Bacillus* spp. in *daqu* fermentation, a complex model of *daqu* fermentation was prepared. In addition to inoculation with strains of *Bacillus* spp., cell or spore suspensions of *A. niger* FUA5001, *M. racemosus* FUA5009, and *P. roqueforti* FUA5012, *S. cerevisiae* FUA4002, *S. fibuligera* FUA4036, and *P. kudriavzevii*

FUA4039 were added to a final cell count of 10^5 CFU/g each and cell suspensions of *K. cowanii* FUA10121 and *W. cibaria* FUA3456 were added to a final cell count of 10^6 CFU/g each. The complex *daqu* samples were prepared and incubated at the same conditions with the simplified *daqu* model as described above.

3.2.9 Determination of microbial population in complex model of *daqu* fermentation by qPCR

Samples from the complex *daqu* model were collected after 1, 3, 6, 10, 13 or 16 days of fermentation were collected. Community DNA was extracted by E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc. Norcross, USA) according to the instruction of manufacturer. The microbial populations were quantified with qPCR using primers targeting *Bacillus* species, all bacteria, and fungi with the primer pairs *srfAA*F and *srfAA*R, 340F and 758R (Juck et al., 2000), and Fnpstr and Rnpstr (Rodríguez et al., 2012), respectively. The assays were carried out on a 7500 Fast instrument (Applied Biosystems) with a commercial QuantiFast SYBR Green PCR kit (Qiagen) according to the instructions of manufacturer. The calibration curves and amplification conditions were constructed as described (Liu et al., 2017).

3.2.10 Extraction and purification of the antifungal lipopeptides from the complex model of *daqu* fermentation

To monitor the production of antifungal lipopeptides in the complex *daqu* fermentation, samples were obtained on the 1st, 3rd, 6th 10th, 13th, and 16th day of fermentation. The extraction procedure for lipopeptides was similar to that described above for extraction from LB cultures, but with an additional homogenization step. Approximately 8.5 g of the *daqu* samples were homogenized in 50 mL distilled water by stomaching for 5 minutes. The samples were then centrifuged at

12,000 × *g* for 20 min to remove solids. Samples were further processed with same procedure as described above for cultures in LB.

3.2.11 Antifungal activity test of peptides produced by *Bacillus* in the complex *daqu* model

To determine the production of antifungal peptides during *daqu* fermentation, the antifungal activity of methanolic *daqu* extracts was determined with *A. niger* FUA5001 as the indicator strain. Serial two-fold dilutions of the peptide extracts and MEB were prepared in 96-well microtiter plates and inoculated with 10 µL of a suspension of conidia *A. niger* FUA5001 to a cell count of 10^6 CFU/mL. The plates were then incubated at 25 °C for 5 d. After incubation, fungal growth was observed visually.

3.2.12 Procedure for LCMS analysis of LB culture and complex model of *daqu* extracts

To qualitatively identify the antifungal lipopeptides produced by *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, and *B. velezensis* FUA2155, each sample was detected first by reverse phase-high performance liquid chromatography followed by coupled to using mass spectrometry (RP-HPLC-MS). Analysis was performed using an Agilent 1200 SL HPLC System with a Phenomenex Aeris XB-C8 column, 3.6 µm, 100 Å, 50 x 2.1 mm with a trap cartridge (Phenomenex, Torrance, USA) with a guard thermostated at 35 °C. A buffer gradient composed of 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B) was used. A 2 µL aliquot of the sample was loaded onto the column at a flow rate of 0.45 mL/min and an initial buffer composition of 95 % mobile phase A and 5 % mobile phase B for 0.5 min was performed to effectively remove the salts. The elution of the lipopeptides was performed using a linear gradient from 5 % to 65 % mobile phase B for 4.8 min, 65 % to 95 % mobile phase B for 1.0 min, 95 % mobile phase B for 0.8 min, then back to the initial buffer conditions in 0.5 min. Mass spectra were acquired in a positive mode of ionization using an Agilent

6220 Accurate-Mass TOF HPLC/MS system (Santa Clara, CA, USA), equipped with a dual sprayer electrospray ionization source; the second sprayer providing a reference mass solution. Mass correction was performed for each individual spectrum using peaks at *m/z* 121.0509 and 922.0098 from the reference solution. The conditions for mass spectrometry are as stated: drying gas 10 L/min at 350 °C, nebulizer at 30 psi, mass range of 100-3200 Da, acquisition rate of ~1.03 spectra/sec, fragmentor voltage at 175 V, skimmer voltage at 65 V, capillary voltage at 4000 V, and instrument state 2 GHz High Dynamic Range. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.07.00 SP2.

3.3 Results

3.3.1 Prediction of antifungal lipopeptides produced by *B. amyloliquefaciens* and *B. velezensis*

The production of antimicrobial peptides in the genomes of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, *B. velezensis* FUA2155 was initially predicted by antiSMASH (Table 3.3). All three strains were predicted to produce multiple antimicrobial lipopeptides. The predicted number and identity of peptides in the two strains of *B. amyloliquefaciens* were identical. *B. velezensis* FUA2155 was predicted to additionally produce three antimicrobial peptides including a second surfactin gene cluster (Table 3.3). The genome of *B. velezensis* FUA2155 also included gene clusters encoding for the synthesis of the antibacterial peptides difficidin and macrolactin H with high identity. Difficidin has both antifungal and antibacterial activities (Im et al., 2020), while macrolactins have broad-spectrum antimicrobial activity (Yuan et al., 2016). Macrolactin H exhibited an antibacterial activity (MIC = 10 mg/L) against *Staphylococcus aureus* (Nagao et al., 2001), but antifungal activity has not been described.

Table 3.3 The prediction of cluster of different antifungal peptides in the genome of three strains of *Bacillus* predicted by antiSMASH.

Secondary Metabolite	Type	Cluster	Similarity		
			Fad We	Fad 82	FUA2155
surfactin	[a]NRP:Lipopeptide	[b]NRPS	82 %	82 %	47 %
surfactin	NRP:Lipopeptide	NRPS	-	-	< 30 %
surfactin	NRP:Lipopeptide	NRPS	-	-	39 %
fengycin	NRP	NRPS, transAT-[c]PKS, betalactone	93 %	93 %	93 %
iturin	NRP, Polyketide	NRPS	77 %	77 %	88 %
bacillaene	transAT-PKS, NRPS, T3PKS	Polyketide + NRP	100 %	100 %	100 %
butirosin A/B	PKS-like	Saccharide	< 30 %	< 30 %	< 30 %
bacilysin	other	Other	100 %	100 %	100 %
bacillibactin	RiPP-like, NRPS	NRP	100 %	100 %	100 %
difficidin	transAT-PKS	Polyketide + NRP	-	-	100 %
Macrolactin H	transAT-PKS	Polyketide	-	-	100 %

[a]NRP: non ribosomal peptides

[b]NRPS: non ribosomal peptide synthetase

[c]PKS: polyketide synthetases

Predictions of the antifungal peptides by antiSMASH were verified by BLASTn (Table 3.4). No significant similarity was found for butirosin A/B and the query coverage for bacillibactin, bacilysin and fengycin biosynthetic genes in the genomes of the three *Bacillus* strains were below 60%. The query coverage and identity of bacillaene, surfactin and iturin in the three strains were high.

Table 3.4 Identification of gene clusters encoding different lipopeptides by BLAST.

Lipopeptides	Accession number	Fad We		Fad 82		FUA2155	
		Query Cover	Per. Ident	Query Cover	Per. Ident	Query Cover	Per. Ident
surfactin	AJ575642.1	91 %	92.89 %	91 %	92.89 %	40 %	98.32 %
fengycin	CP000560.1	56 %	92.83 %	56 %	92.83 %	54 %	97.96 %
iturin	AB050629.1	96 %	90.14 %	96 %	90.14 %	86 %	97.90 %
bacillaene	AJ634060.2	100 %	92.47 %	100 %	92.47 %	100 %	97.98 %
butirosin A/B	AB097196.1	No significant similarity found					
bacilysin	CP000560.1	56 %	92.83 %	56 %	92.83 %	54 %	97.96 %
bacillibactin	AL009126.3	18 %	76.56 %	18 %	76.51 %	29 %	76.36 %
difficidin	AJ634062.2	No significant similarity found		No significant similarity found		100 %	97.88 %
macrolactin H	AJ634061.2	No significant similarity found		No significant similarity found		100 %	98.18 %

3.3.2 Analysis of antifungal lipopeptides in the LB cultures of *Bacillus*

Gene clusters coding for production of three families of antifungal lipopeptides, surfactin, fengycin and iturin were identified in the genomes of the three strains of *Bacillus*. To determine whether these lipopeptides are produced during growth in LB broth, total lipopeptides were extracted from culture supernatants of *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155. As shown in Figure 3.1, the signal intensity of surfactin, iturin A, and fengycin were qualitatively analyzed by HPLC-MS, and the log[signal intensities] are shown in a gradient. Of the three families of lipopeptide tested, surfactins were detected in all three strains of *Bacillus* with a high signal intensity. In LB cultures of *B. amyloliquefaciens* Fad 82, the surfactins were lower than that of the other two strains. Iturins were also produced by all three strains but the log[signal intensities] in extracts from cultures of *B. amyloliquefaciens* Fad We, and Fad 82 was about 100 times lower than that in the *B. velezensis* FUA2155 extracts. Fengycins were not detected in any of the cultures.

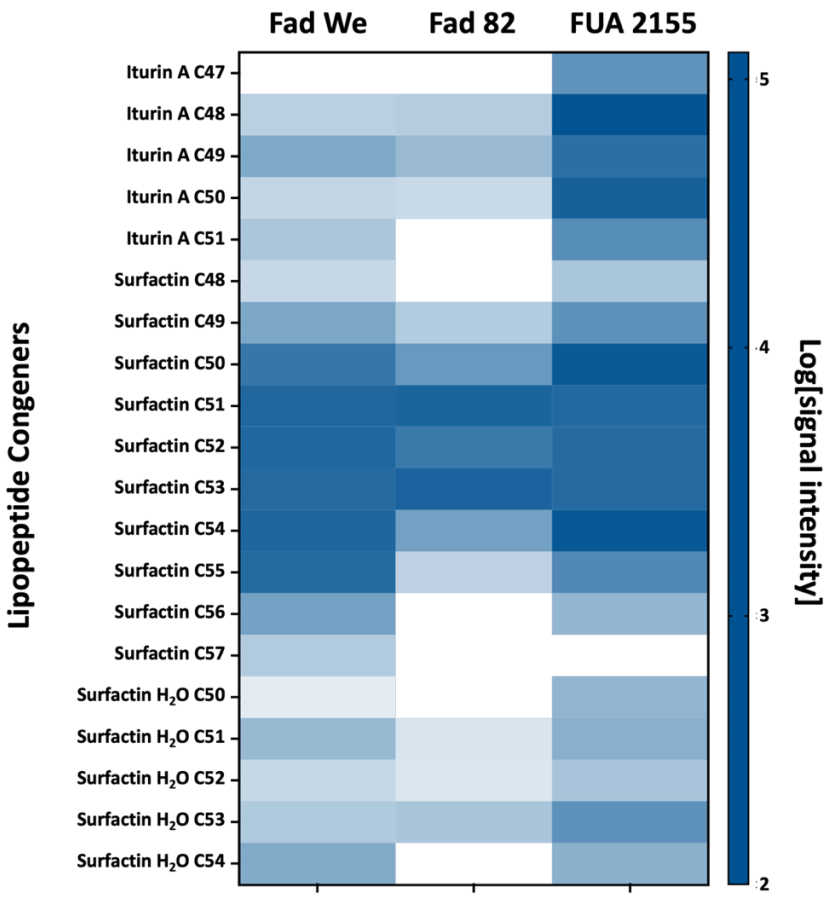


Figure 3.1 Heat map of lipopeptide congeners from *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82, and *B. velezensis* liquid LB broth samples. Samples were analyzed *via* LCMS and the log[signal intensities] are shown in a gradient. Experiments were down in triplicate.

The presence of peptides extracted from LB cultures of *B. amyloliquefaciens* Fad We, Fad 82, and *B. velezensis* FUA2155 were also analyzed by MALDI-TOF previously (data not shown). Based on the MALDI-TOF assay done previously (data not shown), *B. amyloliquefaciens* Fad We and Fad 82 have similar peptide compositions, where surfactin can be found in all the three strains, but at a lower concentration in *B. velezensis* FUA2155. Iturin A with a C15-beta lipid tail can be detected in *B. velezensis* FUA2155. No fengycin can be seen in any of the three strains. The previous results are consistent with the results in this study.

3.3.3 Antifungal activity of surfactin, fengycin, and iturin A

The minimum inhibitory concentration of surfactin, fengycin and iturin A was determined with a critical dilution assay with five mycelial molds and three yeasts as indicator strains. Iturin A inhibited 7 of the 8 indicator strains with an MIC ranging from 10 to 50 mg/L; only *S. fibuligera* was relatively resistant (Figure 3.2). The MIC of surfactin and fengycin against most indicator strains ranged from 300 to 500 mg/L, the highest concentration that was tested. Fengycin inhibited *M. racemosus* FUA5009 and S. *cerevisiae* FUA4002 at a concentration of about 200 mg/L.

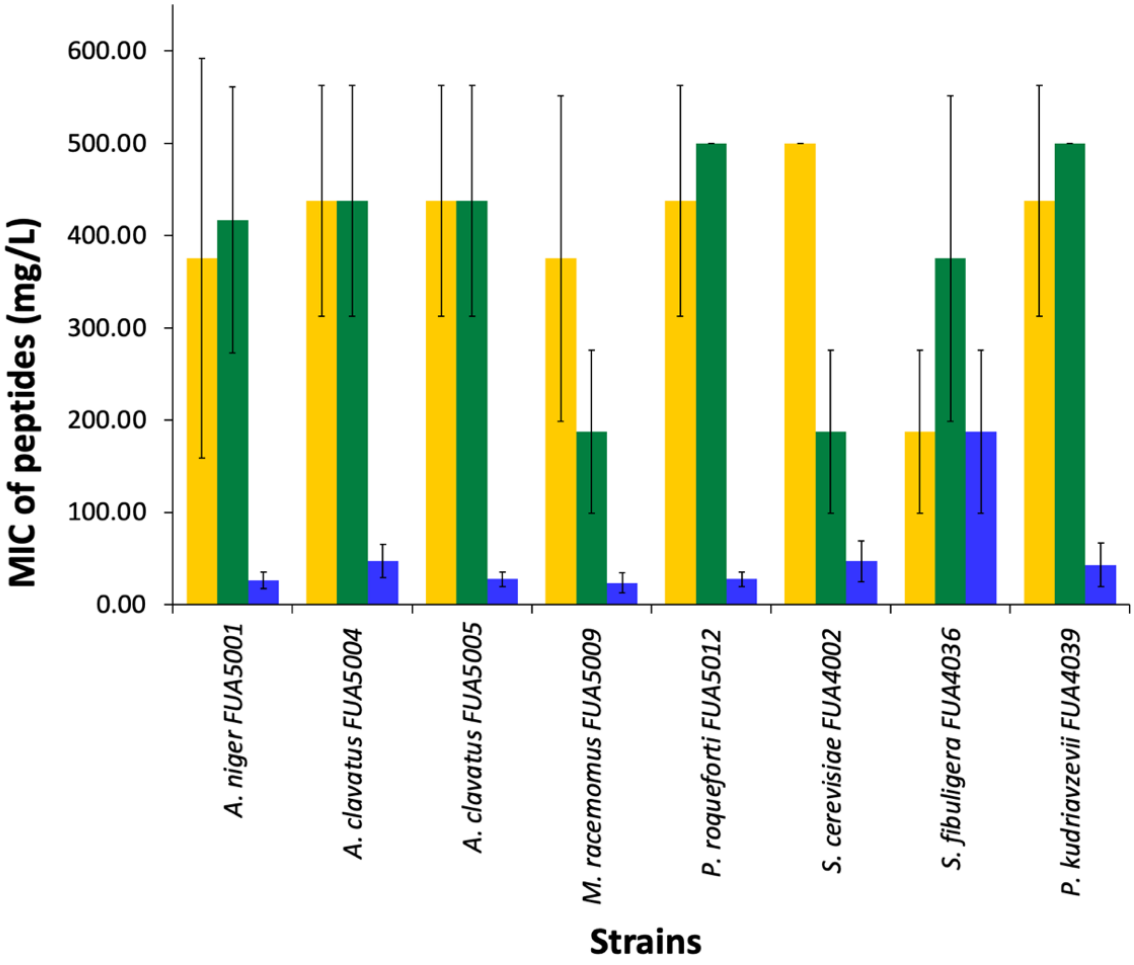


Figure 3.2 Minimum inhibitory concentration of surfactin (yellow bar), fengycin (green bar) and iturin A (blue bar) against filamentous fungi and yeasts. Results were presented as means ± standard deviation of quadruplicate independent experiments.

3.3.4 Performance of *Bacillus* as starter cultures in simplified *daqu* model

Of the thee strains of *Bacillus* species, *B. velezensis* FUA2155 was isolated from *daqu* (Wang et al., 2018) while the two strains of *B. amyloliquefaciens* were isolated from ropy bread (Röcken and Spicher, 1993). All three encode for multiple amylases, which contribute to the spoilage of bread (Li et al., 2020), but are beneficial technological traits in *daqu* fermentations. To determine their suitability as cultures for *daqu,* the performance of three strains was evaluated in a simplified *daqu* model using un-inoculated wheat flour as control. The evolution of pH and viable cell counts during the fermentation is shown in Figure 3.3 and Figure 3.4, respectively. A decrease of the pH from 6.5 to lower than 5.5 was observed in the four *daqu* fermentation, including the control. The pH increased in samples inoculated with *B. velezensis* FUA2155 during the ripening stage (37 °C, rH 95 % for 2 d).

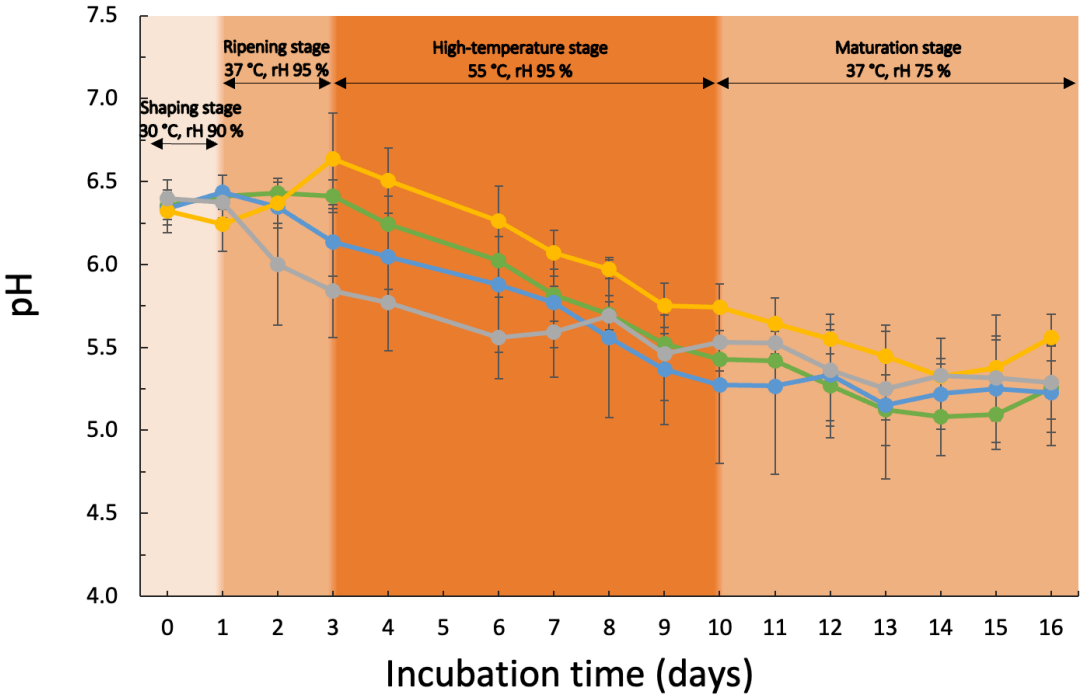


Figure 3.3 The pH tested during the fermentation of the simplified *daqu* model. Different line colors indicate the different strains inoculated in the *daqu* fermentation: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow, *B. velezensis* FUA2155; and gray, control (without addition of *Bacillus* strain). Different stages of fermentation were differentiated by the color of background.

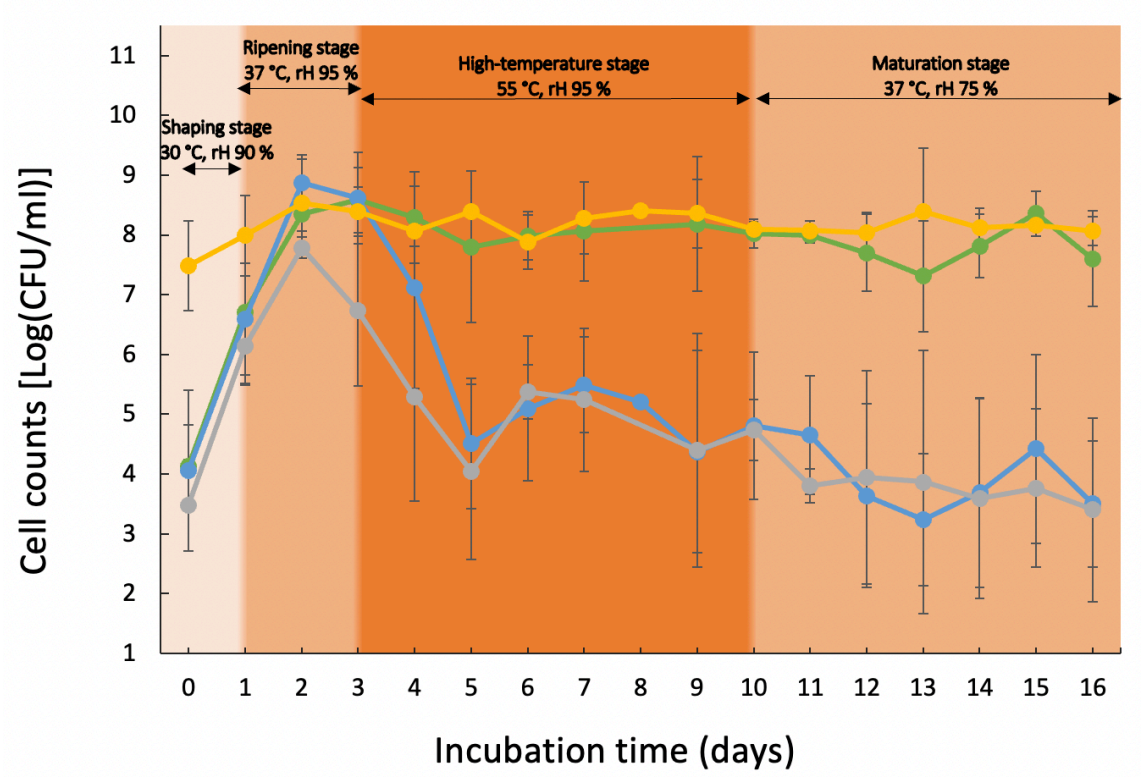


Figure 3.4 The total cell counts tested during the fermentation of the simplified *daqu* model. Different color of the line indicated different strains inoculated in the *daqu* fermentation: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow *B. velezensis* FUA2155; and gray, no inoculation. Different stages of fermentation were differentiated by the color of background. Results are presented as means ± standard deviation for five biological replicates.

The total cell counts during the simplified *daqu* germentation are illustrated in Figure 3.4. *B. velezensis* FUA2155 and *B. amyloliquefaciens* Fad We showed high cell counts of approximately 8 logs after the shaping stage and remained consistent until the end of the fermentation. Cell counts of samples inoculated with *B. amyloliquefaciens* Fad 82 dropped sharply after the ripening stage, and a similar trend was observed in the control. An initial assessment of whether the inocula persisted, relative to flour-derived microorganisms, was based on the observation of colony morphology on LB agar plates. In uninoculated control samples, colonies with a morphology that matched the strains of *Bacillus* that were used as inoculum were minimal

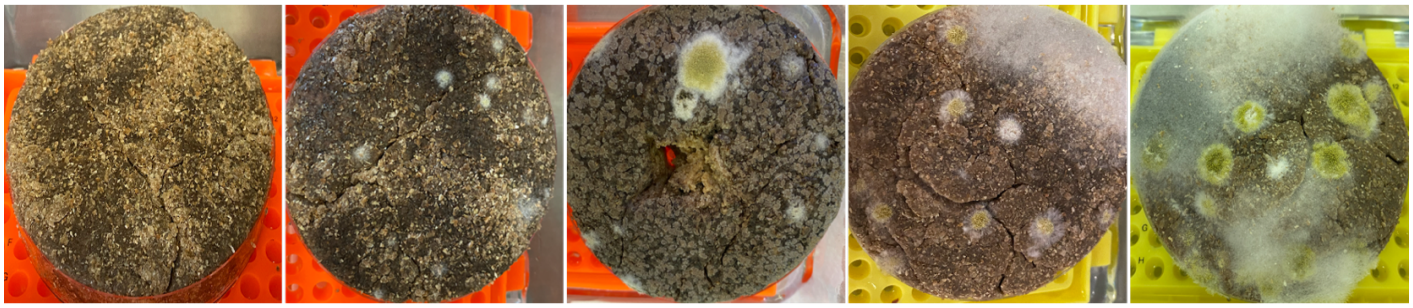
and accounted for less than 10 % of the total colonies throughout the fermentation. In samples inoculated with *B. amyloliquefaciens* Fad 82, about 70 % of colonies had the same morphology as the inoculum. In fermentations inoculated with *B. amyloliquefaciens* Fad We or *B. velezensis* FUA2155, more than 90 % of the colonies had the same morphology as the inoculum. Taken together, cell counts in combination with observation of the colony morphologies suggests that *B. amyloliquefaciens* Fad We or *B. velezensis* FUA2155 were the dominant fermentation microbes in the simplified *daqu* fermentation model.

In addition to bacterial growth, growth of mycelial fungi was observed on the surface of *daqu* samples (Table 3.5). Substantial mold growth was observed in the uninoculated control samples at day 2 and mycelia covered most of the surface by day 3. Mold growth on samples inoculated with *B. amyloliquefaciens* Fad 82 was comparable to the control. Mold growth and formation of conidia was not detected on samples inoculated with *B. amyloliquefaciens* Fad We until day 4, while no visible mold growth was observed on samples inoculated with *B. velezensis* FUA2155. On day 4, samples were transferred to 55 °C which inhibited any further growth of molds irrespective of the inoculum (Table 3.5).

Table 3.5 Degree of the mold growth during the first 4 days of the simplified *daqu* model.

Inoculated strain	Incubation time				
	Day 0	Day 1	Day 2	Day 3	Day 4
Control	-	-	+++	++++	+++++
B. amyloliquefaciens Fad We	-	-	+	+	++
B. amyloliquefaciens Fad 82	-	-	++++	++	+++
B. velezensis FUA2155	-	-	-	-	-

-, no mycelial mold growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % pf the surface covered by mycelium. The reference pictures for defining the fungal growth are indicated in Figure 3.5.



- + ++ +++ ++++

Figure 3.5 Reference pictures for defining fungal growth during the simplified *daqu* fermentation model. -, no mycelial mold growth visible; +, small spots of mycelial growth; ++, spots of mycelial growth and conidia; +++, 25-50 % of the surface covered by mycelium; and ++++, more than 50 % pf the surface covered by mycelium.

3.3.5 Gene expression of *srfAA*, *fenA* and *ituA* at the first, second, and third day of the simplified *daqu* fermentation

To determine whether antifungal lipopeptides are expressed during the growth of bacilli in model *daqu* fermentations, mRNA encoding for *srfAA*, *fenA* and *ituA* was quantified in the samples of simplified *daqu* model by RT-qPCR (Figure 3.6). Relative gene expression was calculated with *gyrB* as the housekeeping gene and exponential cultures in LB broth as reference conditions. All three genes were expressed by all three strains during growth in the *daqu* model. Over-expression of *srfAA* and *fenA* was observed at day 1 and/or day 2 of fermentation. On day 3 of fermentation, *srfAA* was down-regulated in all three strains. *B. amyloliquefaciens* FAD 82 also down-regulated *fenA* and *ituA* on day three of the fermentation (Figure 3.6).

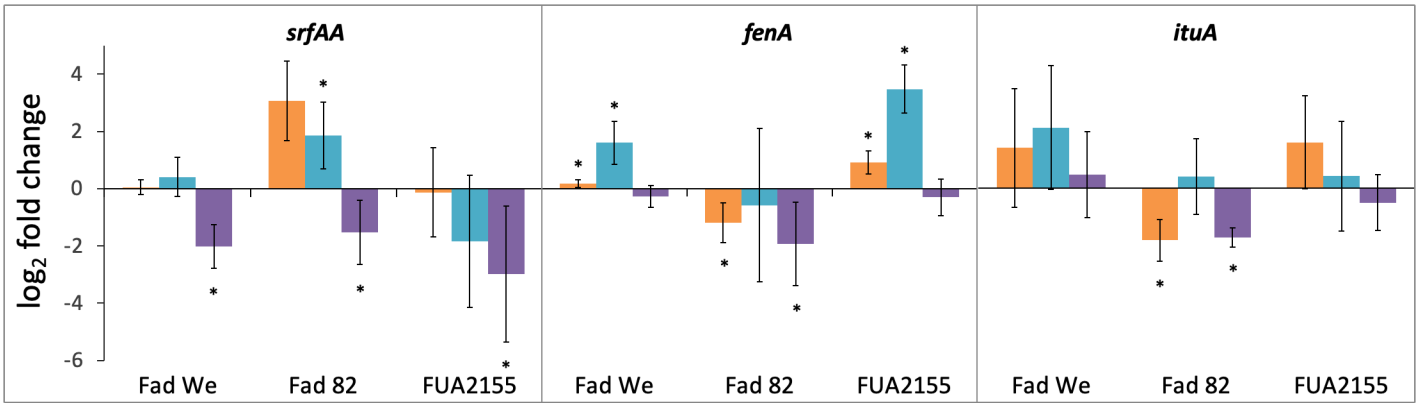


Figure 3.6 Expression of *srfAA*, *fenA* and *ituA* in the samples of the 1st (orange bar), 2nd (blue bar), and 3rd day (purple bar) of the simplified daqu fermentation using *B. amyloliquefaciens* Fad We, *B. amyloliquefaciens* Fad 82 and *B. velezensis* FUA2155. Relative gene expression was quantified by RT-qPCR with *gyrB* as the housekeeping gene and exponential cultures in LB broth as reference conditions. Significant difference between *daqu* fermentation and LB broth conditions was determined by t-test and labeled with asterisks ($P < 0.05$). Data represent means ± standard deviation of the means from three independent experiment.

3.3.6 Antifungal activity of peptides produced by *Bacillus* in the complex *daqu* model

The results of antifungal activity test of antifungal lipopeptides extracted from the complex model of *daqu* fermentation were illustrated in Table 3.6. The *A. niger* FUA5001 was used as the indicator strain. Extracts from control samples inoculated with all 8 fermentation organisms, but not with any *Bacillus* strains, did not inhibit mold growth, indicating that bacilli were the sole or main contributors to antifungal activity. The inhibitory activity of *daqu* extracts obtained after 1 d of fermentation was highly variable. Extracts from *daqu* inoculated with *B. velezensis* FUA2155 showed strong and consistent antifungal activity from the 1st to the 6th day of fermentation. In this period of fermentation, consistent antifungal activity was also observed for extracts obtained from *daqu* fermentations with the two strains of *B. amyloliquefaciens* but the inhibitory activity was weaker when compared to fermentations with *B. velezensis* FUA2155.

Table 3.6 Antifungal activity of peptides extracted from the complex *daqu* model with different strains of *B. amyloliquefaciens* or *B. velezensis*.

Strains	Day 1	Day 3	Day 6	Day 10	Day 13	Day 16
Control	0	0	0	0	0	0
B. amyloliquefaciens Fad We	3 ± 4	1 ± 1	1 ± 0.5	1 ± 0.5	0 ± 0	0.3 ± 0.5
B. amyloliquefaciens Fad 82	0.3 ± 0.5	2 ± 1.7	1 ± 0.8	1 ± 0	1 ± 0	2 ± 2
B. velezensis FUA2155	3 ± 4	3 ± 1	2 ± 1	1 ± 0	1 ± 0.5	1 ± 2

0: no inhibition; 0-0.9: slight inhibition; 1-1.9: moderate inhibition; 2-2.9: strong inhibition; ≥ 3: significant inhibition. Data represent means ± standard deviation of the means from three independent experiment. Experiments were down in triplicate.

To confirm antifungal activity of *Bacillus* strains, gene copies representing *Bacillus*, total bacteria, and fungi in the complex *daqu* fermentation samples were quantified by qPCR (Figure 3.7). An increase of *Bacillus*, total bacteria and fungi was found in each sample in the ripening stage (1st to 3rd day). Compared with other samples, the samples inoculated with *B. velezensis* FUA2155 showed the highest log(copies/g) of *Bacillus* and total bacteria, and the lowest log(copies/g) of fungi during the whole fermentation process. The log(copies/g) of total bacteria count for *B. amyloliquefaciens* Fad We and Fad 82 from the 3rd to 10th day of fermentation ranged from 11-12 but decreased on the 13th day. Additionally, log(copies/g) of fungi remained stable after the 3rd day. Overall, the gene copies of *Bacillus*, total bacteria, and fungi differed among the complex *daqu* samples inoculated with different *Bacillus* strains. *B. velezensis* FUA2155 was more competitive when compared to the other two *Bacillus* strains.

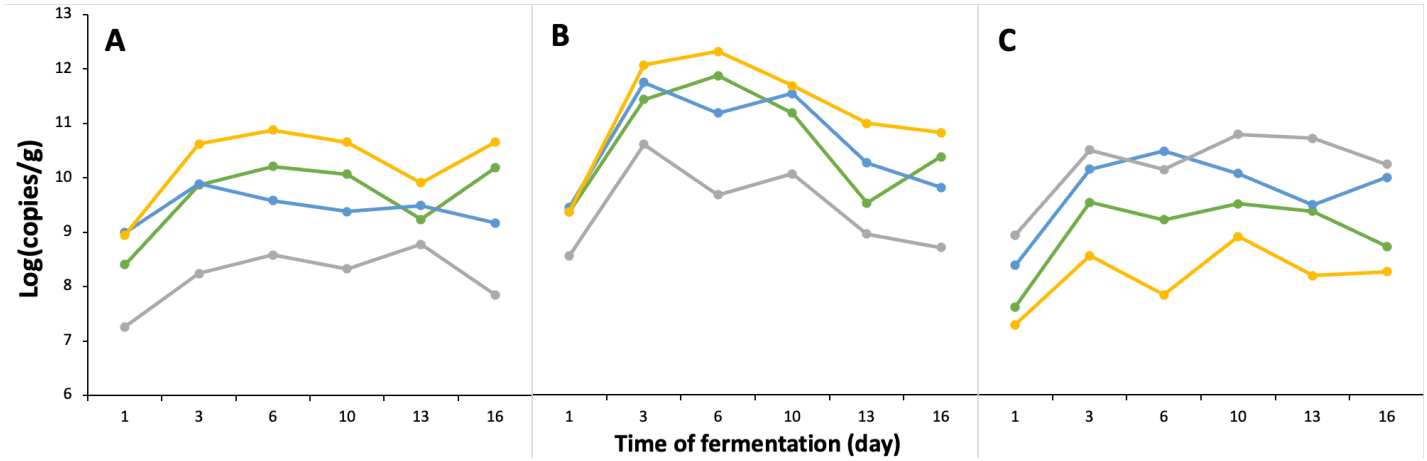


Figure 3.7 Microbiota components analysis by qPCR across the fermentation of the complex *daqu* model samples. **A.** *Bacillus*. **B.** Total bacteria. **C.** Fungi. Different color of the line indicated different strains inoculated in the *daqu* fermentation: green, *B. amyloliquefaciens* Fad We; blue, *B. amyloliquefaciens* Fad 82; yellow *B. velezensis* FUA2155; and gray, no inoculation. The data are based on one replicate of qPCR with one DNA isolation.

3.3.7 Analysis of antifungal lipopeptides in complex model of *daqu* extracts by HPLC-MS

To further compare the production of the lipopeptides from the three strains of *Bacillus* in *daqu* fermentation, the extracts of the complex *daqu* model were also analyzed for the presence of antifungal lipopeptides. The signal intensity of the lipopeptides extracted from different time points of *daqu* fermentation were qualitatively detected by LCMS, and the log[signal intensity] of surfactin, iturin A, and fengycin were summarized in Figure 3.8.

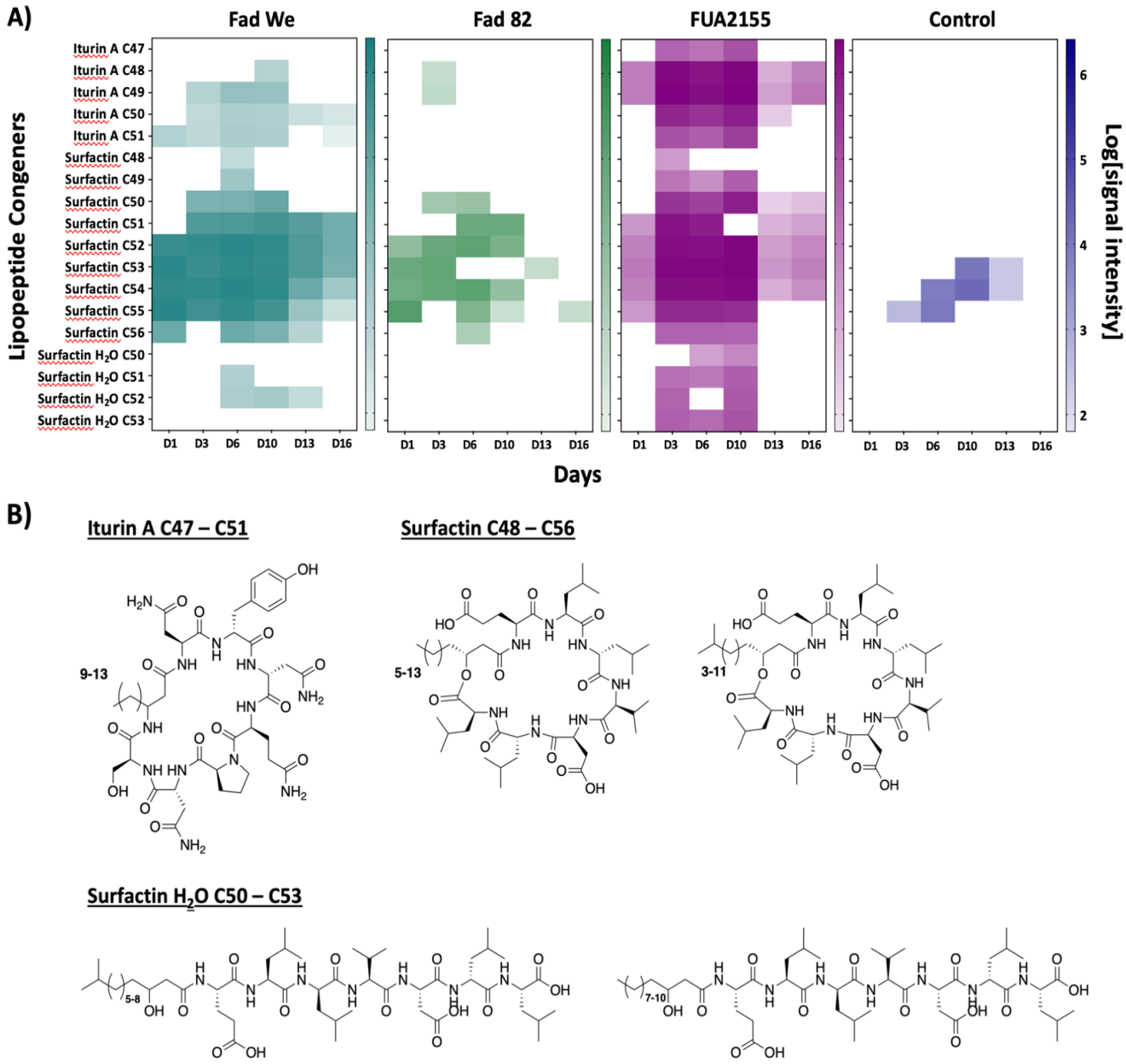


Figure 3.8 Production of lipopeptides during *daqu* fermentation. **A.** Heat map of lipopeptide congeners produced by *B. amyloliquefaciens* FadWe, Fad 82, and *B. velezensis* FUA2155 in *daqu* fermentations inoculated with 8 bacterial and fungal strains, and uninoculated control samples. Samples analyzed via LCMS and the log[signal intensities] are shown in a gradient. **B.** Base structures of antifungal lipopeptide congeners, denoted by the varying alkyl chain lengths. Experiments were done in triplicate.

The signal intensity of surfactin, iturin A, and fengycin in the different *daqu* samples were variable. Surfactins were detected among all the samples including the control group, but mainly in the samples inoculated with *B. amyloliquefaciens* Fad We, and *B. velezensis* FUA2155. The signal intensity of surfactins C52-C55 in *B. amyloliquefaciens* Fad We samples from day 1 to day 10 was relatively high. Surfactin C50-C55 had the highest intensity of surfactins in *B. velezensis* FUA2155 samples from day 3 to day 10. The log[signal intensity] of iturins, on the other hand, demonstrated a substantial difference. Iturin A C47-C51 was found in all *B. velezensis* FUA2155 samples. No iturin A C47 was found in any of the *B. amyloliquefaciens* Fad We samples on any of the days. The signal strength of iturins was low or non-existent in *B. amyloliquefaciens* Fad 82 and control samples. In addition, fengycin was not detectable in any of the samples. Some surfactin isomers were also detected in the negative control samples, which can be explained by the fact that the grains likely contain *Bacillus*, therefore so low levels of the lipopeptides are expected (Figure 3.8).

3.4 Discussion

This study is the first to apply mass spectrometry to detect all surfactin and iturin A isomers in *daqu* matrix samples as well as in LB cultures, whereas previous studies only discovered one or two peptides in *jiuqu*/*baijiu* samples (Zhang et al., 2014; Chen et al., 2020). Experimentation described in this communication expends prior knowledge by providing a comprehensive qualitative analysis of isomers of surfactin, iturin A, and fengycin from *daqu* fermentation matrix were analyzed by LC-MS, as well as by documenting *in situ* antifungal activity in simple and complex *daqu* models.

Lipopeptides synthesized by *Bacillus* strains possess broad spectrum antimicrobial activity. Different lipopeptides have their own unique chemical structures, properties and biological activities. Surfactin is a powerful biosurfactant with emulsifying and foaming properties. Because

of the amphiphilic nature, surfactin is tightly anchored into lipid layers and can thus interfere with biological membrane integrity in a dose-dependent manner (Ongena and Jacques, 2008). Surfactin has antibacterial and antiviral abilities, but no apparent antifungal effects.

Iturins exhibit strong *in vitro* antifungal activities against a wide variety of yeast and fungi but only limited antibacterial and no antiviral activities (Aranda et al., 2005). For example, iturin from *B. pumilus* HY1 inhibited *A. flavus* and *A. parasiticus* with an MIC of 50 mg/L (Cho et al., 2009). This fungitoxicity of iturins has been attributed to membrane permeabilization (Gordillo and Maldonado, 2012). Nevertheless, the underlying mechanism is based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins (Aranda et al., 2005). Based on the results of the MIC assays of surfactin, iturin A and fengycin against several strains of yeasts and fungi in this study, iturin A showed the highest inhibition activity against 7 of the 8 indicator strains with an MIC ranging from 10 to 50 mg/L (Figure 3.2), in agreement to earlier studies (Aranda et al., 2005; Cho et al., 2009).

The action of fengycin is less known compared with other lipopeptides but is also readily interacts with lipid layers and to some extent retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent manner (Deleu et al., 2005). Fengycin was reported to exhibit strong fungitoxic activity, specifically against filamentous fungi (Vanittanakom et al., 1986; Koumoutsi et al., 2004). Recently, the fengycin was proved to mediate the cross-kingdom communication between bacteria and fungi (Venkatesh et al., 2022). Fengycin facilitates bacterial invasion into fungal chlamydospores when comparing the growth of fungi strains and wild type *B. velezensis* or *B. velezensis* Δ*fenD* in a co-culture system.

Additionally, lipopeptides are known to act in an antagonistic or synergistic manner. For example, surfactin showed antagonistic activity with fengycin (Tao et al., 2011). The MIC of fengycin

against *Rhizopus stolonifer* in the presence and absence of commercial surfactin was compared. The MIC of fengycin increased from 0.4 to 2.0 g/L when commercial surfactin was added (Tao et al., 2011). In contrast, a positive synergistic effect was found to be in relation to interactions between iturin A and surfactin. With the addition of iturin A, the haemolytic activity of surfactin was significantly increased (Maget-Dana et al., 1992).

A previous study compared the MIC of the three isolated lipopeptides against *Gibberella zeae.* The results showed the iturins were slightly more inhibitory than the fengycins, while the surfactins demonstrated no activity, even at the highest concentration (1 μM) tested (Dunlap et al., 2011). In addition, in a recent study about the production of lipopeptide in cocultures of *Trichoderma harzianum* and *B velezensis*, the iturin inhibited *T. harzianum* more effectively than fengycin and surfactin (Vassilev et al., 2022). After deletion of the synthesize gene of each lipopeptides respectively, the inhibition zone of each mutant against *T. harzianum* was compared. Iturin, fengycin, and surfactin had inhibition zones ranging in size from small to large. In order to assess their activity in accordance with concentration, 0.5 g/L of each lipopeptide was added separately to the culture of *T. harzianum*. The growth of the fungus was completely inhibited when iturin was added, whereas fengycin had a lower inhibitory effect (~50 %), and surfactin showed a faint inhibition (Vassilev et al., 2022). Both findings indicate that the iturin has a stronger inhibition activity than fengycin and surfactin. Overall, these findings together with the MIC test and mass spectrometry analysis of *daqu* samples in this study, iturin A produced by *B. velezensis* FUA2155 may be the main reason for the higher inhibitory effect compared with the two *B. amyloliquefaciens* strains on the mold growth based on the observation on the surface of *daqu* samples (Table 3.5) (Dunlap et al., 2011; Vassilev et al., 2022).

The interactions between plants and microorganisms are incessant. For example, the vegetative cells and endospores of *Bacillus* are widely present in soil and air. Numerous isolates of the *Bacillus* genus have been developed as endophytes and biological control agents of plant pests and pathogens, while some strains of *Bacillus* have beneficial interactions with plants (Schirawski and Perlin, 2018). The plants serve as sheltered habitats and provide nutrients to *Bacillus*, while the strains of *Bacillus* in soil serve as endophytes that produce antimicrobial peptides to protect the plants against harmful microbes (Szymańska et al., 2016). Therefore, the existence of *Bacillus* endospores in *daqu* relates to the stable occurrence of these organisms as part of commensal microbiota of plants, including wheat (Tilak and Reddy, 2006; Chen et al., 2007; Fan et al., 2011; Jamal et al., 2019), and to the formation of spores which remain viable throughout storage and processing of grains. Lastly, cereal grains and flours generally harbor spores of *Bacillus* species (Needham et al., 2005; Fangio et al., 2010) which makes the contamination rather unavoidable. Due to the open environment and spontaneous fermentation, the microbial communities of *daqu* vary a lot. The different strains of *Bacillus* that may exist in *daqu* produce a variety of lipopeptides with different antimicrobial activities. Our study indicates that the production of different lipopeptides by bacilli influences the community assembly of *daqu* fermentation.

Production of antifungal lipopeptides has been described for multiple strains of *Bacillus* that were isolated from solid-state fermented products (Slivinski et al., 2012; Wu et al., 2015, 2021; Lee et al., 2016a, 2016b; Zhi et al., 2016; Owusu-Kwarteng et al., 2020), but only few investigations detected the presence of these peptides *in situ* during food fermentation (Zhang et al., 2014; Chen et al., 2020). As an example, surfactin was traced in a Moutai liquor making process and was identified and quantified by UPLC–MS/MS (Chen et al., 2020). Surfactin in Moutai liquor was mainly produced by *Bacillus* spp. during the *daqu* making process and stacking fermentation stage,

but only a minute fraction carried over with the distillation into the final liquor (Chen et al., 2020). The isoforms of the surfactins have been analyzed in food fermentation (Lee et al., 2012). However, the antifungal activities of different isoforms are not well studied and remains unknown.

This study is one of few studies that re-model and constitutes the *daqu* microbiota with identified strains of *Bacillus*, fungi and yeasts (Li et al., 2017; Wang et al., 2017; He et al., 2020). Besides, this study is the first to demonstrate the *in situ* antifungal activity of lipopeptides produced by *Bacillus* spp. against molds in the cereal fermentation matrix. The production of antifungal peptides was monitored and analyzed by mass spectrometry during the *daqu* fermentation process. Furthermore, this study provides novel insights into controlling the *daqu* microbiota by selecting antifungal strains for the production of *Baijiu*, which in turn may standardize and improve the quality of the product. Lastly, this research also provides directions applying *Bacillus* for other food fermentations to control fungi.

3.5 Conclusion

The presence of *Bacillus* as endophyte in or on grains make it unavoidable in the *daqu* fermentation. The *Bacillus* spp. in *daqu* fermentation not only affects the accumulation of enzymes in the fermentation process but also dictates the final community composition due to the production of antimicrobial lipopeptides. Hence, it is necessary to study the interaction between the *Bacillus* and fungi in a *daqu* fermentation model to allow a better understanding of the role of *Bacillus* spp. during the fermentation process.

However, the effect of antifungal lipopeptides produced by *Bacillus* spp. in *daqu* fermentation matrix is not well studied and remains unclear. This study compares the antifungal activity of surfactin, iturin A and fengycin against several fungi and yeast strains *in vitro*, where iturin A is shown to display the most prominent antifungal activity. Furthermore, this study illustrates the

antifungal activity of lipopeptides produced by *Bacillus* spp. against molds in a *daqu* fermentation matrix *in situ* and detected the signal intensity of the three peptides by mass spectrometry. The results show that the *B. velezensis* FUA2155 strain exhibit a higher antifungal activity in the *daqu* fermentation model, which can be explained by the production of iturin A. Overall, this study provides an insight on the interaction between the *Bacillus* and fungi in *daqu* fermentation models to better understand the role of *Bacillus* spp. during the *daqu* fermentation.

Chapter 4 Effect of Copy Number of The *spoVA*^{2mob} Operon, Sourdough and Reutericyclin on Ropy Bread Spoilage Caused By *Bacillus* spp.ad Spoilage Caused By *Bacillus* spp.

4.1 Introduction

Bread or steamed bread is a staple of the daily diet in most regions of the world with a temperate climate (Kourkouta et al., 2017). Ropy spoilage of bread is caused by strains of *Bacillus* spp. including *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* (Pepe et al., 2003; Sorokulova et al., 2003; Valerio et al., 2012, 2015). *Bacillus* endospores are present in flour and survive after baking. Their growth and production of amylases and proteases initially generate a typical fruity odor, subsequently result in a sticky and stringy crumb and slime formation, making the bread inedible (Corsetti et al., 2000; Pepe et al., 2003). *Bacillus* endospores have been isolated from bakery environment and also from raw materials, such as wheat flour, yeast and bread improvers (Bailey & von Holy, 1993; Pepe et al., 2003). Contamination of wheat flour with *Bacillus* endospores relates to the stable occurrence of these organisms as part of commensal microbiota of plant including wheat (Vessey and Buss, 2002; Tilak and Reddy, 2006; Chen et al., 2007; Fan et al., 2011; Jamal et al., 2019), and to the presence of *Bacillus* as member of seed-borne endophytic microbial community (Robinson et al., 2016; Shahzad et al., 2016). *Bacillus* endospores remain viable throughout storage and processing of grains (Needham et al., 2005; Fangio et al., 2010) which makes the contamination of flour not accidental but unavoidable.

Endospores of *Bacillus* are heat resistant (Setlow, 2003, 2006) and not inactivated during baking, where the crumb is heated to a maximum temperature of 100 °C for a few minutes. Strains of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* were reported to exhibit comparable heat

resistance, amylase activity and spoilage ability (Setlow, 2006; Valerio et al., 2012). However, a large intra-species variation of heat resistance of *Bacillus* endospores was attributed to the presence the *spoVA*^{2mob} operon (Berendsen et al., 2016a). Strains of *B. amyloliquefaciens* and *B. subtilis* contain up to three copies of the *spoVA*^{2mob} operons and the heat resistance of spores increased with an increase of the copy number (Berendsen et al., 2016a; Wang et al., 2018). A relationship between the *spoVA*^{2mob} operon and the potential to cause ropy spoilage is further suggested by the observation that all strains of *B. subtilis* and *B. amyloliquefaciens* that were isolated from ropy bread (Röcken and Spicher, 1993) were later identified to harbor multiple copies of the *spoVA*^{2mob} operons (Li et al., 2019). A contribution of the *spoVA*^{2mob} operon to the ability of bacilli to survive baking, however, has not been determined experimentally.

Ropy spoilage of bread develops rapidly at warm and humid conditions (Vaičiulytė-Funk et al., 2015), and is prevented by chemical preservatives (Saranraj & Geetha, 2012) or the use of sourdough. In past years, the use of sourdough or sourdough products in baking has been re-established as the default method of bread production (Anonymous, 2022b, 2022a). Sourdough prevents ropy spoilage (Pepe et al., 2003; Sadeghi, 2008) through acidification and the inhibitory activity of undissociated organic acids (Rosenquist and Hansen, 1998).

Sourdough isolates of lactic acid bacteria were reported to produce bacteriocins with activity against rope-forming bacilli (Pepe et al., 2003; Digaitiene et al., 2012), however, their activity in bread is limited (Rosenquist and Hansen, 1998), likely because the bacteriocins are inactivated by proteases and thiol exchange reactions in sourdough (Gänzle, 2014). Some strains of *Limosilactobacillus reuteri*, a stable component of type II sourdoughs (Zheng et al., 2016), produce reutericyclin, a heat stable and antimicrobial tetramic acid with activity against a wide range of gram-positive bacteria including rope-forming bacilli (Gänzle et al., 2000). Reutericyclin is

produced in active concentration in sourdough fermentation (Gänzle and Vogel, 2003), but a possible contribution to bread preservation has not been demonstrated. Therefore, the aim of this study was to determine if ropy spoilage in bread could be inhibited by the use of reutericyclin or sourdough fermented by *L. reuteri* TMW 1.656. To identify relevant spoilage organisms, strains of *Bacillus* spp. that were previously isolated from ropy bread or other cereals products were characterized with respect to the copy number of the *spoVA*^{2mob} operon and their heat resistance during the baking process, and with respect to the presence of extracellular amylases.

4.2 Materials and methods

Table 4.1 Origin of strains used in this study and copy number of the *spoVA*^{2mob} operon in each strain.

Microrganism	Strain; origin	Copy # of *spoVA*^{2mob} operon/genome	Reference
B. velezensis	[a]FUA 2155; *daqu*	0	(Wang et al., 2018)
B. subtilis	FUA 2114; malted oats	0	(Li et al., 2019)
B. subtilis	Fad 110; ropy bread	2	(Röcken and Spicher, 1993)
B. subtilis	Fad 109; ropy bread	2	(Röcken and Spicher, 1993)
B. amyloliquefaciens	FUA 2154; *daqu*	2	(Wang et al., 2018)
B. amyloliquefaciens	FUA 2153; *daqu*	2	(Wang et al., 2018)
B. amyloliquefaciens	Fad 99; ropy bread	3	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad We; ropy bread	3	(Röcken and Spicher, 1993)
L. reuteri (reutericyclin positive)	TMW1.656; sourdough	n/a	(M. Gänzle & Vogel, 2003)
L. reuteri (reutericyclin negative)	TMW1.656Δ*rtcN*Δ*gtfA*	n/a	This study

[a] FUA number, Food microbiology culture collection at the University of Alberta.

4.2.1 Bacterial strains and culture conditions

The strains used in this study and their origin are listed in Table 4.1. Strains were maintained at −80 °C in 20 % glycerol. Strains of *L. reuteri* were incubated in mMRS medium at 37 °C for 24 h. The mutant strain of *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* was constructed by disruption of the reuteransucrase GtfA in *L. reuteri* TMW1.656Δ*rtcN* as described (Lin et al., 2015; Chen et al., 2016). Strains of *Bacillus* were grown aerobically on LB agar plates at 37 °C for 18 h.

4.2.2 Preparation of *Bacillus* endospore suspensions

Strains of *Bacillus* were surface plated on LB plates and incubated at 37 °C for 7 days to ensure the sporulation of more than 90 % of cells. Sporulation was confirmed by staining with the Schaeffer-Fulton method (malachite green solution of 5 % and 0.1 % safranin) which results in green staining of spores and red staining of vegetative cells (Maksong et al., 2017). Microscopic examination of spore suspensions confirmed that 90 to 99 % of the cells contained spores. Spore suspensions were obtained as described elsewhere (Margosch et al., 2004b). In short, flood the surface of plate with 5 mL cold sterile distilled water twice to collect the spores. The spore suspensions were washed with sterile water for four times by centrifugation at 3,000 × *g* for 15 min at 4 °C and resuspended in sterile distilled water. Between the second and third wash cycles, the spore suspensions were treated at 80 °C for 10 min to kill vegetative cells. The spore suspensions were stored at −80 °C until further use (Paidhungat et al., 2002; Li et al., 2019).

4.2.3 Determination of survival of strains of *Bacillus* spp. with different copy number of the *spoVA*^{2mob} operons after baking

To determine the contribution of the *spoVA*^{2mob} operon or multiple copies of the operons on survival after baking, we inoculated wheat dough with spores of 8 strains of *Bacillus* with a

different copy number of the *spoVA*^{2mob} operon and determined the survival after baking. The recipe of wheat bread is shown in Table 4.2. Ingredients were mixed with a KitchenAid 3 Qt mixer for 1 min (slow) and 14 min (fast), followed by a bulk proof at 25 °C for 60 min. The dough was divided into pieces of 150 g and each piece was rolled into a rectangular dough sheet and inoculated by spreading 1.5 mL of spore suspensions of *Bacillus* spp. with a viable spore count of 10^8 spores/mL. Subsequently, the dough was rolled, shaped, proofed for 60 min at 32 °C, and baked at 180 °C for 20 min. The internal temperature of bread during baking was monitored by a thermocouple that was inserted to the bread during baking. The temperature profile during baking process is shown in Figure 4.1. Samples were taken from the dough before baking, after baking and cooling at room temperature for 1 h. Total aerobic plate counts of bread samples were determined by mixing 10 g of bread with 90 mL peptone in a stomacher. Serial dilutions in peptone water were surface plated on LB plates and were incubated at 37 °C for 18 h.

Table 4.2 Wheat bread formula.

Ingredients[a]	Amount [g]
White wheat flour	100.0 minus weight of freeze-dried sourdough
Freeze-dried sourdough	0, 3.0, 6.0, 12.0, or 24.0
Sterile tap water	60.0
Yeast	2.0
Salt	2.0
Sucrose	2.0

[a] White wheat flour (Robin Hood) and baker's yeast (Fleischmann's Active Dry Yeast) were obtained from a local supermarket.

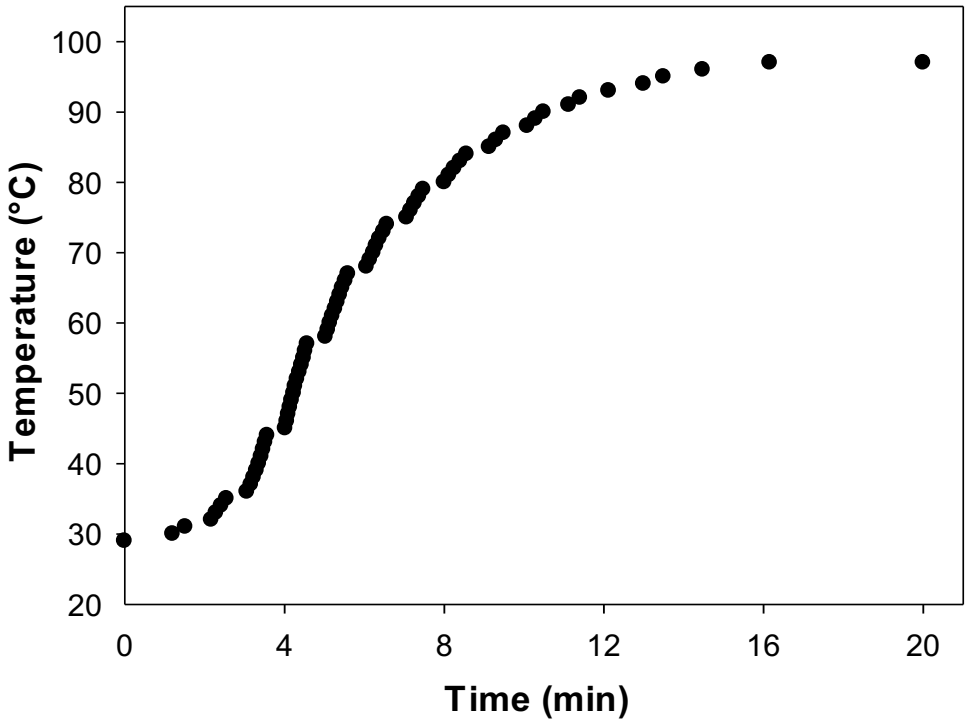


Figure 4.1 The temperature profile during baking process.

4.2.4 Determination of the spoilage phenotype of different strains of *Bacillus* inoculated on dough

To monitor the typical characteristics of rope spoilage, including appearance, aroma, and bacterial growth, two slices of each bread were placed in Petri dishes and stored at 30 °C for 7 days in a sealed container with 10% NaCl solution to maintain a_W at 0.95 throughout the storage process. The degree of spoilage, smell and bacterial growth of the sliced bread were visually examined and recorded by three persons separately each day. Two slices of bread for each sample were analyzed each day, and the experiment was repeated 3 times.

4.2.5 Determination of the spoilage phenotype of different strains of *Bacillus* inoculated on bread

Uncontaminated bread was prepared following a similar procedure as indicated above. After baking and cooling at room temperature, the bread was sliced. Then 30 µL of each spore suspension from 5 strains of *Bacillus* were inoculated at 5 different corners on one bread slice, and 3 replicates of slices were prepared under the same procedure. Uninoculated slices that were incubated at the same conditions served as control.

The inoculated bread slices were placed in petri dishes in containers with 10 % NaCl solution (same as before) and incubated at 30 °C for 5 days. The bacterial growth and characteristics of ropy spoilage such as discoloration, visible lines, soft and sticky crumb, and slime were identified throughout five days of storage. The experiment was repeated 3 times.

4.2.6 Analysis of amylases encoded in the genomes of *Bacillus* spp.

Sequences of extracellular amylases and glucoamylases in *Bacillus* species were retrieved from the Universal Protein Resource (UniProt) (https://www.uniprot.org/) database. These protein sequences were used as query sequences for BLAST analysis of whole genome sequences from the strains of *Bacillus* spp. (Li et al., 2019) used in this study. A different complement of amylases was found in *B. amyloliquefaciens* and *B. subtilis*. Therefore, we further blasted these sequences of amylases against more sequences of *Bacillus* strains, including 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of *B. velezensis* in total (Li et al., 2019).

4.2.7 Preparation of sourdough

Anti-rope activity has been considered as an important characteristic of some lactic acid bacteria (Pepe et al., 2003). The reutericyclin positive *L. reuteri* TMW1.656 was used as reference strain

and its anti-rope activity was compared to *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA*. After incubating the two strains in mMRS medium at 37 °C for overnight, 10 mL of culture were added into a 15 mL centrifuge tube, respectively. The culture was washed by centrifugation at 5000 rpm for 3 min and resuspended in 10 mL sterile tap water. The 10 mL of washed strain culture was mixed thoroughly with 10 grams of reweighed white flour. The mixture was fermented at 30 °C for 24 h, corresponding to a sourdough pH of 4.60 ± 0.02 and 4.57 ± 0.03 for *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN*Δ*gtfA*, respectively. The fermented sourdough was placed at −20 °C overnight and then freeze-dried. After freeze drying, the dry sourdough was ground and stored at −20 °C until use.

4.2.8 Evaluation of the effect of sourdough on ropy spoilage

On the basis of slime production and heat resistance, *B. amyloliquefaciens* Fad 99 was selected as an indicator to determine the antagonistic activities of different sourdough dosage. Sourdough bread was prepared following the recipe in Table 4.2 by adding 0, 3, 6, 12 or 24 % freeze-dried sourdough made with *L. reuteri* TMW1.656, respectively (substitute white flour with freeze-dried sourdough). Each dough was inoculated with 1.5 mL spore suspension of *B. amyloliquefaciens* Fad 99 as described above. Sourdough bread was prepared as described above after inoculation on dough with the spore suspension of *B. amyloliquefaciens* Fad 99. This strain was chosen because, among the strains of *Bacillus* investigated in this study, Fad 99 showed the greatest spoilage potential. The bacterial growth and ropy characteristics were observed over 7 d to determine the dosage of sourdough that inhibits growth of bacilli in bread. This experiment was carried out in three independent experiments.

4.2.9 Detection of the inhibition effect of reutericyclin on ropy bread spoilage caused by *Bacillus* species

The reutericyclin-positive *L. reuteri* TMW1.656 and the isogenic reutericyclin-negative derivative *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* were used to compare the inhibition effect of reutericyclin on ropy spoilage that caused by *B. amyloliquefaciens* Fad 99. The same procedure was applied to produce freeze-dried sourdoughs fermented with *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN*Δ*gtfA*, respectively. One mL spore suspension from *B. amyloliquefaciens* Fad 99 was inoculated on the dough containing 12 % freeze-dried sourdough. The sourdough bread was baked as described before and bacterial growth and different ropy spoilage characteristics were monitored daily. The experiment was performed in three independent replicates with replicated culture preparation and independent baking trials.

4.2.10 Statistical analysis

Experiments were carried out at least in three replicates with different batches of spore suspensions and the same batch of freeze-dried sourdough. Statistical analysis of cell counts of *Bacillus* spp. after baking was carried out by ANOVA with the Holm-Sidak post hoc analysis and an error probability of 5 % ($P < 0.05$).

4.3 Results

4.3.1 Variations of heat resistance of spores from strains of *Bacillus* with different copy number of the *spoVA*^{2mob} operons in bread

Heat resistance of *Bacillus* endospores is dependent on the c(Berendsen et al., 2016a; Li et al., 2019). Since ropy spoilage of bread is dependent on spore survival during the baking process,

bread dough was inoculated with spores of strains of *Bacillus* with a different copy number of the *spoVA*^{2mob} operon and their survival after baking was determined (Figure 4.2).

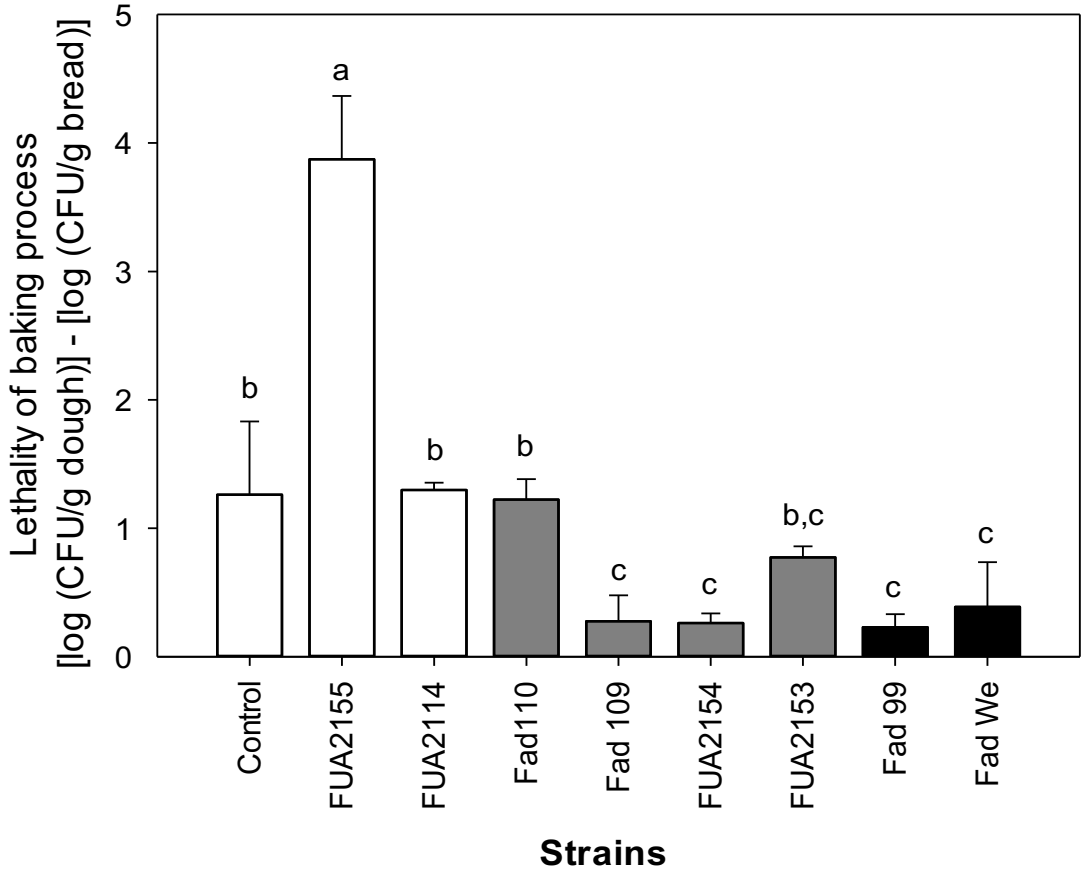


Figure 4.2 Lethality of the baking process [log(N/N_0)] towards spores of strains of *Bacillus* with different copy number of the *spoVA*^{2mob} operons. "N" represents the cell/spore counts after treatment at that time. "N_0" represents the initial cell/spore counts. The color coding of the bars indicates the copy number of the *spoVA*^{2mob} operon as follows: white (0 copies), gray (two copies), and black (three copies). Strain number and species are indicated as follows: *B. velezensis* FUA 2155; *B. subtilis* FUA 2114; *B. subtilis* Fad 110; *B. subtilis* Fad 109; *B. amyloliquefaciens* FUA 2154; *B. amyloliquefaciens* FUA 2153; *B. amyloliquefaciens* Fad 99; *B. amyloliquefaciens* Fad We. Control refers to bread that was not inoculated with *Bacillus* endospores. The data are means of three independent experiments; error bars are standard deviations. Cell counts that do not share a common superscript differ significantly ($P < 0.05$).

Overall, the lethality of the baking process, which exposed spores of *Bacillus* spp. to a temperature of more than 90 °C for about 10 min, was limited. Spore counts of *Bacillus* strains that did not encode for a *spoVA*^{2mob} operon were reduced by more than 1 log (CFU/g) after baking; spores of *Bacillus* strains that encoded three copies of the *spoVA*^{2mob} operons were reduced by less than 0.5 log (CFU/g); spores of *Bacillus* strains with two copies of the *spoVA*^{2mob} operons showed strain-specific survival (Figure 4.2).

4.3.2 Appearance of ropy spoilage phenotype of different strains of *Bacillus* inoculated on dough

B. subtilis and *B. amyloliquefaciens* are recognized as the causative agents of spoilage problem. To determine whether the spoilage phenotype of different strains of *B. subtilis* and *B. amyloliquefaciens* differs, spore suspensions of 5 different strains of *Bacillus* were inoculated at 5 different spots on one bread slice. The inoculation of bread rather than dough was chosen to eliminate the influence of survival after baking. Spoilage characteristics were monitored for 5 days (Figure 4.3). The spores from 2 strains of *B. amyloliquefaciens* spoiled the bread more rapidly when compared to spores from 3 strains of *B. subtilis*. *B. amyloliquefaciens* Fad 99 visibly degraded the crumb after 1 day of incubation and formed slime after 3-4 days; crumb degradation and slime formation by spores from *B. subtilis* was observed only after 3 and 5 days, respectively (Figure 4.3). *B. amyloliquefaciens* Fad 99 was chosen as the spoilage organism for the subsequent experiments.

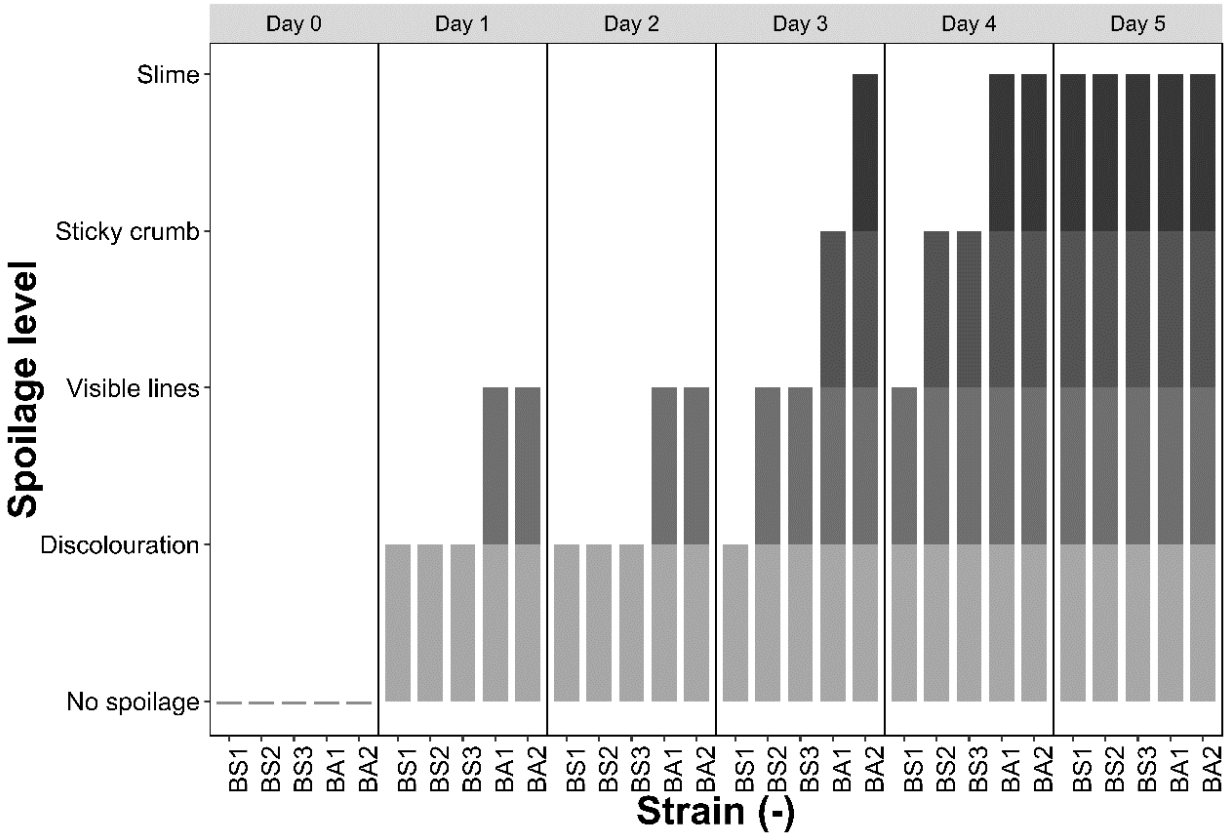


Figure 4.3 Kinetics of appearance of rope spoilage characteristics in breads contaminated with *B. subtilis* and *B. amyloliquefaciens* after baking. Contaminated slices were stored at 30 °C and a_W 0.95 for 5 days. Different colors of gray scale represent different levels of spoilage from low to high. The strain number is labeled as follows: BS1 (*B. subtilis* FUA 2114); BS2 (*B. subtilis* Fad 110); BS3 (*B. subtilis* Fad 109); BA1 (*B. amyloliquefaciens* FUA 2154) and BA2 (*B. amyloliquefaciens* Fad 99). The data represent results from three independent experiments.

4.3.3 Analysis of amylases encoded in genomes of *Bacillus* spp.

To identify putative genetic determinants for the different spoilage phenotypes of *B. amyloliquefaciens* and *B. subtilis* strains, genes coding for extracellular amylases and proteases were identified in the genomes of 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of *B. velezensis* (Li et al., 2019). Amylases were identified by BLAST analysis with protein sequences of extracellular amylases from *Bacillus* spp. that were available in the UniProt database.

Strains of *B. amyloliquefaciens* and *B. subtilis* differed in regard to the amylases that were identified in the genomes. Strains of *B. subtilis* and *B. velezensis* were found to encode for AmyE as sole extracellular amylase while five extracellular amylases including a hyperthermostable α-amylase are encoded in each genome of *B. amyloliquefaciens* (Figure 4.4). All genomes encode for two glucoamylases, but β-amylases were not identified. The different complement of amylases likely relates to the different spoilage phenotype of strains of *B. amyloliquefaciens* and *B. subtilis*.

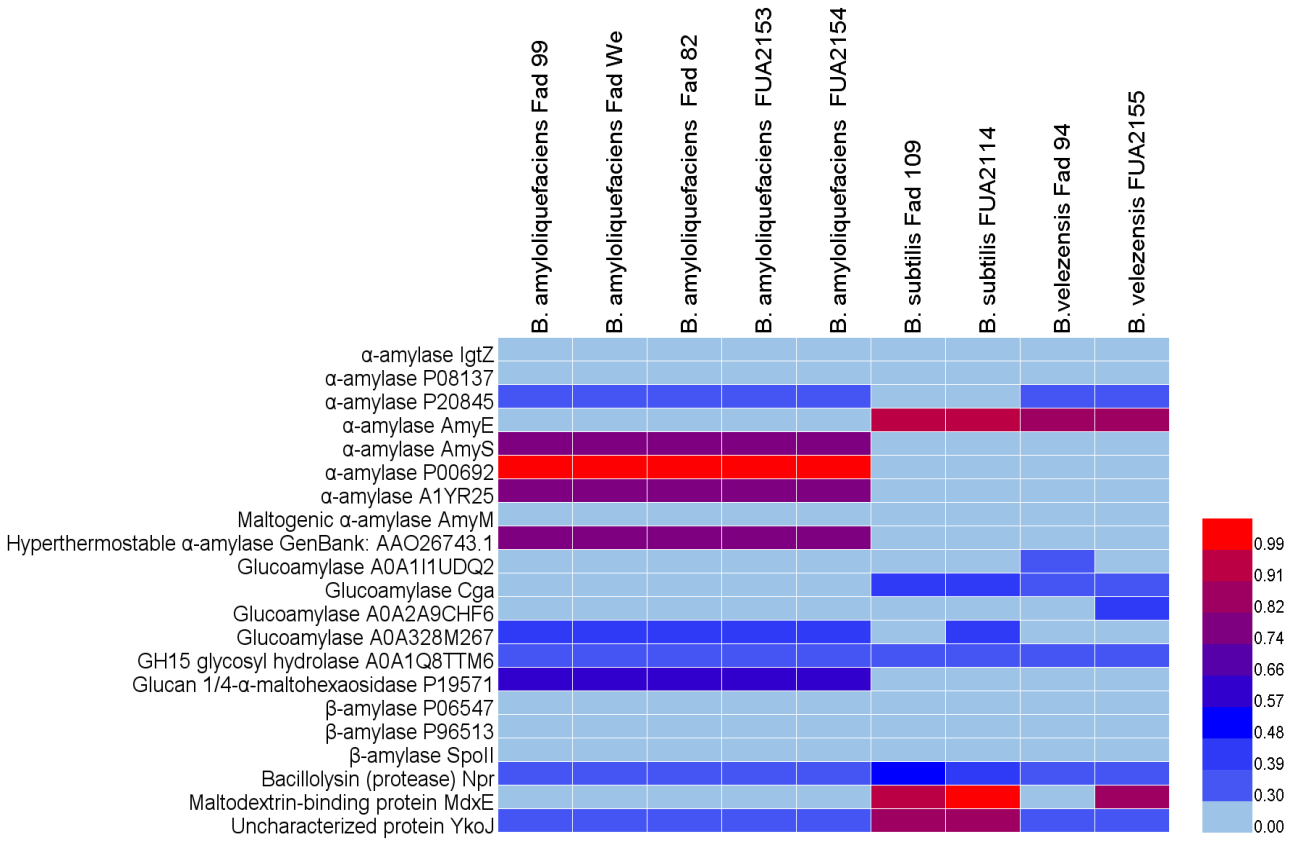


Figure 4.4 Identification of amylases, glucoamylases and other starch-active enzymes encoded in the genomes of 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of *B. velezensis*. With exception of the four glucoamylases, all proteins encode for a signal peptide that is predicted to mediate protein export. Uniprot protein accession numbers are indicated unless specified otherwise. **IgtZ** (A0P8X0) GH119 α-amylase; **P08137** GH13 α-amylase; **P20845** GH13 α-amylase; **AmyE** (P00691) GH13 α-amylase; **AmyS** (P06278 and P06279) GH13 α-amylase;

P00692 GH13 α-amylase; **A1YR25** GH13 α-amylase; **AmyM** (P19531) GH13 maltogenic α-amylase; **GenBank: AAO26743.1** GH13 hyperthermostable α-amylase; **A0A1I1UDQ2** GH15 glucoamylase; **Cga** A0A3S4RT35 GH15 glucoamylase; **A0A2A9CHF6** GH15 glucoamylase; **A0A328M267** GH15 glucoamylase; **A0A1Q8TTM6** GH15 glycosyl hydrolase; **P19571** GH13 glucan 1/4-α-maltohexaosidase; **P06547** GH14 β-amylase; **P96513** GH14 β-amylase; **SpoII** P36924 β-amylase; **Npr** (P29148) M4 family peptidase bacillolysin (protease); **MdxE** (O06989) maltodextrin-binding protein of the bacterial solute-binding protein 1 family. **YkoJ** (O35012) uncharacterized protein (putative protease inhibitor).

4.3.4 Effect of sourdough dosage on ropy spoilage

To determine the effect of sourdough on ropy spoilage, bread doughs were prepared with 0, 3, 6 12 and 24 % of freeze-dried sourdough fermented with the reutericyclin-producing *L. reuteri* TMW1.656. Bread dough was inoculated with a spore suspension of *B. amyloliquefaciens* Fad 99 and the spoilage phenotype was monitored at daily intervals (Figure 4.5). The addition of 3 % sourdough did not affect spoilage, but spoilage was delayed by 2-3 days after the addition of 6 or 12 % sourdough corresponding to a bread pH of 6.2 and 5.9, respectively (Figure 4.5). The bread made with 24 % sourdough did not spoil during the 5 days of observation.

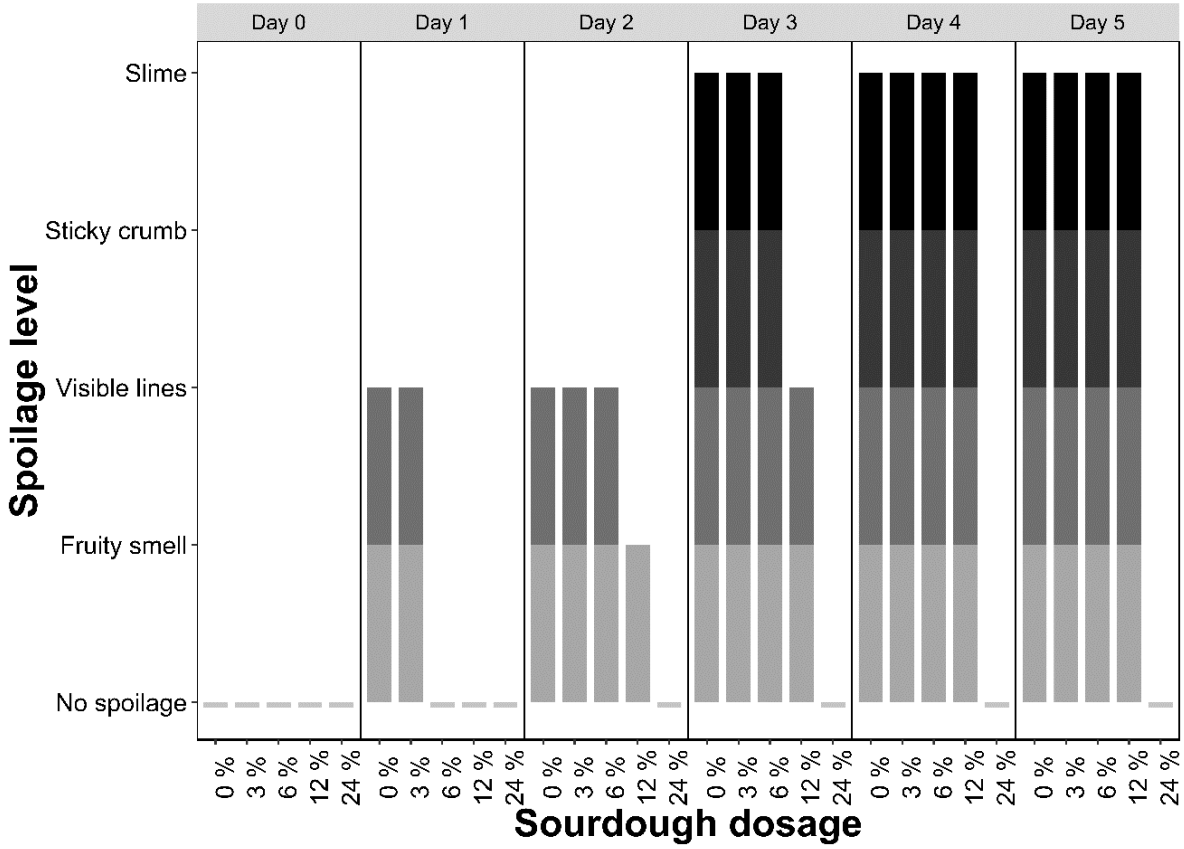


Figure 4.5 Kinetics of appearance of spoilage characteristics in breads baked from doughs contaminated with *B. amyloliquefaciens* (Fad 99) during storage at 30 °C and a_W 0.95 for 5 days. The doughs contained different dosage of sourdough fermented with reutericyclin-positive *L. reuteri* TMW1.656. Different colors of gray scale represent variations in levels of spoilage from low to high. Different sourdough dosage was labeled in the figure. The pH of bread produced with 0, 3, 6, 12 or 24 % sourdough was 6.40 ± 0.044; 6.30 ± 0.019; 6.17 ± 0.024; 5.93 ± 0.041; and 5.53 ± 0.040, respectively. Data represent results from three independent experiments.

4.3.5 Detection of the inhibition effect of reutericyclin produced by *L. reuteri* on ropy bread spoilage caused by *Bacillus*

To compare the inhibition effect of reutericyclin on ropy spoilage caused by *B. amyloliquefaciens* Fad 99, sourdoughs were fermented with the reutericyclin positive *L. reuteri* TMW1.656 and its isogenic, reutericyclin negative *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* (Chen et al., 2016). The GtfA-RtcN double mutant was used to avoid exopolysaccharide formation during sourdough

fermentation, which may confound the assessment of slime formation during ropy spoilage. Bread was prepared with 12 % sourdough, a dosage that delays spoilage by 1-2 d (Figure 4.5), and bread dough was inoculated with spores of *B. amyloliquefaciens* Fad 99. Bread without reutericyclin showed visible line on the third day and became slimy on the fourth day. Bread with reutericyclin showed visible lines only on day five and slime was not observed until day seven (Figure 4.6).

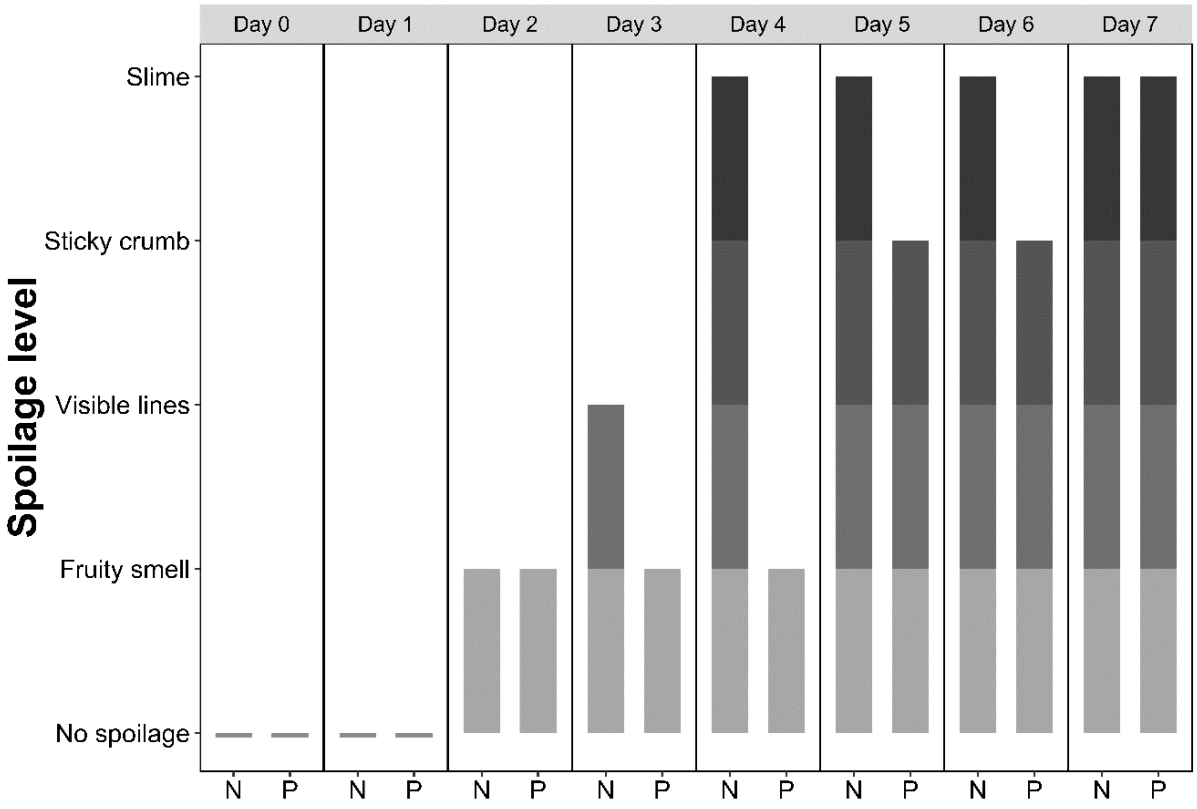


Figure 4.6 Effect of reutericyclin on the kinetics of appearance of ropy spoilage characteristics of breads baked from doughs contaminated with *B. amyloliquefaciens* Fad 99 during storage at 30 °C and a_W 0.95 for 7 days. Sourdough was added at a dosage of 12 %. Different colors of gray scale represent different levels of spoilage from low to high. "N" represented bread produced with sourdough fermented with the reutericyclin-negative *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA*; "P" represented bread produced with sourdough fermented with the reutericyclin-producing *L. reuteri* TMW1.656. The pH of bread produced with sourdough fermented with *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*rtcN*Δ*gtfA* was 5.93 ± 0.041 and 5.93 ± 0.021, respectively. Data represent results from three independent experiments.

4.4 Discussion

Ropy spoilage of bread is frequent in warm and humid climate including Mediterranean countries, Africa as well as Australia (Voysey and Hammond, 1993). *Bacillus* spp., which produce extracellular slimy polysaccharides and possess proteolytic and amylolytic enzymes, are the major cause of ropiness (Corsetti et al., 2000; Pepe et al., 2003). *Bacillus* endospores that are present in raw materials survive during baking (Pepe et al., 2003; Valerio et al., 2012) and lead to ropy spoilage if storage conditions support germination and growth (Vaičiulytė-Funk et al., 2015).

The *spoVA*^{2mob} operon plays an essential role in the heat resistance of *Bacillus* spores; its effect on heat resistance is dependent on the copy number of the operon in genomes of *Bacillus* spp. (Berendsen et al., 2016a; Wang et al., 2018). This study used 8 strains of *Bacillus* isolated from ropy bread and *daqu* (Röcken and Spicher, 1993; Wang et al., 2018) determine the impact of the copy number of the *spoVA*^{2mob} operon on heat resistance during baking process, where the temperature reaches up to 100 °C for several minutes. Viable counts of spores with two or three copies of *spoVA*^{2mob} operons were reduced by less than 1 log (CFU/g); however, the lethality of the baking process towards spores of strains that did not harbor a *spoVA*^{2mob} operon was also limited. Because of this limited inactivation of endospores that do not harbour the *spoVA*^{2mob} operon, the presence of multiple copies of the operons did not further improve survival during baking and appears not to be a prerequisite for the ability of strains to spoil bread.

Strains of *B. subtilis* were most frequently isolated from ropy bread; however, several past studies mis-identified *B. amyloliquefaciens* as *B. subtilis* (Röcken and Spicher, 1993; Pepe et al., 2003; Valerio et al., 2012) because the two species show a high level of similarity of their 16S rRNA gene sequence (Reginensi et al., 2013). This study compared the spoilage phenotype of 3 strains of *B. subtilis* and 2 strains of *B. amyloliquefaciens* and demonstrated that *B. amyloliquefaciens*

caused spoilage of bread 2-3 days earlier than strains of *B. subtilis.* We further investigated the complement of extracellular amylases and proteases that are encoded in genomes of 5 strains of *B. amyloliquefaciens*, 2 strains of *B. subtilis* and 2 strains of the closely related species *B. velezensis* (Li et al., 2019). Strains of *B. amyloliquefaciens* were differentiated from *B. subtilis* and *B. velezensis* by encoding for multiple extracellular α-amylases and gluco-amylases. Moreover, a hyperthermostable α-amylase was identified in each genome of *B. amyloliquefaciens* but not in *B. subtilis* and *B. velezensis*; this enzyme may remain active after baking and initiate starch degradation in bread. The comparison of the genotype of *B. subtilis* and *B. amyloliquefaciens* with the spoilage phenotype strongly suggests that extracellular amylases and proteases contribute to ropy spoilage of bread, however, the contribution of individual enzymes remains subject to future studies.

Rope-forming bacilli are endophytes of plants including wheat, and their presence in seeds mediates vertical transmission of commensal plant microbiota to the offspring (Robinson et al., 2016; Shahzad et al., 2016), therefore, contamination of wheat flour with *Bacillus* spores is unavoidable (Pepe et al., 2003; Saranraj and Geetha, 2012). The use of sourdough as a biopreservative has only limited effect against fungal bread spoilage (Axel et al., 2017; Quattrini et al., 2018), but inhibits growth of rope-forming bacilli (Röcken and Spicher, 1993; Rosenquist and Hansen, 1998; Valerio et al., 2008). The preservative effect of sourdough was mainly attributed to formation of organic acids and acidification of the bread crumb (Rosenquist and Hansen, 1998; Valerio et al., 2008). Acidification to a pH of 3.7 to 4.5 inhibits ropy spoilage (Pepe et al., 2003), and undissociated acetic acid enhances the preservative effect of sourdough (Rosenquist and Hansen, 1998).

Although sourdough has re-claimed its place as the standard process of bread-making, the use of sourdough or sourdough products may not always suffice to inhibit ropy spoilage. Dried sourdough products are standardized on the basis of the concentration of organic acids but typically much of the volatile acetic acid is lost during drying (Brandt, 2007). Moreover, not all sourdoughs are effective at inhibition of rope spores due to their high pH and low concentration of organic acids (Katina et al., 2002). The concentration of organic acids in sourdoughs and bread dough fermented in bakeries is not as readily standardized as for stabilized sourdough products (Brandt, 2007). In addition, low pH and undissociated organic acids may alter the flavor and taste of bread (Hansen and Schieberle, 2005). In this study, addition of 24 % sourdough to a bread recipe effectively inhibited the growth of *B. amyloliquefaciens* but the combined use of organic acid with specific antimicrobial compounds may allow bread preservation without excessive acidity.

Bacteriocins of lactic acid bacteria were evaluated with respect to their activity against *Bacillus* spp. (Pepe et al., 2003; Digaitiene et al., 2012) but nisin seems to be the only bacteriocin which has a general inhibitory effect against strains of *Bacillus* (Rosenquist and Hansen, 1998). The use of nisin as additive or the use of nisin-producing starter cultures in sourdough, however, had no effect on growth of *Bacillus* in bread (Rosenquist and Hansen, 1998). This may relate to inactivation of nisin by proteases and thiol exchange reactions at the dough stage, or to their heat inactivation during baking (M. Gänzle, 2014). Reutericyclin is produced by some strains of *L. reuteri* and *S. mutans*(Lin et al., 2015; Tang et al., 2020). Reutericyclin is a tetramic acid derivative which resists proteolysis in dough and heat inactivation during baking (Gänzle et al., 2000). Reutericyclin exhibits a bactericidal mode of action against *Bacillus* spp., and inhibited germination of spores (Gänzle et al., 2000). We used the same strains of *Bacillus* that were previously characterized with regards to their sensitivity to reutericyclin (Gänzle et al., 2000).

Fermentation of sourdoughs with isogenic strains that produce comparable levels of organic acids but differ with respect to reutericyclin production (this study) (Lin et al., 2015) demonstrated that reutericyclin significantly contributes to inhibition of *Bacillus* growth in bread. The use of reutericyclin-producing strains thus allows the prevention of ropy spoilage with a reduced dosage of sourdough and corresponding reduced levels of acidity (this study). The reutericyclin producing strain *L. reuteri* TMW1.656 was isolated from an industrial sourdough and beneficially impacts bread texture through production of reuteran (X. Y. Chen et al., 2016; M. Gänzle & Vogel, 2003).

In conclusion, multiple copies of the *spoVA*^{2mob} operon made only a limited contribution to the resistance of *Bacillus* spores during baking. The different spoilage phenotype observed after growth of *B. amyloliquefaciens* and *B. subtilis* may relate to the differential presence and expression of extracellular amylases and proteases in these species. Moreover, this study confirmed the inhibitory effect of sourdough and demonstrated that reutericyclin produced at the sourdough stage contributes to inhibition of growth of rope-forming bacilli in bread.

Chapter 5 The *spoVA*^{2mob} Operon Contributes to Pressure Resistance of *Bacillus* Endospores

5.1 Introduction

Endospores (spores) produced by *Bacillus* are resistant to heat, UV irradiation, desiccation and chemical assaults (Setlow, 2003). They can survive in non-thermal or insufficiently heated processed foods (Setlow, 2003). Increasing the intensity of thermal treatment reduces the microbial load, however, the efficacy of treatment might be limited on distinctly heat resistant spores, which cannot be killed by a conventional thermal treatment alone (Berendsen et al., 2016b). Furthermore, high temperatures may negatively impact texture, flavor and nutrient content in a variety of food products (Holdsworth and Simpson, 2016). Accordingly, non-thermal food processing technologies are developed to make up the disadvantages of traditional thermal treatments (Matser et al., 2004).

High pressure processing is among the commercially most successful non-thermal processing technologies; pressure processes are used for the inactivation of vegetative microbial cells (Matser et al., 2004; Farkas, 2016). Pressure-assisted thermal sterilization to inactivate spores by using compression heating for rapid and uniform heating of the food product is still in the development stage. The mechanisms of the spore inactivation by pressure are not fully understood yet and warrant further investigation (Reineke, Mathys, Heinz, & Knorr1, 2013) *Bacillus* and *Clostridium* spp. exhibit a large intra-species variation with regards to the spore resistance to pressure (Margosch et al., 2004b, 2004a; den Besten et al., 2018). Spores of *C. botulinum* Group I (or *C. parabotulinum*, (Smith et al., 2018)) are among the most pressure resistant bacterial spores and

strains of *B. amyloliquefaciens* have been used as pressure-resistant surrogate organisms for *C. botulinum* (Margosch et al., 2006).

The heat resistance of bacilli correlates to the copy number of *spoVA*^{2mob} operon, especially the last gene of *spoVA*^{2mob} operon on chromosome (Berendsen et al., 2016a; Krawczyk et al., 2017; Wang et al., 2018). The *spoVA*^{2mob} operon is present in *Bacillus* spp., including *B. amyloliquefaciens*, *B. subtilis*, *Bacillus cereus, Bacillus licheniformis* and *Bacillus thermoamylovorans* (Berendsen et al., 2016b; Krawczyk et al., 2016; Wang et al., 2018). *Bacillus* strains carrying multiple copies of *spoVA*^{2mob} operons exhibit $D_{100\,°C}$- or $D_{110\,°C}$-values that are up to 100-fold higher when compared to strains of the same species that do not carry this operon (Berendsen et al., 2016a; Wang et al., 2018). The pressure resistance of bacterial spores does not generally correlate to their heat resistance, however, some spore properties, particularly the content of DPA and water, relate to their resistance to high pressure as well as high temperature (Margosch et al., 2004b). The resistance of spores to combined application of high pressure and high temperature may depends on the spores' ability to retain DPA and on the heat resistance of the DPA-free spores (Margosch et al., 2004b; Reineke et al., 2013a). Scholars have different opinions on the mechanism of spore inactivation under different conditions. The most likely mechanisms for spore inactivation with treatment of pressure above 600 MPa and temperature higher than 60 °C are compromises on integrity of the inner spore membrane and the function of some membrane proteins (Pagán and Mackey, 2000; Reineke et al., 2013b). This therefore facilitates Ca-DPA release in the absence of physiological activity (Vepachedu et al., 2007). Proteins encoded by the regular *spoVA* operon, the SpoVAC, SpoVAD and SpoVAEB, relate to DPA uptake during sporulation and release during germination (Vepachedu and Setlow, 2007; Velásquez et al., 2014) and may form parts of DPA channel in the inner membrane (Vepachedu et al., 2007). Therefore,

the copy number of *spoVA*^{2mob} operon per genome may also correlate to the spore DPA content and pressure resistance. Nevertheless, the potential link between the copy number of *spoVA*^{2mob} operons and spore pressure resistance has not been reported.

This study therefore aimed to determine the relationship between copy number of *spoVA*^{2mob} operon and pressure resistance of *Bacillus* endospores. To quantify the copy number of *spoVA*^{2mob} operon in food isolates of *Bacillus* spp., a fast and accurate droplet digital PCR (ddPCR) assay for quantification of the absolute gene copy number was developed. Whole genome sequencing is described as reference to assess the copy number variation of target genes (Zong et al., 2012; Zare et al., 2017). PCR-based methods are also applied as rapid methods for gene copy number determination (Li and Olivier, 2013). The ddPCR system has been utilized as a method that quantifies absolute copy numbers of nucleic acids with high accuracy and precision (Pinheiro et al., 2012; Yang et al., 2014). In ddPCR system, an absolute target sequence is quantified from the ratio of positive and negative partitions estimated by the Poisson distribution (Hindson et al., 2013) without the need for an external calibrant (Hayden et al., 2013; Morisset et al., 2013; Strain et al., 2013). Reports on the use of ddPCR to study the number of gene copies on bacterial chromosomes, however, are scarce.

5.2 Materials and methods

5.2.1 Bacterial strains, preparation of spore suspensions and culture conditions

The *Bacillus* strains used in this study and their origins are listed in Table 5.1. All strains were grown aerobically on LB agar plates at 37 °C for 18 h. Spores were prepared by plating aliquots of 0.1 mL from fresh overnight cultures on LB agar plates. After incubation at 37 °C for 7 days, the Schaeffer-Fulton method which results in the coloring will appear green on the spores, as well as red in the vegetative cells, was used to stain the culture (Maksong et al., 2017).

Table 5.1 Origin of 17 *Bacillus* spp. and copy number of *spoVA*^{2mob} operon in each strain.

Organism	Strain origin	Methods		Reference
		ddPCR-Experimental[b] (Theoretical) copy # of *spoVA*^{2mob} operon/genome	CNOGpro-Copy # of *spoVA*^{2mob} operon/genome (GenBank accession number)	
B. velezensis	Fad 94, ropy bread	0.09 ± 0.06 **(0)**	**0** (SDKF00000000)	(Röcken and Spicher, 1993)
B. pumilus	FUA2024[a], cow vagina	- **(0)**	-	(Wang et al., 2013)
B. subtilis	FUA2114, malted oats	**- (0)**	**0 (**VRTW00000000**)**	Unpublished
B. velezensis	FUA2155, *daqu*	0.24 ± 0.01 **(0)**	**0** (SDKI00000000)	(Wang et al., 2018)
B. cereus	FUA2120, malted oats	1.19 ± 0.10 **(1)** 1.09 ± 0.30 **(1)**[c]	-	Unpublished
B. subtilis	FUA2148, *daqu*	1.24 ± 0.11 **(1)** 1.27 ± 0.43 **(1)**	-	(Wang et al., 2018)
B. amyloliquefaciens	FUA2149, *daqu*	1.18 ± 0.28 **(1)** 1.11 ± 0.18 **(1)**	-	(Wang et al., 2018)
B. subtilis	Fad 110, ropy bread	2.10 ± 0.16 **(2)** 1.93 ± 0.37 **(2)**	-	(Röcken and Spicher, 1993)
B. subtilis	Fad 109, ropy bread	1.94 ± 0.31 **(2)** 1.92 ± 0.51 **(2)**	**2** (SDKE00000000)	(Röcken and Spicher, 1993)

Table 5.1 (continued).

Organism	Strain origin	Methods		Reference
		ddPCR-Experimental[b] (Theoretical) copy # of *spoVA*^{2mob} operon/genome	CNOGpro-Copy # of *spoVA*^{2mob} operon/genome (GenBank accession number)	
B. amyloliquefaciens	Fad 108, ropy bread	1.73 ± 0.08 **(2)** 1.72 ± 0.06 **(2)**	-	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad 11/2, ropy bread	2.14 ± 0.21 **(2)** 1.71 ± 0.47 **(2)**	-	(Röcken and Spicher, 1993)
B. amyloliquefaciens	FUA2153, *daqu*	1.92 ± 0.11 **(2)** 1.94 ± 0.13 **(2)**	**2** (VRTV00000000)	(Wang et al., 2018)
B. amyloliquefaciens	FUA2154, *daqu*	2.08 ± 0.18 **(2)** 2.02 ± 0.34 **(2)**	**2 (**VRTU00000000**)**	(Wang et al., 2018)
B. amyloliquefaciens	Fad We, ropy bread	3.06 ± 0.18 **(3)** 2.64 ± 0.17 **(3)**	**3** (VRTX00000000)	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad 97, ropy bread	2.95 ± 0.23 **(3)** 2.81 ± 0.27 **(3)**	-	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad 99, ropy bread	3.17 ± 0.15 **(3)** 3.01 ± 0.31 **(3)**	**3** (SDKH00000000)	(Röcken and Spicher, 1993)
B. amyloliquefaciens	Fad 82, ropy bread	2.69 ± 0.12 **(3)** 2.35 ± 0.21 **(2)**	**3** (SDKG00000000)	(Röcken and Spicher, 1993)

[a] FUA number, Food microbiology culture collection at the University of Alberta.

[b] Mean ± standard deviation in triplicates.

[c.] The underlined data were obtained from DNA extractions applying bead beating.

As determined by microscopy, making sure 90-99 % of cells contained green spores. Spore suspensions were obtained as described elsewhere (Margosch et al., 2004b). In short, flood the surface of plate with 5 mL cold sterile distilled water twice to collect the spores. After being harvested, the spore suspensions were washed four times by centrifugation at 3,000 × *g* for 15 min at 4 °C and resuspended in sterile distilled water. To kill all the vegetative forms, the spore suspensions were pasteurized at 80 °C for 10 min between the second and third wash cycles. The spore suspensions were stored at -80 °C until used. Cell counts were determined on LB agar. Appropriate dilutions were surface-plated on LB agar and plating was carried out in triplicate from independent proper dilution series. The plates were incubated aerobically for 18 h at 37 °C. CFUs were calculated using Microsoft Excel (Microsoft Corp., Redmond, CA, USA).

5.2.2 Extraction of DNA and restriction digestion of the DNA sample

We applied two methods for DNA extraction. The first method was following the protocol of Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). In the second method, prior to following the protocol of kit, approximately 1 mL of overnight culture of each strain was placed into a 2 mL microcentrifuge tube filled with 0.5 g of silica beads. The samples were smashed by bead beating for 30 seconds repeating 8 times. The concentration of DNA was analyzed by NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific).

Genomic DNA samples were further treated by Fast Digest restriction enzyme HindIII (Thermo Fisher Scientific) at 37 °C for 45 min with subsequent heat-kill at 65 °C for 10 min to cut the intact genomic DNA into fragments and separate linked copies of the *spoVA*^{2mob} operon efficiently. Then DNA samples were stored at -20 °C until further use. Prior to ddPCR analysis, DNA was encapsulated into droplets (Hindson et al., 2011; Miotke et al., 2014).

5.2.3 Primer and probe design

In this study, we applied TaqMan hydrolysis probes as the reporter fluorophores. Each ddPCR reaction contained duplex TaqMan probes for the region of interest (ROI)-the last gene on the *spoVA*^{2mob} operon and the region of reference (REF)-DNA gyrase subunit B (*gyrB*), respectively. In this study, we chose *gyrB* gene as the REF gene because this gene is highly conserved and single-copy housekeeping gene (la Duc et al., 2004). We extracted several nucleotide sequences of *Bacillus* strains from the National Center for Biotechnology Information (NCBI) database (Table 5.3) and aligned them with MUSCLE website (https://www.ebi.ac.uk/Tools/msa/muscle/). Afterwards, we examined the aligned sequences to determine conserved regions suitable as targets. Primers and probes were first designed using IDT PrimerQuest (http://www.idtdna.com/Primerquest/Home/Index) and then adjusted manually following the instructions of the Droplet Digital™ PCR Application Guide (Bio-Rad Laboratories, Inc., CA). Primers and probes are shown in Table 5.2.

Table 5.2 Sequences of primers and probes.

Gene	Primer/Probe	Sequence (5'→3') (name)	*T*m[a] (°C)	Product size (bp)
last gene of *spoVA*^{2mob} operon	*spoVA*^{2mob}-F	AACCACTAGCCACGATTG	59	169
	spoVA^{2mob}-R	AAGGGTCTTTCTTGTGGG	59	
	spoVA^{2mob}-probe	/FAM[b]/ACGAAGTCGGGCTTGGCTACA/	68	
gyrB gene	*gyrB*-F	ATCGTCGACAACAGTATTG	57	205
	gyrB-R	CTTTATATCCGCTTCCGTC	57	
	gyrB-probe	/HEX[c]/CCCTGCGGTTGAAGTCATCATGA/	66	
front part of *spoVA*^{2mob} operon	*spo*-F	AAGGTCGAGCAAAGACTG	59	1516
	spo-R	ACCTGTAGCCACAACTAAC	59	

[a] *Tm*, melting temperature. [b] FAM, 6-carboxy fluorescein. [c] HEX, reporter dye.

Table 5.3 Strains of *Bacillus* used in sequence alignment of *spoVA*^{2mob} operon.

Species	Strain name	NCBI reference sequence	Reference
B. amyloliquefaciens	SRCM101267	NZ_CP021505.1	Unpublished
B.amyloliquefaciens	UMAF6639	NZ_CP006058.1	(Magno-Pérez-Bryan et al., 2015)
B. cereus	ATCC 14579	NC_004722.1	(Ivanova et al., 2003)
B. cereus	B4116	NZ_LJKF00000000.1	(Hayrapetyan et al., 2016)
Bacillus sp.	ABP14	NZ_CP017016.1	Unpublished
Bacillus sp.	HBCD-sjtu	NZ_CP025122.1	Unpublished
B. subtilis	CW14	NZ_CP016767.1	Unpublished
B. subtilis	NCIB 3610	NZ_CP020102.1	Unpublished
B. velezensis	SCGB 574	NZ_CP023431.1	Unpublished

5.2.4 Quantification of the copy number of *spoVA*^{2mob} operon by ddPCR

All experiments were performed using a QX200^{TM} Droplet Digital PCR system (Bio-Rad). Each ddPCR reaction consisted of 12.5 µL of 2X ddPCR SuperMix for probes (no dUTP) (Bio-Rad), 100 pg of template DNA, 840 nM of each forward and reverse primers of *gyrB*, 720 nM of each forward and reverse primers of *spoVA*^{2mob}, and 400 nM of both probes. The final volume was filled up with nuclease free water to 25 µL. The entire reaction mixture was loaded into a DG8 cartridge (Bio-Rad) together with 70 µL of droplet generation oil (Bio-Rad) and placed in the QX200^{TM} droplet generator (Bio-Rad). Approximately 20,000 droplets were then generated in the droplet generator. The generated droplets were transferred to a new 96-well PCR plate and the plate was subjected to amplification in a C1000 Touch^{TM} thermal cycler (Bio-Rad). The thermal cycling conditions consisted of a 5 min activation period at 95 °C, followed by 40 cycles at 95 °C for 20 s and 56 °C for 60 s, 1 cycle at 94 °C for 10 min, and ending at 4 °C. After amplification, fluorescence measurement was detected by QX200^{TM} droplet reader (Bio-Rad) with the following settings: ROI was detected in the FAM channel and REF was detected in the HEX channel. The

raw fluorescence was analyzed with QuantaSoft™ software (Bio-Rad). In this experiment, positive droplets which contain at least one copy of *spoVA*^{2mob} operon exhibited a fluorescence compared to negative droplets without *spoVA*^{2mob} operon. The copy numbers of the ROI gene were calculated by multiplying the known copy number of REF gene by the ratio of the ROI:REF concentration.

5.2.5 Quantification of the copy number of *spoVA*^{2mob} operon by genome sequencing and CNOGpro

The genomes of *B. subtilis* Fad 109, *B. amyloliquefaciens* Fad 82 and Fad 99, and *B. velezensis* Fad 94 and FUA2155 were sequenced using a Hi-seq 2500 on high output. Sequencing was performed as fee-for-service by Genome Quebec (Montreal, QC, Canada). *B. subtilis* FUA2114, *B. cereus* FUA2120, and *B. amyloliquefaciens* FUA2153, FUA2154 and Fad We were sequenced by Illumina next-generation sequencing by MicrobesNG (Edgbaston, Birmingham, United Kingdom). The accession number of each strain is listed in Table 5.1. The 125 bp reads were assembled using SPAdes (Bankevich et al., 2012) and the resulting contigs were scaffolded with the Medusa server (Bosi et al., 2015). Afterwards the scaffold containing the *spoVA*^{2mob} was subjected to read depth analysis using CNOGpro (Brynildsrud et al., 2015) and the copy number for the region of the scaffold containing the *spoVA*^{2mob} operon was determined.

5.2.6 Determination of pressure resistance of *Bacillus* endospores

Spores were diluted in sterile distilled water to obtain the working spore suspension with a cell count of approximately 5.0×10^7 to 4.7×10^8 CFU/mL. 120 µL of spore suspension was transferred into a polypropylene tube (Fisher Scientific) which was sealed on both sides, avoiding inclusion of air bubbles. The samples were treated with a High Pressure Micropump MP5 system (High Pressure Physics, Polish Academy of Sciences, Warsaw, Poland) at 600 MPa and 80 °C. The pressure vessel was immersed in a water bath that was maintained at 80 °C. The temperature

profiles for treatments at 600 MPa and 80 °C are shown in Table 5.4. Adiabatic heating during compression to 600 MPa increased the temperature in the pressure vessel by less than 6.5 °C. Samples were held on ice before and after pressure treatment (Margosch et al., 2006). Viable spore counts were determined after surface plating on LB agar.

Table 5.4 Pressure-temperature profiles for treatment at 600 MPa and 80 °C.

Process stage	Time	Pressure (MPa)	Temperature (°C)
Compression	0	0	79.0
	42 sec	100	84.9
	65 sec	200	85.3
	82 sec	300	85.5
	96 sec	400	85.6
	108 sec	500	85.7
	118 sec	600	84.6
Pressure holding time	1/2/4/8 min	600	80.6
Decompression	0	600	80.6
	114 sec	0	73.3

5.2.7 Determination of spore DPA content by fluorescence spectrometry

To evaluate the amount of DPA present in the spores of the individual bacterial species, 2 mL of spore suspensions were standardized to an OD_{600} of 2 and autoclaved for 60 min at 121.1 °C, to inactivate spores and ensure complete DPA release (Hofstetter et al., 2013a). A terbium-DPA fluorescence assay was carried out as described (Kort et al., 2005; Hofstetter et al., 2013a). Briefly, untreated as well as autoclaved spore suspensions were centrifuged at 10,600 × *g* for 4 min, and 150 µL of the supernatant were mixed with 150 µL of 20 mM Terbium (III) chloride solution in non-transparent 96-well microtiter plates (Corning® 96 well NBS™ Microplate, Corning, Inc., Corning, NY, USA). Fluorescence intensity was determined using a fluorescence spectrophotometer (Varioskan Flash, Thermo Electron Corp., Nepean, Canada) with excitation

and emission wavelengths of 270 and 545 nm, respectively. A calibration standard curve was recorded, using analytical grade DPA (Sigma Aldrich, St. Louis, MO, USA) in the range of 0-150 µM, and linearity was ensured (R^2 > 99.6 %). All measurements were carried out in quadruplicate technical repeats from three independent spore suspensions. DPA release was expressed as the difference between the DPA concentration prior and subsequent to autoclaving. Further, spore enumeration was performed by serial dilution in sterile distilled water and spread plating on LB agar plates. In order to obtain comparable values, DPA release was interrelated to spore numbers, as molar DPA release per spore (Hofstetter et al., 2013a).

5.2.8 Determination of spore DPA released by high pressure treatment as a percentage of total DPA content

To evaluate the correlation between the *spoVA*^{2mob} operon and the amount of DPA released by pressure treatment, 4 strains with different copy numbers of the *spoVA*^{2mob} operon were tested: *B. velezensis* Fad 94 with 0 copies, *B. amyloliquefaciens* FUA2149 with 1 copy, *B. amyloliquefaciens* Fad 99 with 2 copies, and *B. amyloliquefaciens* Fad 108 with 3 copies of the *spoVA*^{2mob} operon per genome. Spore suspensions were standardized to an OD_{600} of 2 and divided into aliquots of 200 µL. The DPA release was quantified after pressure treatment at 600 MPa and 80 °C for 1, 2, 4, and 8 min. The total content of DPA was quantified after autoclaving for 60 min at 121.1 °C. DPA release was expressed as the percentage of DPA amount after high pressure treatment to the total DPA content in spores determined after autoclaving.

5.2.9 Statistical analysis

The independent experiments were repeated at least three times (biological replicates). Statistical analysis was performed with RStudio 3.4.3 (RStudio Inc., Boston, MA, USA) software using a

mixed model with copy number and strain treated as the fixed factor and random factor, respectively. A P value of ≤ 0.05 was considered statistically significant.

5.3 Results

5.3.1 Variation of the copy number of *spoVA*^{2mob} operon determined by ddPCR

To obtain strains of *Bacillus* spp. differing with respect to the copy number of *spoVA*^{2mob} operon, 17 strains of *Bacillus* isolated from spoiled foods, fermented foods, and the environment were selected. Nine strains of *Bacillus* were previously isolated from ropy bread, and resisted pressure treatment (Röcken and Spicher, 1993; Margosch et al., 2004b); two strains were isolated from malted oats; one strain originates from the cow vagina (Wang et al., 2013); and five strains from *daqu*, a solid-state cereal fermentation (Zheng et al., 2013; Wang et al., 2018). Presence or absence of the *spoVA*^{2mob} operon was determined by PCR amplification of the last gene of the *spoVA*^{2mob} operon. Nevertheless, since all *spoVA*^{2mob} positive *B. cereus* strains for which genome sequences are available in Genbank possess a 3' truncated version of the operon, a second pair of primers was used to confirm that the 3' end of the operon was present in *B. cereus* FUA2120. The copy number of *spoVA*^{2mob} operon in these 17 *Bacillus* strains was determined by ddPCR (Table 5.1). All strains with 2 or 3 copies of the *spoVA*^{2mob} belong to *B. amyloliquefaciens* or *B. subtilis*; neither of the two *B. velezensis* strains carried *spoVA*^{2mob} operon; in addition, one strain of *B. cereus* carried a single copy of the operon (Table 5.1).

5.3.2 Variation in ddPCR data with DNA extractions from two different methods

Multiple copies of a gene are accounted for a single copy in ddPCR assays if they are on the same DNA molecule. Therefore, the fragmentation of DNA during DNA isolation may influence the results. We employed bead beating as a method that produces highly fragmented DNA and a

genomic DNA isolation kit that allows isolation of high molecular weight genomic DNA. For most strains, the two methods of DNA extraction provided the same copy number of the *spoVA*^{2mob} operon. For *B. amyloliquefaciens* Fad 82, the copy number determined after DNA isolation with bead beating was 3 *spoVA*^{2mob} copies per genome, while the corresponding result of analysis of high molecular weight DNA was 2 copies per genome (Table 5.1).

5.3.3 Variation of the copy number of *spoVA*^{2mob} operon determined by genome sequencing and CNOGpro

The genomes of 9 strains of *Bacillus* were sequenced to validate the ddPCR assay. Genome sequencing confirmed the absence of the *spoVA*^{2mob} in *B. velezensis* Fad 94, FUA2155 and *B. subtilis* FUA2114. The genome of *B. subtilis* Fad 109, *B. amyloliquefaciens* FUA2153 and FUA2154 harbored 2 *spoVA*^{2mob} copies; 3 copies of *spoVA*^{2mob} operons were identified in *B. amyloliquefaciens* Fad 99, Fad 82 and Fad We, matching the results of ddPCR (Table 5.1).

5.3.4 Variation in pressure resistance of *Bacillus* strains

To determine the pressure resistance of spores from the 17 *Bacillus* strains, spores were subjected to treatment at 600 MPa and 80 °C (Figure 5.1). A high variation in pressure resistance was observed among the 17 strains and their pressure resistance levels were positively correlated with copy numbers of *spoVA*^{2mob} operon (Figure 5.1). Viable spore counts of four strains lacking the *spoVA*^{2mob} operon were reduced by 6-8 log CFU/mL after 4 min. The pressure resistance of the three strains with a single copy of *spoVA*^{2mob} operon was variable. *B. subtilis* FUA2148 and *B. amyloliquefaciens* FUA2149 were more resistant to pressure than *B. cereus* FUA2120. All 6 strains containing 2 copies of *spoVA*^{2mob} operons, two strains of *B. subtilis* and four strains of *B. amyloliquefaciens*, had a similar resistance to high pressure. Pressure treatment for 8 min reduced viable spore counts by between 1 to 3 log CFU/mL. An inactivation by less than 2 log

CFU/mL was observed for the corresponding treatment of the 4 strains of *B. amyloliquefaciens* with 3 copies of *spoVA*^{2mob} operons.

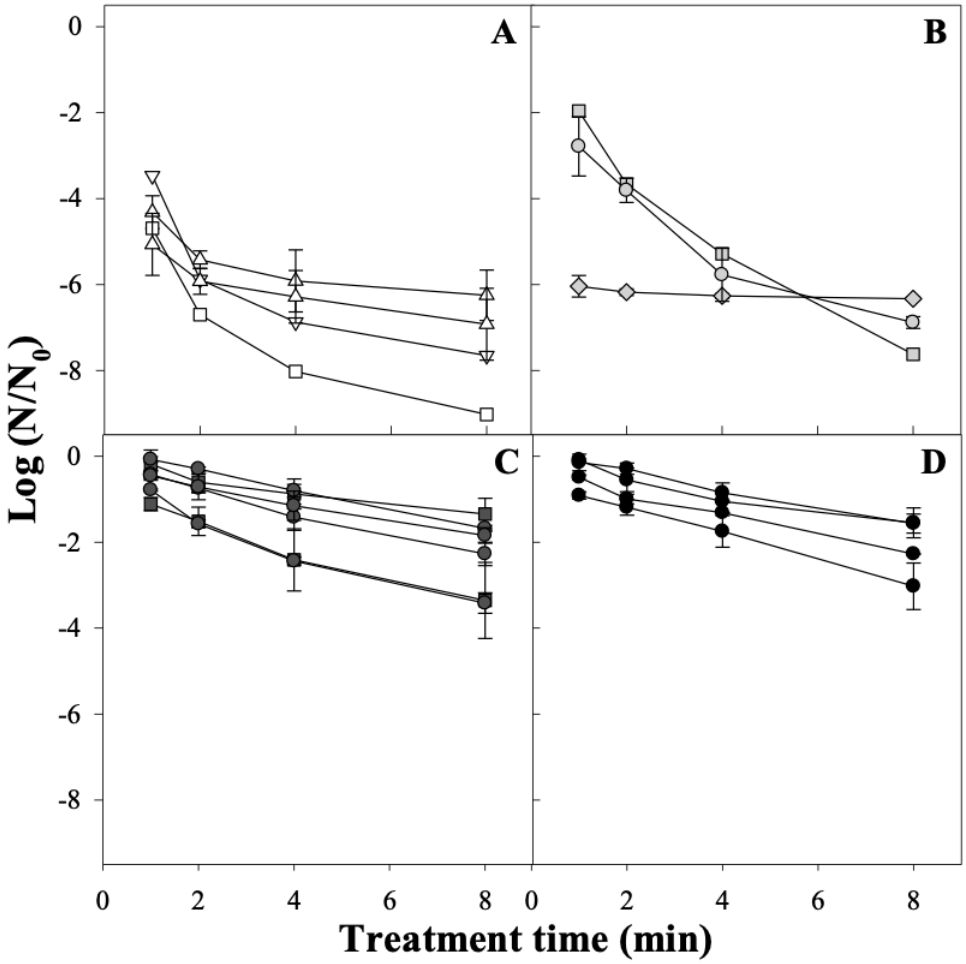


Figure 5.1 Viable spore counts [log(N/N$_0$)] of 17 *Bacillus* strains after treatment at 600 MPa and 80 °C. The panel A, B, C and D represent strains without a *spoVA*^{2mob} operon copy (4 strains), or with 1 (3 strains), 2 (6 strains) or 3 copies (4 strains) of the *spoVA*^{2mob} operon per genome, respectively. Species are differentiated by different symbols as follows: (▲), *B. velezensis*; (▼), *B. pumilus*; (■), *B. subtilis*; (◆), *B. cereus*; (●), *B. amyloliquefaciens*. Data are shown as means ± standard deviation of three independent experiments.

5.3.5 Variation of spore DPA content

Because SpoVA proteins were linked to the DPA content of spores (Vepachedu and Setlow, 2007), we determined whether the copy number of the *spoVA*^{2mob} operon was associated with the DPA

content in spores, which is a significant factor of spore pressure resistance. While the spore DPA content differed among strains, there was no relationship between DPA content and the copy number of the last gene on *spoVA*^{2mob} operon (Figure 5.2).

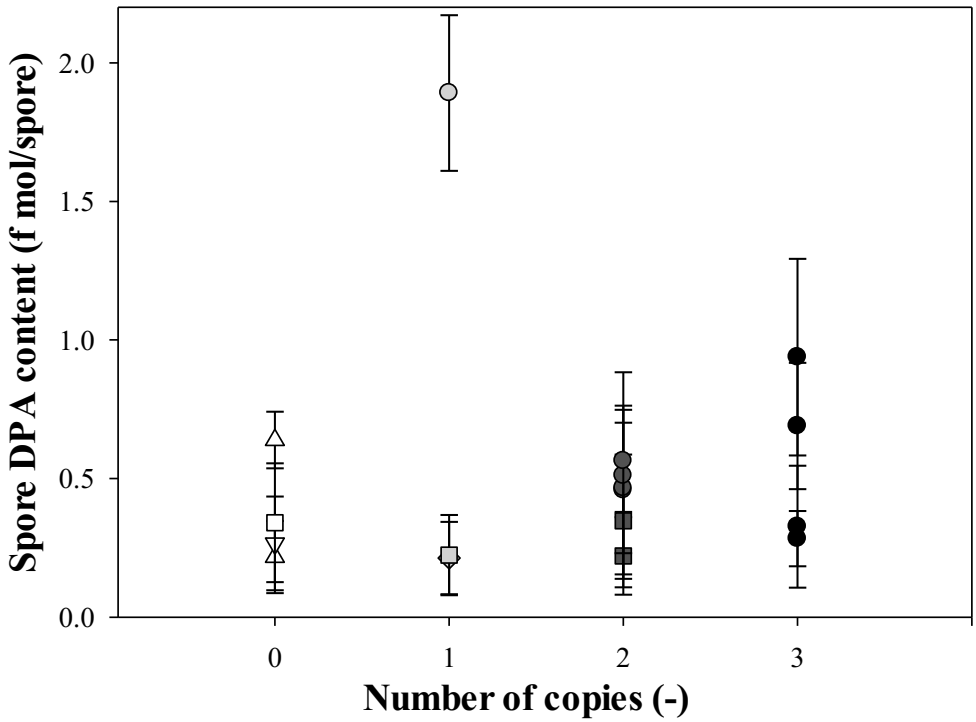


Figure 5.2 DPA content of *Bacillus* spores. Species are differentiated by different symbols as follows: (▲), *B. velezensis*; (▼), *B. pumilus*; (■), *B. subtilis*; (◆), *B. cereus;* (●), *B. amyloliquefaciens*. White, light gray, dark gray, and black symbols represent strains with 0, 1, 2 and 3 copies of *spoVA*^{2mob} operon per genome. The data are means from three independent experiments; error bars are standard deviations.

5.3.6 Variation of spore DPA released by high pressure treatment as a percentage of total DPA content

To evaluate the correlation between *spoVA*^{2mob} operon and DPA release, 4 strains with different copies of *spoVA*^{2mob} operons (0-3 copies) were used. As shown in Figure 5.3, when spores were

treated for 1 and 2 min at 600 MPa and 80 °C, the percentage of DPA released from the spores with 2 or 3 copies was lower than the spores containing 0 or 1 copy of *spoVA*^{2mob} operon. After treatment for 1 min, the spores with 0 or 1 copy of *spoVA*^{2mob} operons released more than 85 % of DPA, whereas the spores with 2 or 3 copies released around 65-72 % of DPA. When treated for 4 and 8 min, all the spores released more than 80 % of DPA.

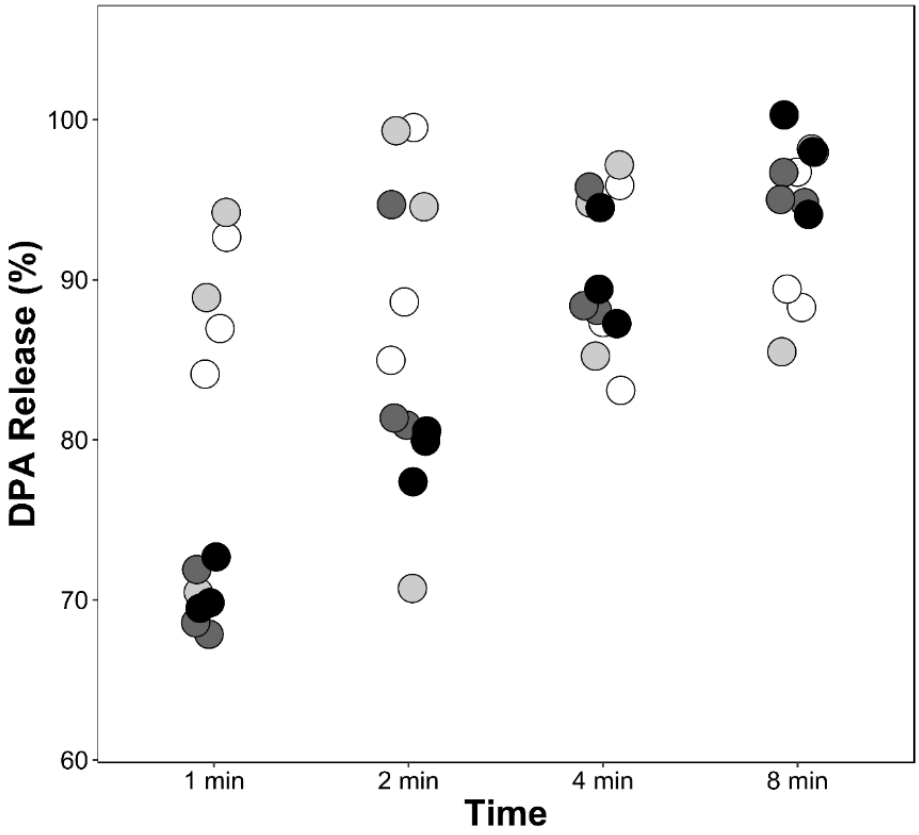


Figure 5.3 Percentage of DPA release from *Bacillus* spores after high pressure treatment at 600 MPa and 80 °C to the total DPA content in spores determined after autoclaving. Strains are differentiated by different colors as follows: (white ○) *B. velezensis* Fad 94 with 0 copy; (light gray ●) *B. amyloliquefaciens* FUA2149 with 1 copy; (dark gray ●) *B. amyloliquefaciens* Fad 99 with 2 copies; (black ●), *B. amyloliquefaciens* Fad 108 with 3 copies of *spoVA*^{2mob} operons per genome.

5.3.7 Correlation of *spoVA*^{2mob} operon copy number and endospores resistance to pressure

Correlation of the *spoVA*^{2mob} operon copy number and the pressure resistance was assessed by comparing the cell counts of strains after 8 min of treatment at 600 MPa and 80 °C Spores with 0 or 1 *spoVA*^{2mob} operon were less resistant to pressure than spores with 2 or 3 copies ($P < 0.001$) but the pressure resistance of strains carrying 0 and 1 copy of *spoVA*^{2mob} operon, or 2 and 3 copies of the operon was not different (Figure 5.4). When the analysis excluded *B. cereus*, the species that is phylogenetically most distant from other species used in this study, spores of strains with a single copy of the *spoVA*^{2mob} operon exhibited intermediate resistance when compared to strains with 0 or 2 copies (Figure 5.1).

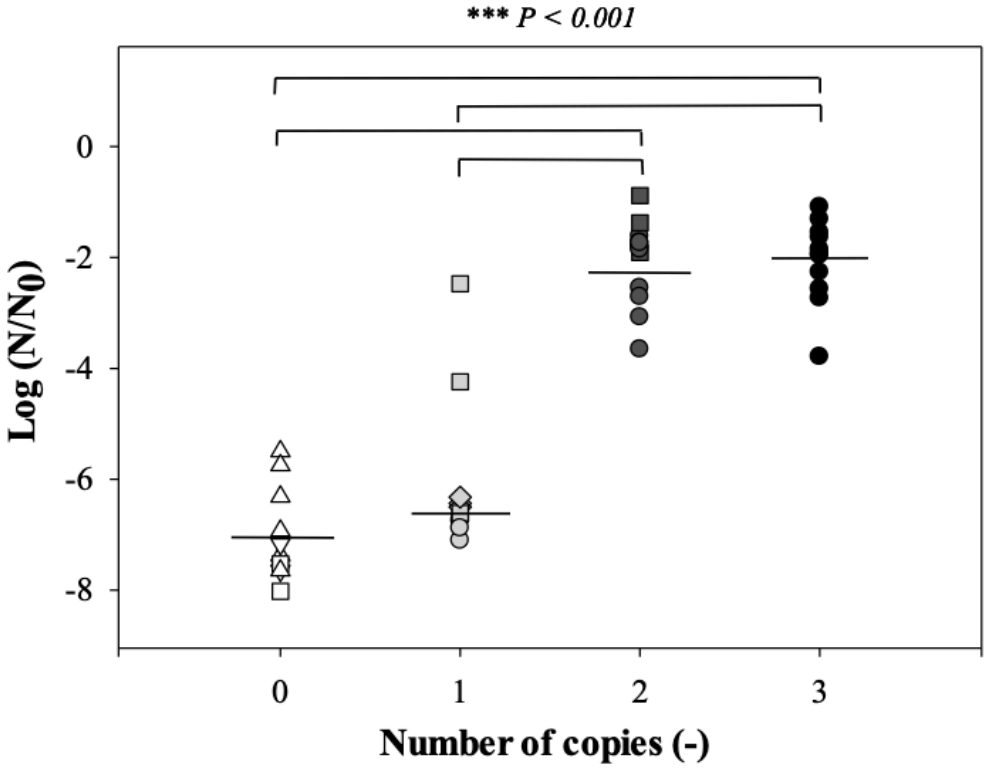


Figure 5.4 Viable spore counts [log(N/N$_0$)] of spores with different copy number of *spoVA*^{2mob} operon after treatment at 600 MPa and 80 °C for 8 min. Species are differentiated by different symbols as follows: (▲), *B. velezensis*; (▼), *B. pumilus*; (■), *B. subtilis*; (◆), *B. cereus;* (●), *B. amyloliquefaciens*. White, light gray, dark gray, and black symbols represent strains with 0, 1, 2 and 3 copies of *spoVA*^{2mob} operon per genome, respectively. Mean values for strains with the same copy number of *spoVA*^{2mob} operon are highly significantly different ($P < 0.001$).

5.4 Discussion

This study reports the impact of the *spoVA*^{2mob} operon on pressure resistance of *Bacillus* endospores. The ddPCR rapidly quantified the gene copy number of the *spoVA*^{2mob} operon in bacilli and results obtained by ddPCR were validated by whole genome sequencing of 9 of the 17 isolates. Spores containing 2 or more copies of *spoVA*^{2mob} operons showed a higher level of pressure resistance than spores with 0 or 1 copy of the operon.

In the previous studies, the researchers inserted one copy of the *spoVA*^{2mob} operon into *B. subtilis* 168 to study its role to heat resistance and germination of spores (Berendsen et al., 2016a; Krawczyk et al., 2016, 2017), but they didn't explore the effect of redundant *spoVA*^{2mob} operons in that strain. Here, we used 17 wild type *Bacillus* strains isolated from spoiled foods, fermented foods, and the environment to illustrate the effects of different copies of *spoVA*^{2mob} operons on pressure resistance. The large amount of *Bacillus* strains we used and the statistically significant results are able to make up this deficiency.

5.4.1 Comparison of ddPCR and other methods for detecting gene copy number

When compared to whole genome sequencing, the reference method for determination of the copy number of a gene in a bacterial genome, PCR methods including quantitative PCR (qPCR) and droplet digital PCR offer the advantage of rapid and high-throughput analysis of multiple samples. qPCR has been widely used for quantification of nucleic acids (Raso and Biassoni, 2014) but the accuracy of qPCR is limited because the qPCR quantification is indirect and requires an external standard (Yang et al., 2014). The accuracy and precision of copy number estimates detected by qPCR may differ because factors such as the physicochemical state of DNA can affect the efficiency of qPCR and consequently the cycle threshold value (Aldhous et al., 2010; Yang et al., 2014). ddPCR more precisely estimates the copy number because the target gene is directly

compared to a reference gene which is analyzed in the same PCR reaction (Pinheiro et al., 2012). The copy number of the *spoVA*^{2mob} operon in the 5 *daqu* isolates was previously analyzed by qPCR (Wang et al., 2018). When matched against genome sequencing as reference, ddPCR analyses provided a higher accuracy and precision than qPCR (Table 5.1) (Wang et al., 2018).

The copy number of a gene in a bacterial genome is an integer, however, ddPCR did not provide integers. The deviation is partially caused by experimental error; in addition, if two copies of a gene are located on the same DNA molecule, they are accounted for as single copy in the ddPCR assay. Accordingly, cell lysis by bead beating, which shears DNA to fragments of less than 10 kbp, provided a higher copy number when compared to a gentler isolation method that retains DNA strands up to 50 kbp. To validate the ddPCR data, we further used CNOGpro to analyze the copy number of *spoVA*^{2mob} operon by the sequencing of 9 *Bacillus* strains corroborating the ddPCR result (Table 5.1). Using ddPCR to detect copy numbers thus has a comparable accuracy as whole genome sequencing but offers the advantage of rapid analysis of multiple samples and more economic.

5.4.2 Relationship between the copy number of *spoVA*^{2mob} operon and pressure resistance of *Bacillus* endospores

A comparison of the pressure resistance of multiple strains of *B. amyloliquefaciens* and *B. subtilis* revealed a high intra-species variation in pressure resistance; in particular, isolates from ropy bread were highly resistant to pressure (Margosch et al., 2004b). These strains were subsequently used as pressure-resistant surrogate organisms for *C. botulinum* in multiple studies (Margosch et al., 2006). Our data on pressure resistance of *B. amyloliquefaciens* and *B. subtilis* confirms prior data obtained with the same strains; furthermore, our data demonstrates that all the rope-forming and pressure resistant strains of *B. amyloliquefaciens* and *B. subtilis* contained 2 or 3 copies of

spoVA^{2mob} operons (Chapter 4). The copy number variation thus explains most of the difference in pressure resistance between the rope-forming *B. amyloliquefaciens* and *B. subtilis* strains and other *Bacillus* spp. It is possible that other *Bacillus* spp. with redundant *spoVA*^{2mob} operons show higher pressure resistance but isolates are not available (this study, and Margosch, Gänzle, et al., 2004; Smith et al., 2018). The replication with strains of diverse species differing in copy number of *spoVA*^{2mob} operon demonstrates that the number of copies of the *spoVA*^{2mob} operon also correlates to spore pressure resistance.

Surprisingly, spores of *B. cereus* FUA2120 with a single copy of the *spoVA*^{2mob} operon were relatively pressure sensitive, particularly when resistance was assessed after 1 min of treatment at 600 MPa and 80 °C. We further tested the heat resistance of this strain at 100 °C for 0, 1 2 and 4 min, showing that this strain was also heat sensitive (Figure 5.5). *B. subtilis, B. amyloliquefaciens,* and *B. velezensis* are phylogenetically closely related but *B. pumilis* and *B. cereus* are more distant to other *Bacillus* spp. analyzed in this study (Huang et al., 2012). Spore properties unrelated to the *spoVA*^{2mob} operon that are shared by spores of the *B. subtilis* group but not by *B. cereus* may thus account for this discrepancy. Of the 1019 genomes of strains of *B. cereus* that were available on GenBank, 19 contained a truncated version of the *spoVA*^{2mob} operon but none carried the full operon. PCR analysis targeting sequences close to the 3' and 5' ends of the operon confirmed, however, that *B. cereus* FUA2120 contains the full length *spoVA*^{2mob} operon. The low frequency of the *spoVA*^{2mob} operon and the relatively low heat and pressure resistance of the *spoVA*^{2mob} positive *B. cereus* FUA2120 suggest that the operon may be occasionally acquired from strains of the *B. subtilis* group but does not provide a competitive advantage to *B. cereus*.

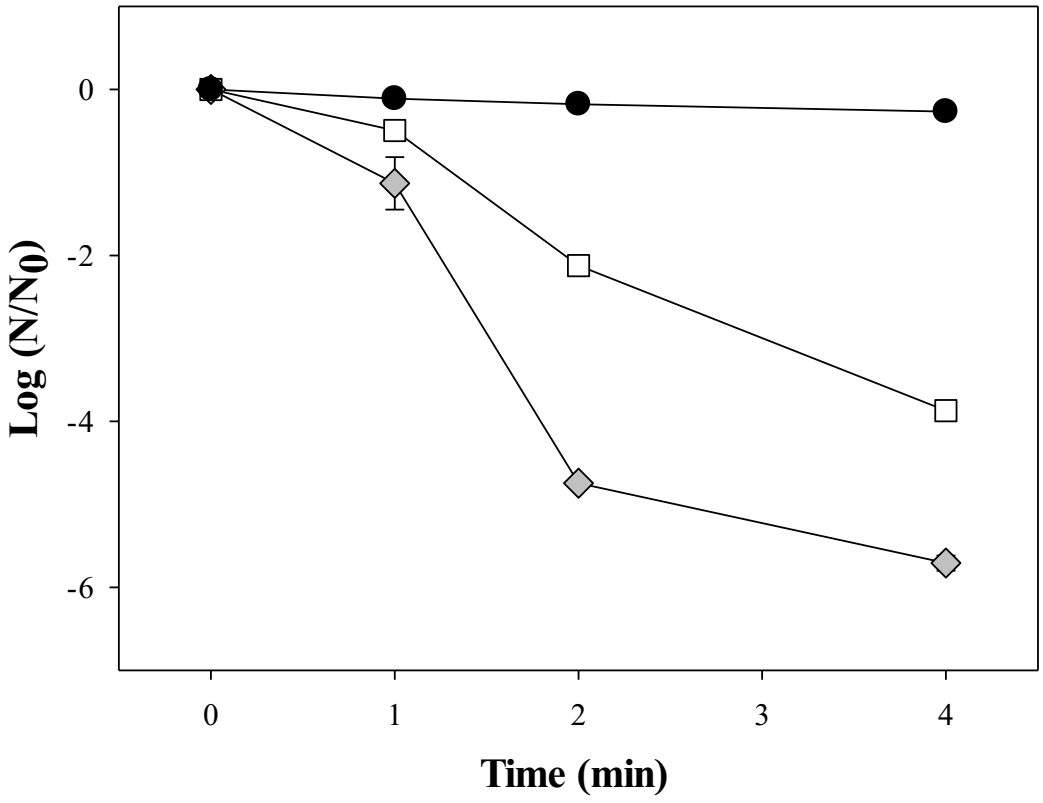


Figure 5.5 Viable spore counts [log(N/N_0)] of 3 *Bacillus* strains after treatment at 100 °C. Species are differentiated by different symbols as follows: (■), *B. subtilis* FUA2114; (◆), *B. cereus* FUA2120*;* (●), *B. amyloliquefaciens* Fad We. White, light gray, and black symbols represent strains with 0, 1, and 3 copies of *spoVA*^{2mob} operon per genome.

5.4.3 Potential mechanism of *spoVA*^{2mob} operons influencing the germination of endospores during high pressure treatment

A thorough understanding of spore pressure resistance is important, but the mechanism varies depending on the exact pressure value applied. With moderately high pressure of less than 300 MPa, the germination of spores was induced *via* the nutrient-triggered germination pathway by activating the nutrient germinant receptors (Wuytack et al., 2000; Paidhungat et al., 2002; Black et al., 2007). On the contrary, very high pressure (400-800 MPa) may directly trigger the release

of Ca-DPA, which is a key event in the spore germination process. In previous studies, the researchers illustrated that the *spoVA*^{2mob} operon can decrease the release of Ca-DPA through SpoVA channel on the inner membrane and slow the germination of spores (Krawczyk et al., 2016, 2017). Slower germination is likely caused by the inner membrane proteins SpoVAC, SpoVAD, and SpoVAEB, which are encoded by the regular *spoVA* operon (Vepachedu et al., 2007; Krawczyk et al., 2017; Wang et al., 2018). These proteins are associated with mechanosensitive channel on spore inner membrane (Velásquez et al., 2014) and mediate DPA uptake during sporulation as well as Ca-DPA release during germination (Setlow, 2003; Krawczyk et al., 2017). It remains unclear, however, whether the presence of multiple copies of the *spoVA*^{2mob} operon incrementally impacts DPA uptake and release. The present study demonstrated a role of the copy number of *spoVA*^{2mob} operon in pressure resistance and pressure induced DPA release. SpoVAC^{2mob}, SpoVAD^{2mob}, and SpoVAEB^{2mob} share 55 %, 49 %, and 59 % amino acid identity with the respective proteins encoded in the regular *spoVA* operon and attenuated the germination of spores (Krawczyk et al., 2016).

The mechanisms of spore resistance to pressure depend on the pressure level that is applied, and the spore response to processes used for pressure-assisted thermal sterilization is unrelated to physiological germination (Margosch et al., 2004b; Reineke et al., 2013b). Pressure of less than 300 MPa at ambient temperature induces germination of spores *via* the nutrient-triggered germination pathway by activating the nutrient germinant receptors (Wuytack et al., 2000; Paidhungat et al., 2002; Black et al., 2007; Reineke et al., 2013a). A pressure of 400 to 800 MPa at ambient temperature triggers release of Ca-DPA to induce germination independent of nutrient receptors (Reineke et al., 2013a). Treatment at 600 MPa and 80 °C or higher inactivates spores without triggering physiological processes involved in germination of spores (Margosch et al.,

2006; Black et al., 2007; Hofstetter et al., 2013b; Reineke et al., 2013a). Previous studies hypothesized that the insertion of *spoVA*^{2mob} operon into *B. subtilis* 168 resulted in higher DPA concentrations in spores and consequently a high-level heat resistance (Berendsen et al., 2016b; Krawczyk et al., 2016). Our results do not confirm a correlation of the copy number of the *spoVA*^{2mob} operon with the DPA concentration of spores but suggest that the *spoVA*^{2mob} operon reduced the pressure-induced but germination-independent DPA release.

In conclusion, this study demonstrated that the pressure resistance of *Bacillus* spores is dependent on the presence and the copy number of the *spoVA*^{2mob} operon. Some of the strains used in this study were previously identified as pressure resistant surrogates for validation of the lethality of pressure-assisted thermal pasteurization against *C. botulinum* (Margosch et al., 2006). Our study contributes to the understanding of pressure resistance at the molecular level; it provides a rationale why rope-forming bacilli exhibit high pressure resistance (den Besten et al., 2018), thus, the *spoVA*^{2mob} operon can be used as a genetic marker to identify the risky spores and improve risk assessments in actual food production. This progress enables further developments of high pressure thermal sterilization in the food industry.

Chapter 6 The Germination and Resistance Properties of Spores from *Bacillus subtilis* With or Without The *spoVA*^{2mob} Operon

6.1 Introduction

Bacillus is one of the most robust organisms in the world mainly due to its ability to form endospores. They inevitably enter the food chain because of their ubiquity in nature. Some *Bacillus* spp. can cause serious safety and spoilage-related issues within the food industry (Ehling-Schulz and Messelhäusser, 2014). The endospores are much more resistant to adverse conditions, including heat, high pressure, UV irradiation, desiccation and chemical assaults than their vegetative counterparts (Setlow, 2003). The resistance of spores enables their survival during food processing, such as heating processes that are commonly applied. The survival of spores in food, followed by germination and outgrowth, can cause food spoilage or food-borne illness depending on the species (Postollec et al., 2012; Kumari and Sarkar, 2016; Griffiths and Schraft, 2017).

The resistance of spores to different circumstances varies. The spores include a large content of pyridine-2, 6-dicarboxylic acid (DPA) in a 1:1 chelate with calcium ions (Ca^{2+}-DPA) within their core (Paidhungat et al., 2000). The SpoVA proteins (encoded by the regular *spoVA* operon) are required for DPA uptake during sporulation and DPA release during germination (Vepachedu and Setlow, 2007; Li et al., 2012; Velásquez et al., 2014). Due to the low water content in the core, which is largely caused by the high DPA content, the dormancy and resistance of spores are significantly impacted (Berendsen, 2016; Setlow and Johnson, 2019). The high DPA content and low water content are part of the reason why DNA in the core is protected (Berendsen, 2016).

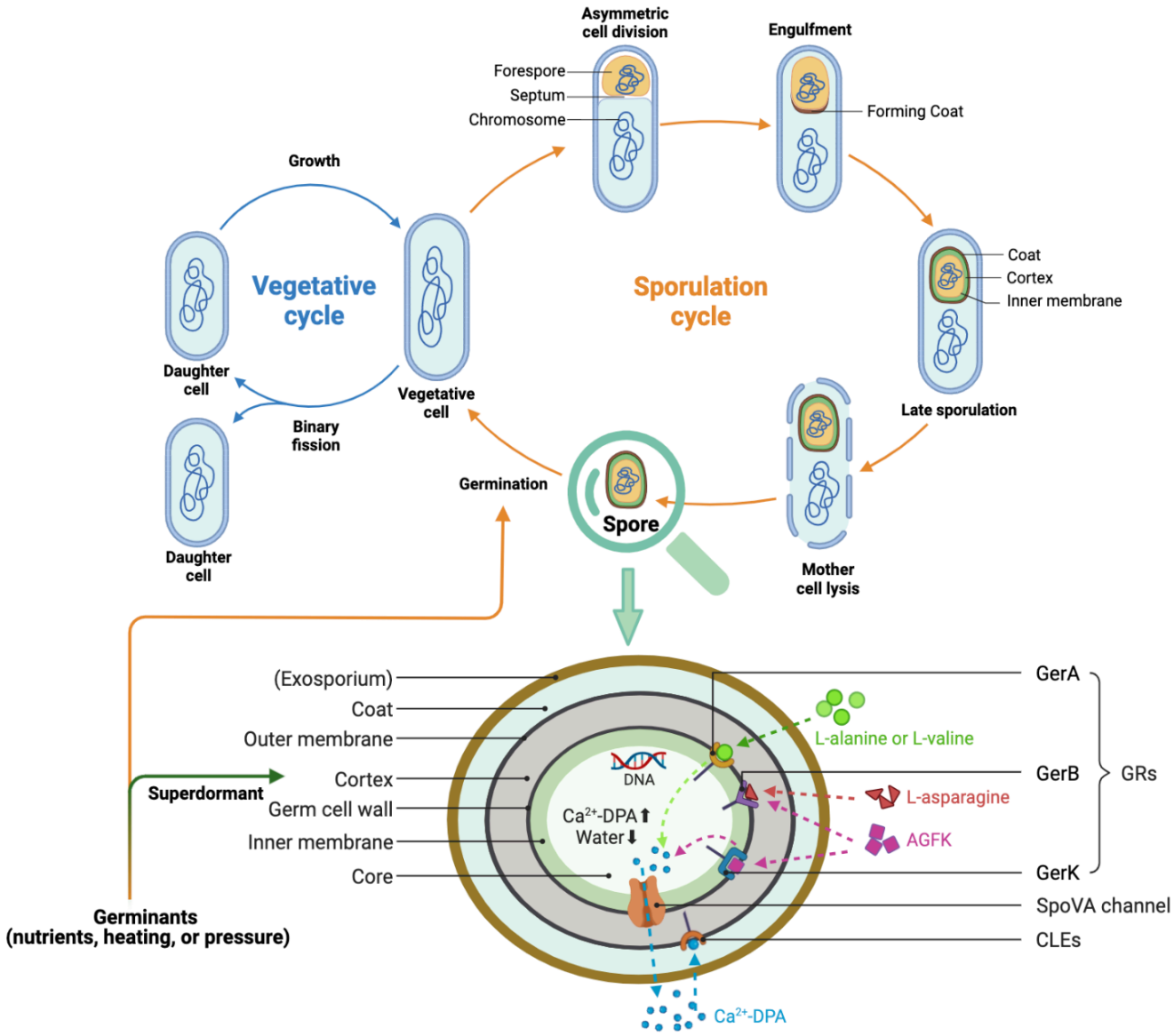


Figure 6.1 Life cycle of *Bacillus* spore and vegetative cell, and schematic illustration of the dormant spore structure and some of its characteristic molecules. The high content of the spore characteristic molecule DPA is partially responsible for the low water content. The spores' DNA in the core is protected from damage by the binded small, acid-soluble proteins (SASPs). The inner membrane contains GRs including GerA, GerB, and GerK, or Ca^{2+}-DPA channels built from SpoVA proteins, which are located in the inner membrane. GerA responds to L-alanine or L-valine. A mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (AGFK) activates the GerB and GerK receptors. GerB responds primarily to L-asparagine. Cortex lytic enzymes (CLEs), which degrade the cortex with the inducement of Ca^{2+}-DPA, are found in the cortex. Note that not all species have an exosporium and the structures are not scaled. AGFK = L-asparagine, D-glucose, D-fructose and potassium. This figure was adapted from Wang et al, 2015.

During the germination of spores, the DPA is released from the spore core *via* the SpoVA channel that is located on the inner membrane of spores (Figure 6.1) (Vepachedu and Setlow, 2004, 2007). This initiates rehydration of the spore core, followed by degradation of the protective peptidoglycan cortex layer by cortex lytic enzymes (CLEs) including CwlJ and SleB, which further leads to water uptake and the completion of germination (Chirakkal et al., 2002).

Furthermore, the heat and pressure resistance properties of *Bacillus* spores vary greatly between species or strains (Margosch et al., 2004b; den Besten et al., 2018). In recent years, it has been reported that a mobile genetic element known as *spoVA*^{2mob} was linked to elevated DPA content by negatively influencing the release of Ca^{2+}-DPA and is responsible for the heat and pressure resistance of *Bacillus* spores (Berendsen et al., 2016a, 2016b; Li et al., 2019). The regular *spoVA* operon has seven genes: *spoVAA*, *spoVAB*, *spoVAC*, *spoVAD*, *spoVAEb*, *spoVAEa* and *spoVAF*, of which the first five genes are essential for the completion of sporulation (Tovar-Rojo et al., 2002). While the novel *spoVA*^{2mob} operon also contains 7 genes: *spoVAC*^{2mob}, *spoVAD*^{2mob} and *spoVAEb*^{2mob} as well as four other genes that encode proteins with unidentified functions. The SpoVAC, SpoVAD and SpoVAEb proteins encoded in the *spoVA* and *spoVA*^{2mob} loci share 55%, 49% and 59% amino acid identity, respectively (Berendsen et al., 2016a). SpoVAC, SpoVAD and SpoVAEb proteins are required for Ca^{2+}-DPA uptake into the developing spore during sporulation and these three proteins alone allow normal Ca^{2+}-DPA uptake in sporulation (Luo et al., 2021). In contrast to the regular conserved *spoVA* operon, *spoVA*^{2mob} operon does not produce homologs of the SpoVAEa and SpoVAF proteins, which are thought to be crucial for DPA release during germinant receptors (GR) dependent germination of spores but not for DPA uptake during spore formation (Perez-Valdespino et al., 2014). Therefore, the generally accepted theory about *spoVA*^{2mob} is that its products might not be able to assist DPA transfer during germination

(Berendsen, 2016). In contrast, they may compete with the regular SpoVA channels or interfere with the SpoVA proteins and/or GRs by either directly or indirectly modifying the characteristics of the spore inner membrane (Wang et al., 2015).

To inactivate the spoilage-related spores, high thermal loads are needed, however, which in turn result in significant food quality losses. Therefore, alternate spore inactivation methods are required to retain important food quality attributes. Recently, the germination-inactivation strategy has shown promise in bridging the gap between food quality and safety (Nerandzic and Donskey, 2010; Setlow, 2014; Zhang and Mathys, 2019). This strategy aims to trigger the germination of spores to reduce their resistance, and then eliminate the spores which lost their extreme resistance during germination with a mild inactivation step (Zhang and Mathys, 2019). The germination of spores can be artificially triggered by a variety of stimuli, including nutrient germinants, Ca^{2+}-DPA, heat activation (generally 60-80 °C), and high pressure *via* two different pathways based on the pressure level (Figure 6.2) (Setlow, 2003; Abee et al., 2011; Reineke et al., 2013a). Among them, high pressure which is one of the most successful non-thermal processing technologies, clearly outperforms other triggers: food products can be treated more homogenously, chemicals addition is not necessary, and food quality retains better than the heat sterilization in terms of nutritional value, color and other sensorial attributes (Matser et al., 2004; Doona et al., 2016; Farkas, 2016; Sevenich and Mathys, 2018; Zhang and Mathys, 2019).

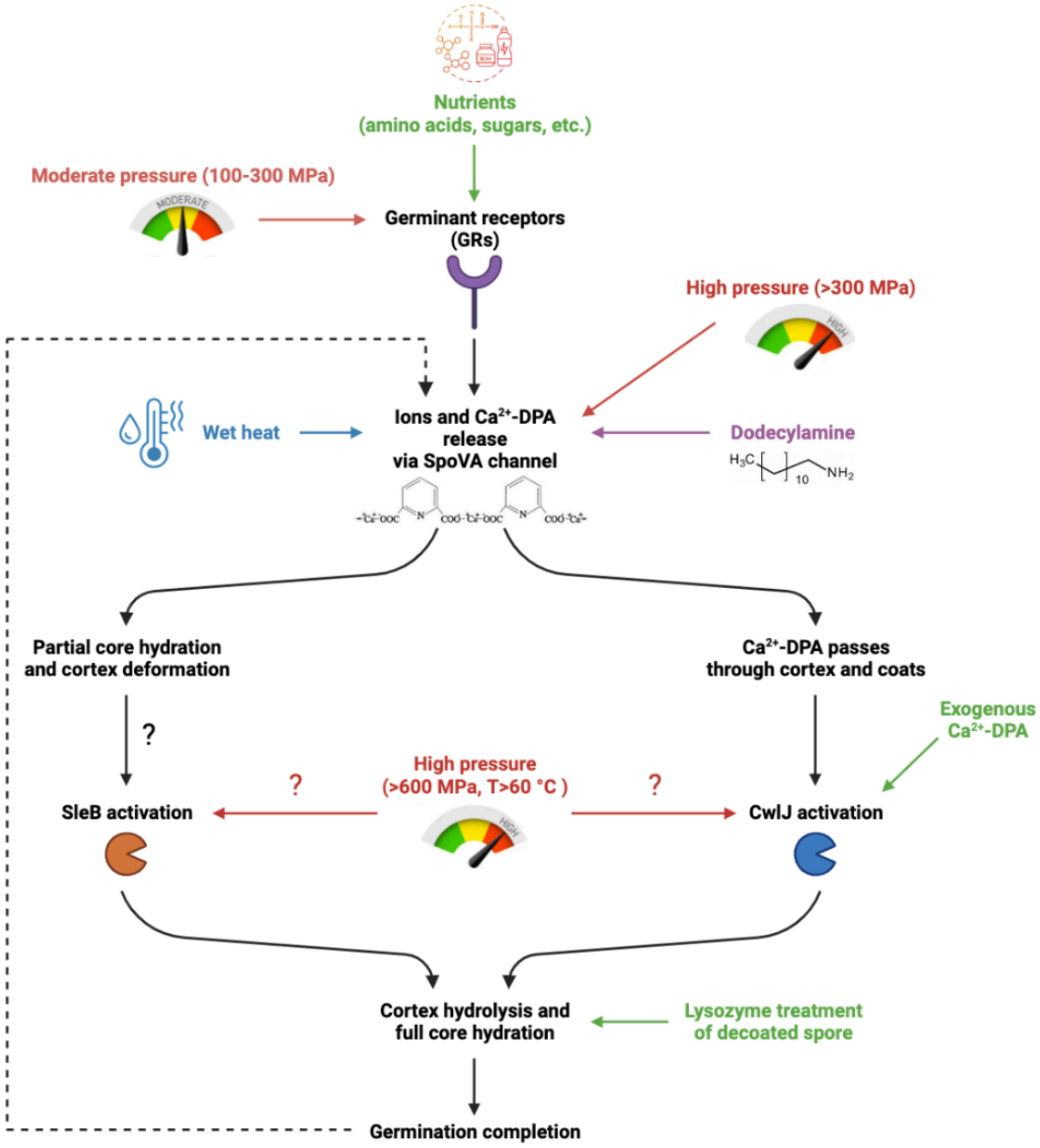


Figure 6.2 Overview of proposed germination pathways of *Bacillus* spores. Nutrients activate germinant receptors, resulting in the release of ions and Ca^{2+}-DPA from the spore core, which triggers activation of CLEs (CwlJ and SleB). CLEs then promote cortex hydrolysis, full core hydration, and completion of germination. Wet heat activation leads to the release of ions and Ca^{2+}-DPA directly. Pressures trigger the germination of spores in two pathways: moderate high pressures (100-300 MPa) trigger germination by activating the GRs, while very high pressure (300-600 MPa) trigger germination independent of the GRs, directly causing Ca^{2+}-DPA release. Exogenous Ca^{2+}-DPA activates CwlJ, which then causes cortex hydrolysis and full core hydration, and this process might be amplified by the consequent release of endogenous Ca^{2+}-DPA. Lysozyme treatment also causes cortex lysis. This figure was adapted from Zhang and Mathys, 2019.

In this study, the extensively studied laboratory strain *Bacillus subtilis* 168 was applied to create the isogenic strain with the insertion of *spoVA*^{2mob} operon, to study the role of *spoVA*^{2mob} operon in the pressure resistance and germination of spores triggered by different germinants. This study compared the percentage of DPA release from spores with and without the *spoVA*^{2mob} operon after germination induced by pressure, heat activation and nutrients germinants. The findings presented in this study have great implications for the food industry, where heat treatments are commonly used to inactivate pathogenic and spoilage microbes, including *Bacillus* spores.

6.2 Materials and methods

6.2.1 Bacterial strains, plasmids, media, reagents, and enzymes used in this study

All bacterial strains and plasmids used in this study are listed in Table 6.1. The strains of *B. subtilis* 168 and *B. subtilis* 168 harboring pJOE8999 plasmid were obtained from The *Bacillus* Genetic Stock Center (BGSC) (http://www.bgsc.org/). For DNA cloning, *Escherichia coli* DH5α (Thermo Fisher Scientific) was used as a general cloning host. All strains were grown aerobically on LB agar plates. The derivative cells containing pJOE8999 were incubated at 30 °C (Altenbuchner, 2016). Otherwise, the strains of *Bacillus* and *E. coli* were incubated at 37 °C. For strains of *E. coli* DH5α, kanamycin was used at a final concentration of 30 μg/mL in LB agar. To test the existence of plasmids in *B. subtilis* 168, kanamycin was supplemented at a final concentration of 5 μg/mL in LB agar. To induce the Cas9 expression, 0.2 % of mannose was added to the medium.

Table 6.1 List of origin and purpose of strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source
B. subtilis 168	Wild type	BGSC
B. subtilis 168 harboring pJOE8999		BGSC
E. coli DH5α		Thermofisher
pJOE8999	Vector	(Altenbuchner, 2016)
pJOE8999.1	pJOE8999 containing the 20 bp sequence of sgRNA (*trpC*)	This study
pJOE8999.1+*spoVA*^{2mob}	pJOE8999.1 with the inserted *spoVA*^{2mob}	This study
E. coli DH5α harboring pJOE8999.1		This study
E. coli DH5α harboring pJOE8999.1+ *spoVA*^{2mob}		This study
B. subtilis 168 *trpC*::*spoVA*^{2mob}	Isogenic strain of *B. subtilis* 168	This study

PCR amplifications for cloning purposes were performed with Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific), and PCR amplifications for screening purposes were performed with DreamTaq Hot Start Green PCR Master Mix (Thermo Fisher Scientific). GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific) was used to purify DNA from the PCR products. All oligonucleotides were gained from Integrated DNA Technologies. All plasmid extractions were achieved using the QIAprep Spin Miniprep Kit (Qiagen). All Fast Digest Enzymes used in this study were obtained from Thermo Fisher Scientific.

6.2.2 Creation of the isogenic strain of *B. subtilis* 168

The creation of the isogenic strain of *B. subtilis* 168 was prepared as described in the following steps: construction of the plasmid by Gibson Assembly, transformation of the constructed plasmid into *E. coli* DH5α, plasmids extraction, and electro-transformation into *B. subtilis* 168.

The sgRNA targeting the *trpC* gene on the chromosome of *B. subtilis* 168 was designed based on the Design CRISPR Guides on the Benchling website (https://benchling.com) and the sequence

with the highest on-target score was chosen as the sgRNA for CRISPR Cas9 system used in this study. *B. subtilis* 168 was applied as the reference genome and "NGG" was used as the protospacer adjacent motif (PAM). The *trpC* gene was chosen because the *trpC* locus is inactivated due to the triplet ATT deletion which leads to tryptophan auxotrophy in *B. subtilis* 168 (Albertini and Galizzi, 1999). Besides, no other significant differences in growth characteristics and competence are reported between *B. subtilis* 168 and the wild type strains (Zeigler et al., 2008). Circular polymerase extension cloning (CPEC) was applied to insert the sequence of sgRNA in the pJOE8999 (Figure 6.3). Oligonucleotides used for the CPEC were shown in Table 6.2. To amplify the segments for CPEC, the sequence of sgRNA was added to the forward primer of segment 1 (S1) and reverse primer of segment 2 (S2), respectively. CPEC reaction was set up on ice and run following the procedure as described elsewhere (Quan and Tian, 2014).

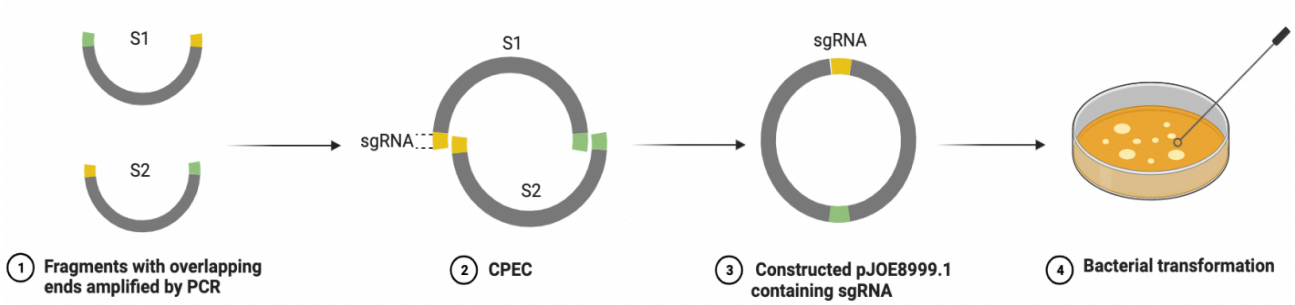


Figure 6.3 The schematic diagram of inserting the sgRNA to pJOE8999 by CPEC. The sequence of sgRNA is added on the forward primer of segment 1 (S1) and reverse primer of segment 2 (S2), respectively. The S1 and S2 share overlapping regions at the ends. In each CPEC cycle, after denaturation and annealing, the hybrid S1 and S2 extend with each other as templates until they complete a whole circle. The assembled plasmid can be used for transformation into competent cells directly.

To construct the vector plasmid with the *spoVA*^{2mob} operon, Gibson Assembly was applied (Figure 6.4).

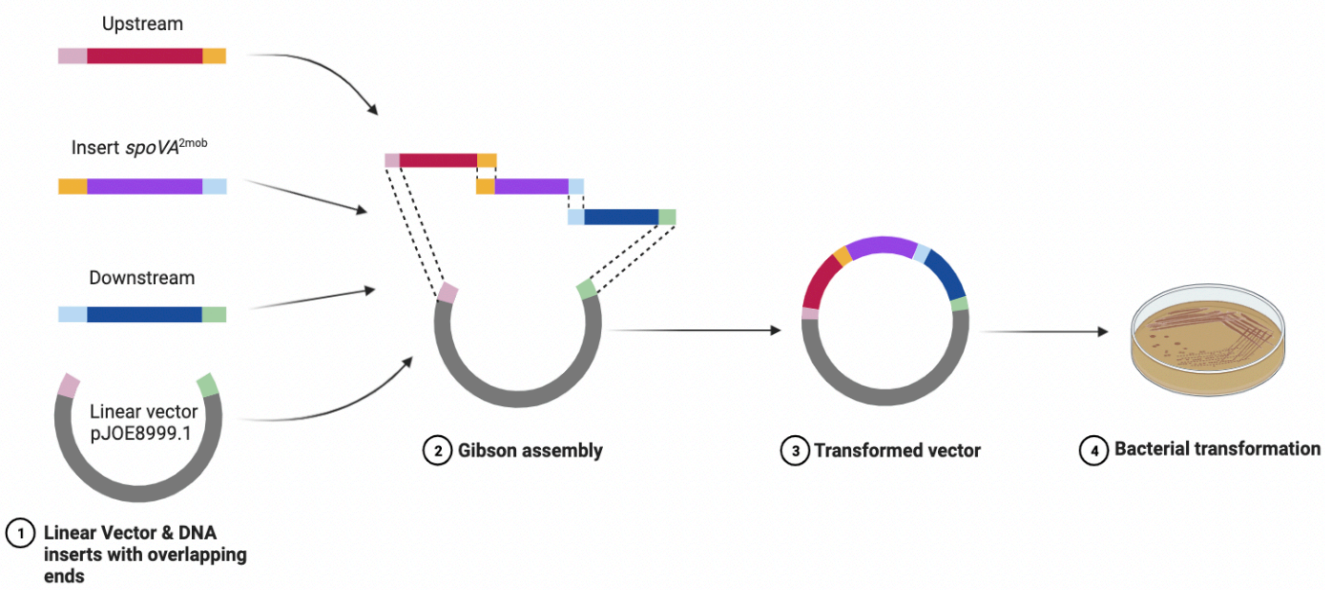


Figure 6.4 The schematic diagram of construction of the plasmids by Gibson Assembly.

The pJOE8999.1 (pJOE8999 containing the 20 bp sequence of sgRNA of *trpC*) were digested by Fast Digestion *XbaI* enzyme (Thermo Fisher Scientific) and then purified as the vector for construction. The oligonucleotides used for amplification of PCR fragments were shown in Table 6.2. The upstream homolog fragment of *trpC* was amplified with oligonucleotides F-up *trpC* and R-up *trpC*, while the downstream homolog fragment was amplified using oligonucleotides F-down *trpC* and R-down *trpC*. Between the amplified upstream and downstream fragments, the *spoVA*^{2mob} operon was amplified using oligonucleotides F-*spoVA*^{2mob} and R-*spoVA*^{2mob}. The vector and fragments shared around 20 base pairs overlap one by one. The vector and three PCR fragments were fused using the Gibson assembly Cloning Kit (New England Biolabs).

Table 6.2 List of oligonucleotides used in this study.

Name	Sequence (5'-3')	Purpose
F-S1 sgRNA	ctccgattcttcttgattccGTTTTAGAGCTAGAAATAGC	To insert the sgRNA in pJOE8999 by CPEC
R-S1	GCTTATCATGAGAAATATCC	To insert the sgRNA in pJOE8999 by CPEC
F-S2	CTCCACTTGCGTTAATAGGG	To insert the sgRNA in pJOE8999 by CPEC
R-S2 sgRNA	ggaatcaagaagaatcggagCGTAGGTACATTTTACTC	To insert the sgRNA in pJOE8999 by CPEC
F-up *trpC*	GGCCAATAAGGCCTTTCTAGTG ATATTCCGGCACACATTCCC	To amplify the upstream *trpC* (left homologous arm)
R-up *trpC*	CCTCCTTCTTCCATATTG TTAATCGGCGAGGTGC	To amplify the upstream *trpC* (left homologous arm)
F-*spoVA*^{2mob}	AACAATATGGAAGAAGGAGGAAA TTATCATGACAGTAATAAATGACG	To amplify the *spoVA*^{2mob} operon
R-*spoVA*^{2mob}	GGAGCGGATGCGTCATGTATGAATC	To amplify the *spoVA*^{2mob} operon
F-down *trpC*	ATACATGACGCATCCGCT CCGATTCTTCTTGATTCCTC	To amplify the downstream *trpC* (right homologous arm)
R-down *trpC*	AAGATTATTTCTTAATCTAGTTCAGCCG AAGCACGTTATGTTTGTATCAAGC	To amplify the downstream *trpC* (right homologous arm)
F-up 168 chromosome	CGGATATGGATACATATCGGTTCATCC	To check the integration of *spoVA*^{2mob} operon on the chromosome of *B. subtilis* 168 (left cross-over)
R-up *spoVA*^{2mob} chromosome	GATTACCATTACATCCTGATGCGAAAC	To check the integration of *spoVA*^{2mob} operon on the chromosome of *B. subtilis* 168 (left cross-over)
F-down *spoVA*^{2mob} chromosome	GATGAACCACTAGCCACGATTG	To check the integration of *spoVA*^{2mob} operon on the chromosome of *B. subtilis* 168 (right cross-over)
R-down 168 chromosome	GAACGGTATTTAATCTGCTTGGGC	To check the integration of *spoVA*^{2mob} operon on the chromosome of *B. subtilis* 168 (right cross-over)

The sequence underlined was sgRNA of *trpC*.

After Gibson Assembly, the constructed plasmids were transformed into competent cells of *E. coli* DH5α using the chemical transformation heat shock method. Then the plasmids were extracted from the strain of *E. coli* DH5α to gain plasmids with a higher concentration.

Electrocompetent cells of the *B. subtilis* 168 used were prepared as described before (Xue et al., 1999). In brief, an overnight culture of *B. subtilis* 168 was diluted to get an initial OD_{600nm} of 0.1, and then grew at 37 °C, 200 rpm to an OD_{600nm} of 0.75-0.95. Then the cell culture was cooled on ice-water for 10 min and then harvested by centrifugation at 4 °C and 5000 × *g* for 10 min, followed by washing four times with the prechilled electroporation solution (0.5M sorbitol, 0.5M mannitol and 10 % glycerol) and suspended the cells in 1 mL electroporation medium. Aliquots of 50 μL were stored at –80 °C until use. The electroporation was carried out at 400 Ω, 25 μF, 2.0 kV and 4.5 ms in 0.2 cm cuvettes. After recovering at 30 °C and 200 rpm for 2 h, the culture was plated on LB agar containing 5 μg/mL kanamycin, then the plates were incubated at 30 °C. The colonies shown in 2 days were selected for colony PCR to screen the target gene on chromosome by primers listed in Table 6.2. Then for the mutant strain candidates, the plasmids were cured by increasing the incubation temperature to 45 °C for one day and 50 °C for another day because the pJOE8999 plasmids were temperature sensitive.

6.2.3 Preparation of spore suspensions

Spore suspensions of the *B. subtilis* 168 and isogenic *B. subtilis* 168 *trpC*::$spoVA^{2mob}$ were prepared following the same procedure as indicated elsewhere (Li et al., 2019). Briefly, 0.1 mL of overnight cultures of both strains were spread on LB agar plates and incubated at 37 °C for 10 d. The sporulated spores were harvested after 90-99 % of the cells contained spores checked by Schaeffer-Fulton staining method (Maksong et al., 2017). Then the surfaces of plates were flooded with 5 mL of cold sterile distilled water twice to collect the spores. The spore suspensions were

washed four times by centrifugation at 3,000 × *g* for 15 min at 4 °C and resuspended in sterile distilled water. To kill all the vegetative forms, the spore suspensions were pasteurized at 80 °C for 20 min between the second and third wash cycles. Then the spore suspensions were stored at −80 °C for future use.

6.2.4 Determination of the resistance of spores to high pressure

The spore suspensions of *B. subtilis* 168 and *B. subtilis* 168 *trpC*::*spoVA*^{2mob} were diluted in sterile distilled water to around 10^7 spores/mL. Aliquots of 120 μL of spore suspension were heat sealed in polypropylene tubes without air bubbles. The samples were treated at 600 MPa and 80 °C for 1, 2, 4, and 8 mins with a High Pressure Micropump MP5 system (High Pressure Physics, Polish Academy of Sciences, Warsaw, Poland). Samples sealed in tubes but without high pressure treatment were used as control group. All the samples were held on ice before and after pressure treatment. Viable spore counts were determined by serial dilution and surface plating on LB agar and incubation at 37 °C after 18 h.

6.2.5 Determination of the germination of spores induced by different germinants

To better investigate the role of *spoVA*^{2mob} operon in the germination of *Bacillus* spores *via* different pathways: pressure, heating, and nutrients, different germination tests were applied in this study. For all the germination tests, the spore suspensions of *B. subtilis* 168 and *B. subtilis* 168 *trpC*::*spoVA*^{2mob} were diluted with 25 mM K-HEPES buffer (pH 7.4) to a concentration of around 10^7 spores/mL.

For the pressure-induced germination test, two different pressures were chosen because of the two different inducement pathways of pressure (Figure 6.2): (1). 150 MPa, 37 °C for 0 min, 5 min and 10 min; (2). 550 MPa, 55 °C for 0 min, 1 min, 3 min and 5 min. Based on previous studies on the

selection of high-pressure temperatures for *Bacillus* spores, 37 °C was the optimal germination temperature for germination at 150 MPa (Black et al., 2005). For germination at 500 MPa, the optimum temperature is ~60 °C (Luu et al., 2015). After pressurizing in heat-sealed sterile polyethylene tubes with the High Pressure Micropump MP5 system, the samples were kept in ice for at least 15 min. The compression rate was approximately 100 MPa/15 sec, while decompression was immediate.

For the heat-induced germination test, the diluted spore suspensions were heat activated in water bath at 75 ℃ for various times (0 h, 1 h, 2 h, 4 h and 6 h), and then cooled in an ice bath for more than 15 min. The experiment was performed in three independent replicates with replicated spore suspensions.

For the pressure and heat induced germination tests, the percentage of germination of spores was determined by comparing the amount of DPA release after treatment with the total DPA amount in spores. The total DPA amounts of the two strains were obtained by autoclaving at 121.1 °C for 60 min. Then the autoclaved spores and the pressure- or heat-treated spores were mixed with an equal amount of 25 mM K-HEPES buffer (pH 7.4) containing 50 mM terbium (III) chloride solution in nontransparent 96-well plates, respectively. Fluorescence intensity of Tb^{3+}-DPA was measured using a fluorescence spectrophotometer with 270 nm excitation and emission wavelengths of 545 nm, appropriate wavelengths for the Tb^{3+}-DPA complex (Yang and Ponce, 2009). Data were obtained from the difference between the DPA release from the pressure- or heat-treated spores and the total DPA released after autoclaving. The TbCl solution mixed with an equal amount of 25 mM K-HEPES buffer (pH 7.4) was used as a blank.

For the nutrient-induced germination test, the percentage of germination of spores was determined by comparing the amount of DPA released at various time points with the total DPA content in

spores, which was determined by autoclaving. Four different kinds of nutrients were chosen to induce the germination of spores: (1). mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK); (2). L-alanine (Ala); (3). L-valine (Val); and (4). L-asparagine (Asn). The concentration of each germinant was 10 mM in 25 mM K-HEPES buffer (pH 7.4) for all the solution. The concentration of each germinant was chosen to be 10 mM, because germination with 40 mM germinants in initial experiments resulted in a less than 15 % increase in germination rates (Luu et al., 2015). Then 90 μL of spore suspensions of both strains were mixed with 90 μL of 50 mM TbCl solution and 10 μL of each germinant in the nontransparent 96-well plates, respectively. In addition, the fluorescence of Tb^{3+}-DPA was read by the fluorescence spectrophotometer every 10 min for 360 min. The TbCl solution mixed with an equal amount of 25 mM K-HEPES buffer (pH 7.4) was used as a blank. The experiment was performed in three independent replicates with replicated spore suspensions.

6.2.6 Determination of the germination of spores by cell counts

To better compare the difference in spore germination between the two isogenic strains, the percentages of germinated spores of two isogenic strains were determined by cell counts. Three different treatments of spores were applied in this experiment: treated at 75 °C for 1 h, 150 MPa for 5 min, and 550 MPa for 3 min, respectively. After treatment, samples were serially diluted to appropriate concentrations and plated on LB agar plates. The plates were incubated at 37 °C for 18 h. To determine the percentage of germinated spores, aliquots of the pressure- or heat-treated spore suspensions were subjected to a heat treatment at 80 °C for 10 min to kill the germinated cells, subsequently serially diluted, plated on LB agar plates, and incubated for 18 h at 37 °C again. The difference between the two sets of data was calculated to distinguish the heat-sensitive (germinated) and heat-resistant (ungerminated) spores. The initial spore counts of the spore

suspensions for each strain without any treatment were used as control. The experiments were repeated for triplicates. The heat treatment of 10 min at 80 °C was found not to kill ungerminated native spores but to kill the germinated cells. The percentage of germinated spores was expressed as indicated in the formula below.

$$\text{Percentage of germinated spores} = \frac{\text{Colonies after pressure or heat treatment} - \text{colonies after 80 °C heating}}{\text{Total colonies of control group}} \times 100\%$$

6.2.7 Statistical analysis

Experiments were performed in at least three replicates with different batches of spore suspensions. For the pressure resistance test, statistical analysis of the reduction of cell counts for the two isogenic *Bacillus* strains after high pressure treatment was carried out by t-test. Statistical analysis of the percentage of DPA release by the two isogenic *Bacillus* strains in the spore germination assays induced by different germinants was carried out using t-test. A P value ≤ 0.05 was considered statistically significant.

6.3 Results

6.3.1 Variation in pressure resistance of *B. subtilis* 168 and *B. subtilis* 168 trpC::*spoVA*^{2mob}

To determine the pressure resistance of spores with and without *spoVA*^{2mob} operon, spores from *B. subtilis* 168 and *B. subtilis* 168 *trpC*::*spoVA*^{2mob} were subjected to treatment at 600 MPa and 80 °C. A difference in pressure resistance was observed between wild type and mutant type strain (Figure 6.5). A difference ($P < 0.05$) of around 1 log CFU/mL was found after high pressure treatment for 4 min. While after 8 min, the difference decreased, which may be due to the condition being severe and most spores from both wild type and mutant are inactivated.

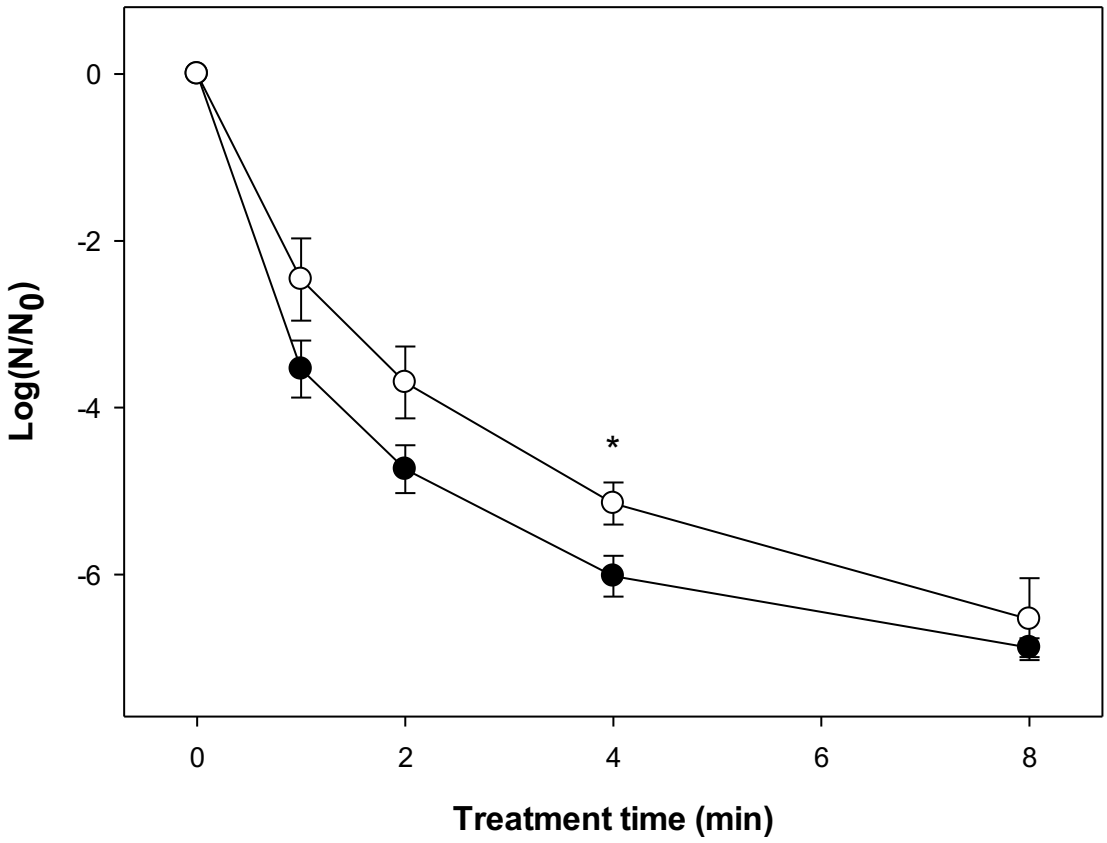


Figure 6.5 Variation in spore reduction [log(N/N_0)] of *B. subtilis* 168 and *B. subtilis* 168 *trpC*::*spoVA*^{2mob} after treatment at 600 MPa and 80 °C for different times. Strains are differentiated by different symbols: (●), *B. subtilis* 168; (○), *B. subtilis* 168 *trpC*::*spoVA*^{2mob}. The data are means of three independent experiments; error bars are standard deviations. Data labeled with an asterisk indicates the data at that time is significantly different based on a t-test.

6.3.2 Germination of spores induced by high pressure

Figure 6.6A and 6.6B show the different results for the percentage of DPA release after treatment with two different pressure parameters (150 MPa, and 550 MPa), respectively. The DPA release from wild type *B. subtilis* 168 was higher than that of mutant type *B. subtilis* 168 *trpC*::*spoVA*^{2mob} after treatment at 150 MPa and 37 °C for 10 min: the DPA released from spores of wild type

B. subtilis 168 reached 80 %, while the release from spores of *B. subtilis* 168 *trpC*::*spoVA*^{2mob} was about 50 % (Figure 6.6A). The percentages of DPA released by *B. subtilis* 168 and *B. subtilis* 168 *trpC*::*spoVA*^{2mob} under pressure treatments at 550 MPa and 55 °C were different at 1 min, 3 min and 5 min ($P < 0.05$). For *B. subtilis* 168 *trpC*::*spoVA*^{2mob}, the percentage of DPA release after high pressure treatment increased with the treatment time. The percentage of DPA released by *B. subtilis* 168 was above 80 %, but the released amount after 5 min of treatment was lower than after 3 min of treatment (Figure 6.6B).

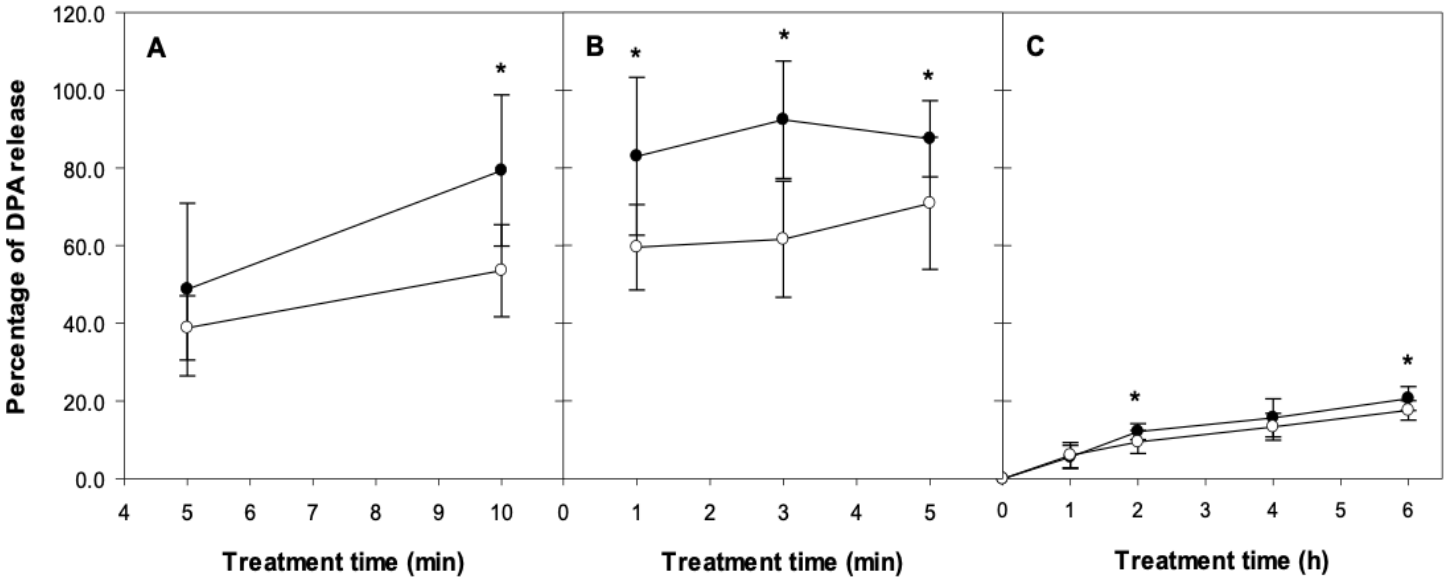


Figure 6.6 Germination of spores induced by different germinants. **A.** Pressure treatment at 150 MPa and 37 °C. **B.** Pressure treatment at 550 MPa and 55 °C. **C.** Heating at 75 °C for various times. The different strains were indicated by the different colors of symbols: (●), *B. subtilis* 168; (○), *B. subtilis* 168 *trpC*::*spoVA*^{2mob}. The data are means of three independent experiments; error bars are standard deviations. Data labeled with an asterisk indicates the data at that time is significantly different based on a t-test.

6.3.3 Germination of spores induced by heat

Figure 6.6C shows the percentages of DPA release after heating at 75 °C for various times. After heating for 1 h, the percentages of DPA released from the spores of the two strains were almost the same. After 2 h, 4 h, and 6 h of heat activation, DPA increased linearly in both groups and had almost the same slope. The slope of wild type *B. subtilis* 168 and mutant *B. subtilis* 168 *trpC*::*spoVA*^{2mob} is 0.051 and 0.043, respectively. The heat did not lead to high percentages of germination. After 6 hours of heat treatment, the DPA release of *B. subtilis* 168 was only 20 %, while the mutant *B. subtilis* 168 *trpC*::*spoVA*^{2mob} was only 17.5 %.

6.3.4 Germination of spores induced by different nutrients

The effect of different germinants on DPA release from the two isogenic strains is shown in Figure 6.7. Among the four different types of nutrients induced spore germination, L-alanine has the best induction effect on both strains of *Bacillus*, followed by AGFK. For L-alanine, the proportion of DPA released by the two groups of spores increased significantly from 10 min to 120 min, and then showed a gentle increase trend. After 6 h of L-alanine induced germination, the spores of wild type *B. subtilis* 168 released about 95 % DPA, and the spores of mutant *B. subtilis* 168 *trpC*::*spoVA*^{2mob} released about 80 % DPA. The amount of DPA release induced by AGFK was faint. Using L-valine and L-asparagine as germinant, no increase in DPA release from either strain. The use of L-valine or L-asparagine as germinant did not increase DPA release from either of the two isogenic strains.

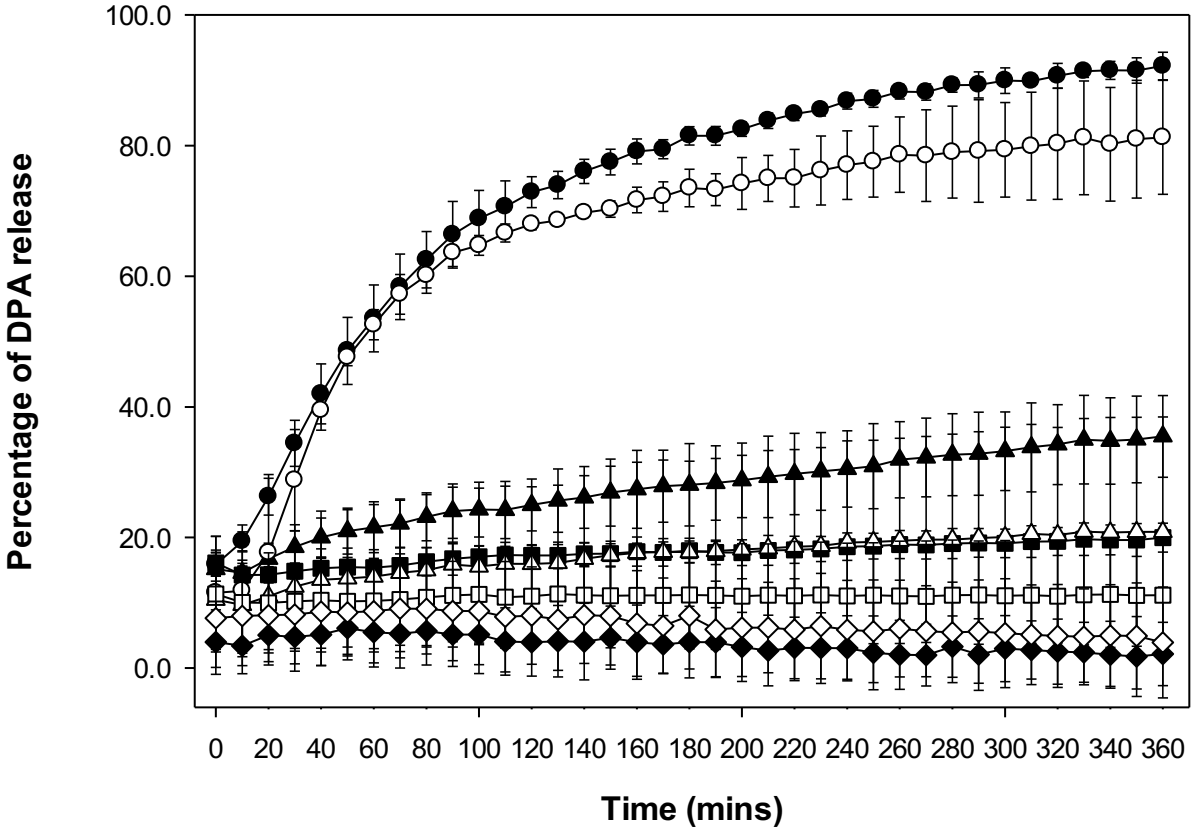


Figure 6.7 Germination of spores induced by different nutrients. The different strains were indicated by the different colors of symbols: Black, wild type *B. subtilis* 168; white, mutant type, *B. subtilis* 168 *trpC*::*spoVA*^{2mob}. Different shapes of symbols represent different nutrients: ▲, AGFK; ●, Ala; ■, Val; ◆, Asn. The data are means of three independent experiments; error bars are standard deviations.

6.3.5 Differentiation of the germination of spores tested by cell counts

To determine the degree of spore germination, aliquots of the spore suspensions after pressure or heat induced germination were subjected to a heat treatment at 80 °C for 10 min to kill the germinated cells. The percentage of germinated spores from the two isogenic strains were

compared. As shown in Figure 6.8, after pressure pr heat induced germination of spores, no difference was found between the two isogenic strains.

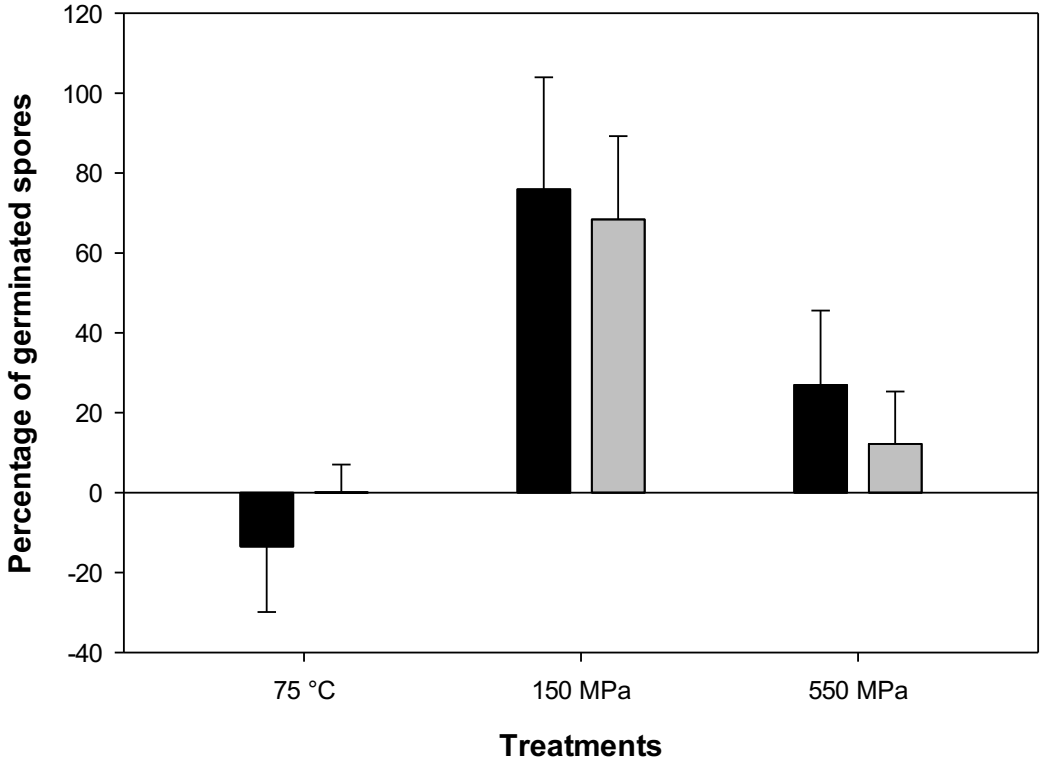


Figure 6.8 Difference of spore germination induced by pressure or heat. The difference was determined by cell counts with and without inactivation of germinated cells at 80 °C for 10 min. Black and gray bars indicated the *B. subtilis* 168 and *B. subtilis* 168 *trpC*::*spoVA*^{2mob}, respectively. The data are means of three independent experiments; error bars are standard deviations.

6.4 Discussion

In this study, the isogenic mutant strain was created by inserting the *spoVA*^{2mob} operon on the chromosome of *B. subtilis* 168, which is the most well-studied laboratory strain of *Bacillus*. Three functioning germinant receptors, GerA, GerB, and GerK, are present in strain *B. subtilis* 168 (Paidhungat and Setlow, 2000). As shown in Figure 6.1 and Figure 6.2, the nutrients induce the

germination of *Bacillus* spores by activation of the GRs. However, different nutrients activate different GRs. GerA responds to L-alanine or L-valine. The AGFK activates the GerB and GerK receptors. Even though GerB responds primarily to L-asparagine and GerK responds to sugars, neither of these receptors is capable of initiating germination on its own (Atluri et al., 2006; Setlow, 2014; Krawczyk et al., 2017).

As shown in Figure 6.7, the percentage of DPA released during the germination of spores induced by various nutritional germinants is different. The release of DPA was most effectively induced by L-alanine. Even though L-alanine and L-valine both trigger GerA, the effect of inducement of L-valine to both isogenic strains was very limited. This might be due to the lack of prior heat activation step in the nutrients-induced germination test. In previous studies, nutrients-induced spore germination experiments were typically performed after a heat activation step of 30 min at 75 °C (Black et al., 2005). Interestingly, recent research investigated that no increase was observed in germination under high pressure when prior heat activation was applied (Delbrück et al., 2021b). Besides, the preheating activation directly affects the GRs proteins because of the differences in preheating activation requirements of individual GRs (Luu et al., 2015; Krawczyk et al., 2017). A previous finding indicated that the preheating for inactivation of spores can disable several germination pathways, particularly the pathway associated with GerA receptor and L-alanine (Krawczyk et al., 2017). Therefore, no heat inactivation was applied in this study. However, no relevant test about heat activation and L-valine germination has been done, and whether the preheating is essential for L-valine induced germination of spores is unknown. A recent study showed that L-valine-induced germination of spores carrying the *spoVA*^{2mob} operon was significantly reduced when the regular *spoVA* operon was absent, but even in the presence of the *spoVA* operon, L-valine-induced germination was slow (Luo et al., 2021).

Various germinant receptor types in the heat resistant *Bacillus* spores containing the *spoVA*^{2mob} operon differ in their heat activation requirements and thermal stabilities (Krawczyk et al., 2017). Spore germination mediated by GerA, which is induced by L-alanine, was optimally activated at lower temperatures than germination induced by the AGFK mixture, which is mediated by the action of GerB and GerK cooperatively (Krawczyk et al., 2017).

The increased wet-heat resistance and decreased germination rates *via* heat activation, nutrients, exogenous Ca^{2+}-DPA, and cationic surfactant dodecylamine by *spoVA*^{2mob} operons have been reported (Luu et al., 2015; Berendsen et al., 2016a; Krawczyk et al., 2016, 2017; Setlow and Johnson, 2019; Kanaan et al., 2022). However, limited studies were found about the high pressure resistance or germination of spores triggered by pressure. Generally, high pressure triggers germination of spores *via* two pathways, depending on the pressure parameter: activating GRs and causing Ca^{2+}-DPA release (100-300 MPa) or causing Ca^{2+}-DPA release directly (300-600 MPa) (Figure 6.2). In this study, the percentage of DPA release from mutant *B. subtilis* 168 *trpC*::*spoVA*^{2mob} was lower than that of *B. subtilis* 168, regardless of whether it was treated with ultra-high pressure (550 MPa) or medium-high pressure (Figure 6.6), indicating the *spoVA*^{2mob} operon significantly delayed the germination of spores induced by high pressure.

Because of the seven proteins encoded in the regular *spoVA* operon compared to only three proteins encoded by *spoVA*^{2mob} operon, and the large sequence differences between the SpoVA proteins encoded by the two operons (Berendsen et al., 2016a), it is not surprising that signal transduction between GRs and the SpoVA channel would be severely compromised with the SpoVA^{2mob} proteins (Setlow, 2003). However, it is not clear whether the *spoVA*^{2mob} encoded proteins are complementary to the SpoVA proteins, interact with SpoVA proteins, or function independently (Berendsen, 2016). Since elevated levels of one GR can decrease the function of other GRs

(Stewart et al., 2012; Luo et al., 2021), it is likely that the products of *spoVA*^{2mob} can compete or interfere with the function of regular SpoVA proteins and/or GRs, delay the germination of spores, and further increase resistance to harsh conditions (Wang et al., 2015).

Based on previous studies, the extreme heat or pressure resistance of *Bacillus* spores was observed in strains with multiple copies (two or three) of the *spoVA*^{2mob} operons on chromosome. The isogenic mutant strains with two and three *spoVA*^{2mob} operon will be helpful to study the role of *spoVA*^{2mob} opreon.

6.5 Conclusion

Due to the wide presence of *Bacillus* spores in plants and the environment, and their potential impact on food spoilage, inactivation of the spores is necessary to achieve commercial sterility. However, thermos sterilization as the traditional inactivation method can result in losses in nutrients, color, or flavor. The novel germination-inactivation strategies is gaining popularity because they allows better maintenance of important food quality attributes.

Based on this study, the *spoVA*^{2mob} operon increases the pressure resistance of *B. subtilis* 168 and decreases germination of spores induced by high pressure significantly. The operon also reduced the percentage of DPA release after germination triggered by nutrients. This progress enables further development of germination-inactivation strategies, including the pressure assisted thermal sterilization in the food industry.

Chapter 7 General Discussion

Bacilli are abundant in nature, particularly in soil, where they form stable symbiotic associations with plants and occur in the rhizosphere or as endophytes (Robinson et al., 2016; Shahzad et al., 2016). The association of bacilli with plants in combination with the extreme resistance of spores make it unavoidable that bacilli are present in plant foods. Depending on the food in question, *Bacillus* species contribute to food fermentations in some spontaneous food fermentations, or are regarded as spoilage organisms in other food products. In some cases, extracellular enzymes produced by *Bacillus* are beneficial for saccharification and hydrolysis of fermentation substrates but the same enzymes can also cause product quality issues. Moreover, species of the *B. cereus* group are toxigenic or pathogenic. Therefore, the different roles of *Bacillus* can be partly explained by the hydrolytic enzymes with various amount at different situation.

7.1 The role of enzymes produced by *Bacillus* at different situations

Hydrolytic activity of extracellular enzymes produced by *Bacillus* play essential and beneficial roles in some food fermentations. For instance, amylases are the primary contributor to the liquefying and saccharifying processes of *daqu* fermentation, which have a significant effect on the quality of *daqu* (Liu et al., 2018). Based on previous metatranscriptomic analysis, α-amylase exhibited the second highest expression level with 465.7 reads per kilobase per million among enzymes related to starch metabolism (Huang et al., 2017). Furthermore, thermotolerant amylases produced by some strains of *Bacillus* are important in *daqu* as the high temperature during fermentation (Jin et al., 1990). In *natto* fermentation, extracellular proteolytic enzymes including neutral proteases, alkaline protease, and esterase are secreted in large amounts by *B. subtilis* var. *natto* (Uehara et al., 1974). They break down soybean proteins into amino acids, which provide taste, flavor, and serve as substrates producing a viscous texture and specific smell of *natto* (Kada

et al., 2013). These proteases are responsible for a marked increase in water-soluble nitrogen during fermentation of *natto* which plays an important role in the characteristic flavor of fermented *natto* (Hu et al., 2010).

Conversely, amylolytic and proteolytic enzymes produced by bacilli cause spoilage in bakery and dairy products. For example, ropy bread spoilage caused by *Bacillus* spp. is characterized by an unpleasant fruity odor followed by enzymatic degradation resulting in sticky and stringy bread crumb and slime formation (Valerio et al., 2015). The microbiological quality of heat-treated dairy products has been associated with the proliferation and metabolic activity *Bacillus* spp., including *B. licheniformis*, *B. mycoides*, *B. megaterium B. subtilis,* and *B. cereus* (Ledenbach and Marshall, 2009; de Jonghe et al., 2010; Montanhini et al., 2013; Arslan et al., 2014). Undesirable technological and sensorial changes in dairy products resulting from enzymatic (amylase, protease, lipase and phospholipases) activities of *Bacillus* spp. include coagulation of milk casein, fat degradation, off-flavors and off-odors (Ledenbach and Marshall, 2009; de Jonghe et al., 2010; Montanhini et al., 2013).

7.2 Hazards of *B. cereus* in food industry

The pathogenic soil-dweller *B. cereus* is commonly encountered in raw milk and subsequent dairy products. *B. cereus* can cause off-flavors such as bitty cream and sweet curdling as a result of its enzymatic activities. The bitty cream is formed by a creamy layer on the milk due to the action of lecithinase (Skovgaard, 2004). Sweet curdling is caused by the action of proteolytic enzymes (Coorevits et al., 2010). In addition, *B. cereus* can produce emetic toxin and diarrheal enterotoxins, which cause two types of gastrointestinal diseases: emesis and diarrhea (Schoeni and Lee Wong, 2005). Cereulide is a heat stable cyclic dodecadepsipeptide and cause the emetic type of symposium (Ehling-Schulz et al., 2005). Cereulide is produced by *B. cereus* cells growing in

contaminated food and causes nausea and vomiting within 1-5 h of ingestion (Schoeni and Lee Wong, 2005). Although the exact number of *B. cereus* cells required to produce adequate toxin leading to disease has not been determined, levels of 10^3-10^{10} CFU/g have been detected in foods, which have caused outbreaks (Logan and Rodríguez-Díaz, 2006). Three heat-labile enterotoxins that cause diarrheal type of symposium are hemolysin BL, nonhemolytic enterotoxin, and cytotoxin K. The cytotoxin K is highly cytotoxic and the major virulence factor of *B. cereus* diarrhea and may also be necrotic and hemolytic (Arslan et al., 2014). The main symptoms of *B. cereus*-related diarrhea include abdominal cramps and watery diarrhea at 8-16 h after ingestion (Ehling-Schulz et al., 2005). The infective dose of the diarrheal disease has been described as 10^5-10^8 CFU/g of food, although lower or higher counts of viable cells or spores have been reported (Logan and Rodríguez-Díaz, 2006). Consequently, *B. cereus* strains are potential extracellular enzyme and enterotoxin producers in dairy products, which not only cause economic losses, but also endanger the health of consumers.

7.3 Toxins and antimicrobial compounds: *B. cereus* group *vs. B. subtilis* group

Figure 2.4 depicts a clear distinction between the production of toxins and antifungal lipopeptides by the *B. cereus* group and *B. subtilis* group. According to the BLAST result, toxins are widely produced in the *B. cereus* group, but lipopeptide production is not predicted. Furthermore, antifungal lipopeptides are widely expressed among the strains of *B. subtilis* group, but no toxin production is shown.

The *B. cereus* group includes several *Bacillus* species with closely related phylogeny and their differentiation is cumbersome but essential notably in term of epidemiology. The most well-studied members of the group, *B. cereus*, *B. anthracis*, and *B. thuringiensis* are known for their pathogenic potential and can be a source of human infection (EFSA Panel on Biological Hazards

(BIOHAZ), 2016; Ehling-Schulz et al., 2019). Depending on virulence gene presence and expression, strains of these species can cause anthrax or gastrointestinal illness in humans or insects (Chapter 2) (Ehling-Schulz et al., 2019). Anthrax is caused by the *B. anthracis*. *B. cereus* strains are generally recognized as opportunistic food poisoning agents that cause two forms of food poisoning, characterized by either nausea and vomiting or abdominal pain and diarrhea. Certain strains of *B. thuringiensis* are insect pathogens and have been commercialized for use worldwide as biopesticides, while some strains have been reported to infect immunocompromised individuals, and cause the same gastrointestinal illness that is caused by *B. cereus* (Arslan et al., 2014; Ehling-Schulz et al., 2019).

B. subtilis group, including *B. subtilis*, *B. amyloliquefaciens*, *B. mojavensis*, *B. paralicheniformis*, *B. pumilus*, *B. licheniformis* and *B. velezensis* are frequently isolated from food fermentations (Harwood et al., 2018). *B. subtilis* group strains have generated the interest as food biopreservatives, therapeutic agents and biopesticides because they are known to produce a wide range of secondary metabolites mediating antibiosis (Caulier et al., 2019). It is estimated that at least 4-5 % of the genome of any given strain of the *B. subtilis* group is devoted to production of antimicrobial compounds. Most of these molecules are antimicrobial peptides (Stein, 2005). Based on the biosynthetic pathways and their chemical nature, the antimicrobial compounds within the *B. subtilis* group can be classified as ribosomal peptides (RPs) (bacteriocins and enzymes), polyketides (PKs), non-ribosomal peptides (NRPs) and volatiles (Caulier et al., 2019).

Overall, the *B. subtilis* group provides a large number of antimicrobial compounds with a wide range of biological functions. This enormous versatility increases the industrial and environmental interest of *B. subtilis* strains, but also requires consideration of their history as spoilage organisms and their safe use in food.

7.4 Practical implications, limitations and future works

Fermented foods are consumed throughout the world and substantially contribute to food security. Many fermented products rely on the participation of *Bacillus* spp. but their contribution to food quality is not well documented. Chapter 2 well summarized the *Bacillus* spp. associated food fermentations. *Bacillus* also cause food spoilage, including bread and dairy products. Additionally, some *Bacillus* spp. produce toxins that are harmful to humans. Chapter 2 highlighted the metabolic properties that relate to food quality and food safety. These metabolic traits of *Bacillus* also provide opportunities for use in food fermentations where they do not occur traditionally. However, the bioinformatic analysis of a genome dataset restricted to only type strains indicated that toxin production is limited to the *B. cereus* clade (Chapter 2), while many useful technological traits such as proteases, amylases, polyglutamates, and lipopeptides, are limited to, or much more frequent in the *B. subtilis* clade. This approach does not inform on strain specific differences. The difference should be confirmed with analyses that employ a larger set of genomes to improve the control of food spoilage and food fermentation in the future.

Bacillus spp. are not avoidable in spontaneous *daqu* fermentation as they reside in or on cereal grains as endophytes. During *daqu* fermentation, hydrolytic enzymes are produced by *Bacillus* and fungi. *Bacillus* spp. also produce a wide range of antifungal lipopeptides. The direct evidence for an impact of antifungal lipopeptides from *Bacillus* spp. on community assembly in *daqu* is lacking. Chapter 3 studied the impact of *Bacillus* produced lipopeptides on fungi growth in a *daqu* fermentation model and provided a new perspective on the role of *Bacillus* lipopeptides during *daqu* fermentation. It is also helpful for further application of *Bacillus* spp. in food fermentations or food productions to help control mold growth. In Chapter 3, the morphology of mold growth on the samples was observed and the signal intensities of the peptides were detected by mass

spectrometry. However, monitoring the cell counts of *Bacillus*, fungi, and other bacteria may be preferable to demonstrate the predominant role of *Bacillus* during *daqu* fermentation.

Due to the wide existence of *Bacillus* in raw ingredients and bakery environments, and the ability of *Bacillus* spores to survive during baking, the concern of heat resistant spores cannot be fully addressed by sanitary prevention and heat treatment during baking process. Based on the findings in Chapter 4, the application of sourdough and reutericyclin effectively enhanced thermal inactivation of *Bacillus* endospores and inhibited rope spoilage in bread, which can be considered as a natural bio-preservative in bread industry. In addition, the study in Chapter 4 illustrated a difference in the phenotype of ropy spoilage and the genotype of *B. amyloliquefaciens* and *B. subtilis.* This finding suggests that variations in the composition of extracellular amylases and proteases in *B. amyloliquefaciens* and *B. subtilis* may contribute to different activity in causing ropy spoilage. However, the detailed variations of composition and the exact role of enzymes in Figure 4.4 have not been well studied and warrant further investigation.

The *spoVA*^{2mob} operon has been proven to lead to the heat resistance of endospores, and redundant copies of the operon increase the heat resistance. However, in Chapter 4, the presence of multiple copies of the *spoVA*^{2mob} operon helps spores survive during baking but is not a prerequisite for the ability of strains to spoil bread, and the lethality of the baking process towards endospores that did not harbor a *spoVA*^{2mob} operon was limited as well. This finding needs to be tested with more wild type strains of *Bacillus* and isogenic strains with various copy number of *spoVA*^{2mob} operon in the future.

Bacillus endospores also exhibit a strong variation in resistance to pressure, but the underlying mechanisms of endospore resistance to pressure are not fully understood. In Chapter 5, the effects of *spoVA*^{2mob} operon on high pressure resistance in *Bacillus* spores were determined. The copy

numbers of *spoVA*^{2mob} operon in 17 strains of *Bacillus* were determined via ddPCR. The results showed these strains contained between 0 to 3 copies of *spoVA*^{2mob} operons. The pressure resistance of spores from the 17 strains of *Bacillus* was tested. The results indicated that the redundant *spoVA*^{2mob} operons in *Bacillus* contributed to higher pressure resistance of spores. Overall, we found that the *spoVA*^{2mob} operon contributes to pressure resistance of *Bacillus* endospores, improving. This finding improved the understanding of pressure resistance mechanisms in *Bacillus* spp. and informed the development of high-pressure sterilization in food processing.

Nevertheless, limited differences were observed in the isogenic strain with the insertion of *spoVA*^{2mob} operon on chromosome, which responded to high pressure, heating and different nutrients induced germination of spores (Chapter 6). According to previous research, *Bacillus* strains with multiple copies (two or three) of the *spoVA*^{2mob} operons on chromosome exhibit significantly higher heat or pressure resistance (Berendsen et al., 2016a, and Chapter 5). Therefore, isogenic mutant strains with two and three *spoVA*^{2mob} operons will be more helpful to investigate the role and mechanisms of *spoVA*^{2mob} in heat/pressure resistance and germination of spores. However, it is extremely difficult to create mutant strains with two or three copies of the *spoVA*^{2mob} operon due to the large size of the operon (around 4 kb) and technical reasons. In our study, the mutant strain with two copies of *spoVA*^{2mob} was successfully created and isolated two times. However, after subculture and stocking at −80 °C, the strains in the stock lost the two copies of *spoVA*^{2mob} operon. This may be due to the impurity of the strains in stock, which is a mixture of mutant and wild type strains. During the freezing process or the re-streaking process, the wild-type strain of *Bacillus* overcame the mutant strain and became dominant. In future studies, efforts are still to be made to create mutants with multiple copy numbers of *spoVA*^{2mob} operon. Additionally,

with the creation of isogenic strains, the variation of DPA content in the isogenic strains needs to be compared to help better understand the mechanisms of pressure resistance and spore germination in the future.

Bibliography

Abee, T., Groot, M. N., Tempelaars, M., Zwietering, M., Moezelaar, R., and Voort, M. van der (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: diversity and role of germinant receptors. *Food Microbiol* 28, 199–208. doi: 10.1016/J.FM.2010.03.015.

Achi, O. K., and Akubor, P. I. (2000). Microbiological characterization of yam fermentation for 'Elubo' (yam flour) production. *World Journal of Microbiology and Biotechnology 2000 16:1* 16, 3–7. doi: 10.1023/A:1008980912708.

Ahaotu, I., Anyogu, A., Njoku, O. H., Odu, N. N., Sutherland, J. P., and Ouoba, L. I. I. (2013). Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. *Int J Food Microbiol* 162, 95–104. doi: 10.1016/J.IJFOODMICRO.2013.01.001.

Albertini, A. M., and Galizzi, A. (1999). The sequence of the *trp* operon of *Bacillus subtilis* 168 (*trpC2*) revisited. *Microbiology (N Y)* 145, 3319–3320. doi: 10.1099/00221287-145-12-3319.

Aldhous, M. C., Bakar, S. A., Prescott, N. J., Palla, R., Soo, K., Mansfield, J. C., et al. (2010). Measurement methods and accuracy in copy number variation: failure to replicate associations of beta-defensin copy number with Crohn's disease. *Hum Mol Genet* 19, 4930–4938. doi: 10.1093/hmg/ddq411.

Alitubeera, P. H., Eyu, P., Kwesiga, B., Ario, A. R., and Zhu, B.-P. (2019). Outbreak of cyanide poisoning caused by consumption of cassava flour-Kasese District, Uganda, September 2017. *Morbidity and Mortality Weekly Report* 68, 308–311. doi: 10.15585/MMWR.MM6813A3.

Altenbuchner, J. (2016). Editing of the *Bacillus subtilis* genome by the CRISPR-Cas9 system. *Appl Environ Microbiol* 82, 5421–5427. doi: 10.1128/AEM.01453-16.

Álvarez-Cao, M. E., Cerdán, M. E., González-Siso, M. I., and Becerra, M. (2019). Optimization of *Saccharomyces cerevisiae* α-galactosidase production and application in the degradation of raffinose family oligosaccharides. *Microb Cell Fact* 18, 1–17. doi: 10.1186/S12934-019-1222-X/FIGURES/9.

Amoa-Awua, W. K. A., and Jakobsen, M. (1995). The role of *Bacillus* species in the fermentation of cassava. *Journal of Applied Bacteriology* 79, 250–256. doi: 10.1111/J.1365-2672.1995.TB03134.X.

André, S., Vallaeys, T., and Planchon, S. (2017). Spore-forming bacteria responsible for food spoilage. *Res Microbiol* 168, 379–387. doi: 10.1016/j.resmic.2016.10.003.

Anonymous (2022a). Bread market - growth, trends, COVID-19 impact, and forecasts (2022 - 2027). Available at: https://www.mordorintelligence.com/industry-reports/bread-market [Accessed July 29, 2022].

Anonymous (2022b). Sourdough market - growth, trends, COVID-19 impact, and forecasts (2022 - 2027). Available at: https://www.mordorintelligence.com/industry-reports/sourdough-market [Accessed July 29, 2022].

Aranda, F. J., Teruel, J. A., and Ortiz, A. (2005). Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. *Biochim Biophys Acta* 1713, 51–56. doi: 10.1016/J.BBAMEM.2005.05.003.

Arslan, S., Eyi, A., and Küçüksari, R. (2014). Toxigenic genes, spoilage potential, and antimicrobial resistance of *Bacillus cereus* group strains from ice cream. *Anaerobe* 25, 42–46. doi: 10.1016/J.ANAEROBE.2013.11.006.

Ashiuchi, M., Kamei, T., Baek, D. H., Shin, S. Y., Sung, M. H., Soda, K., et al. (2001). Isolation of *Bacillus subtilis* (chungkookjang), a poly-γ-glutamate producer with high genetic competence. *Applied Microbiology and Biotechnology 2001 57:5* 57, 764–769. doi: 10.1007/S00253-001-0848-9.

Atluri, S., Ragkousi, K., Cortezzo, D. E., and Setlow, P. (2006). Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. *J Bacteriol* 188, 28–36. doi: 10.1128/JB.188.1.28-36.2006/FORMAT/EPUB.

Aulitto, M., Strazzulli, A., Sansone, F., Cozzolino, F., Monti, M., Moracci, M., et al. (2021). Prebiotic properties of *Bacillus coagulans* MA-13: production of galactoside hydrolyzing enzymes and characterization of the transglycosylation properties of a GH42 β-galactosidase. *Microb Cell Fact* 20, 1–17. doi: 10.1186/S12934-021-01553-Y/TABLES/4.

Axel, C., Zannini, E., and Arendt, E. K. (2017). Mold spoilage of bread and its biopreservation: A review of current strategies for bread shelf life extension. *Crit Rev Food Sci Nutr* 57, 3528–3542. doi: 10.1080/10408398.2016.1147417.

Bader, J., Albin, A., and Stahl, U. (2012). Spore-forming bacteria and their utilisation as probiotics. *Benef Microbes* 3, 67–75. doi: 10.3920/BM2011.0039.

Bailey, C. P., and von Holy, A. (1993). *Bacillus* spore contamination associated with commercial bread manufacture. *Food Microbiol* 10, 287–294. doi: 10.1006/fmic.1993.1033.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology* 19, 455–477. doi: 10.1089/cmb.2012.0021.

Barnes, S. (2010). The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. *Lymphat Res Biol* 8, 89. doi: 10.1089/LRB.2009.0030.

Barus, T., Kristani, A., and Yulandi, A. D. I. (2013). Diversity of amylase-producing *Bacillus* spp. from "Tape" (fermented cassava). *Hayati* 20, 94–98. doi: 10.4308/HJB.20.2.94.

Barus, T., and Wijaya, L. N. (2011). Mikrobiota dominan dan perannya dalam cita rasa tape singkong. *Biota : Jurnal Ilmiah Ilmu-Ilmu Hayati* 16, 354–561. doi: 10.24002/BIOTA.V16I2.119.

Berendsen, E. M. (2016). Heat resistance of *Bacillus* spores: natural variation and genomic adaptation. *Rijksuniversiteit Groningen*.

Berendsen, E. M., Boekhorst, J., Kuipers, O. P., and Wells-Bennik, M. H. J. (2016a). A mobile genetic element profoundly increases heat resistance of bacterial spores. *ISME J* 10, 2633–2642. doi: 10.1038/ismej.2016.59.

Berendsen, E. M., Koning, R. A. A., Boekhorst, J., de Jong, A., Kuipers, O. P. P., and Wells-Bennik, M. H. J. H. J. (2016b). High-level heat resistance of spores of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* results from the presence of a spoVA operon in a Tn1546 transposon. *Front Microbiol* 7, 1912. doi: 10.3389/fmicb.2016.01912.

Bhatia, S., Singh, A., Batra, N., and Singh, J. (2020). Microbial production and biotechnological applications of α-galactosidase. *Int J Biol Macromol* 150, 1294–1313. doi: 10.1016/J.IJBIOMAC.2019.10.140.

Black, E. P., Koziol-Dube, K., Guan, D., Wei, J., Setlow, B., Cortezzo, D. E., et al. (2005). Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. *Appl Environ Microbiol* 71, 5879–5887. doi: 10.1128/AEM.71.10.5879-5887.2005/FORMAT/EPUB.

Black, E. P., Wei, J., Atluri, S., Cortezzo, D. E., Koziol-Dube, K., Hoover, D. G., et al. (2007). Analysis of factors influencing the rate of germination of spores of *Bacillus subtilis* by very high pressure. *J Appl Microbiol* 102, 65–76. doi: 10.1111/j.1365-2672.2006.03062.x.

Blin, K., Shaw, S., Kloosterman, A. M., Charlop-Powers, Z., van Wezel, G. P., Medema, M. H., et al. (2021). antiSMASH 6.0: improving cluster detection and comparison capabilities. *Nucleic Acids Res* 49, W29–W35. doi: 10.1093/NAR/GKAB335.

Bosi, E., Donati, B., Galardini, M., Brunetti, S., Sagot, M.-F., Lió, P., et al. (2015). MeDuSa: a multi-draft based scaffolder. *Bioinformatics* 31, 2443–2451. doi: 10.1093/bioinformatics/btv171.

Brandt, M. J. (2007). Sourdough products for convenient use in baking. *Food Microbiol* 24, 161–164. doi: 10.1016/j.fm.2006.07.010.

Bravo, A., Likitvivatanavong, S., Gill, S. S., and Soberón, M. (2011). *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochem Mol Biol* 41, 423. doi: 10.1016/J.IBMB.2011.02.006.

Brynildsrud, O., Snipen, L. G., and Bohlin, J. (2015). CNOGpro: detection and quantification of CNVs in prokaryotic whole-genome sequencing data. *Bioinformatics* 31, 1708–1715. doi: 10.1093/bioinformatics/btv070.

Budhwar, S., Sethi, K., and Chakraborty, M. (2020). Efficacy of germination and probiotic fermentation on underutilized cereal and millet grains. *Food Production, Processing and Nutrition* 2, 1–17. doi: 10.1186/S43014-020-00026-W/FIGURES/2.

Cairns, J. R. K., and Esen, A. (2010). *β*-Glucosidases. *Cellular and Molecular Life Sciences* 67, 3389–3405. doi: 10.1007/S00018-010-0399-2.

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., and Mahillon, J. (2019). Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. *Front Microbiol* 10, 302. doi: 10.3389/FMICB.2019.00302/BIBTEX.

Chandrasekara, A., and Josheph Kumar, T. (2016). Roots and tuber crops as functional foods: a review on phytochemical constituents and their potential health benefits. *Int J Food Sci* 2016. doi: 10.1155/2016/3631647.

Chen, X. H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., et al. (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat Biotechnol* 25, 1007–1014. doi: 10.1038/nbt1325.

Chen, X. Y., Levy, C., and Gänzle, M. (2016). Structure-function relationships of bacterial and enzymatically produced reuterans and dextran in sourdough bread baking application. *Int J Food Microbiol* 239, 95–102. doi: 10.1016/j.ijfoodmicro.2016.06.010.

Chen, Y., Gu, F., Li, J., He, S., Xu, F., and Fang, Y. (2015). Involvement of colonizing *Bacillus* isolates in glucovanillin hydrolysis during the curing of *Vanilla planifolia* Andrews. *Appl Environ Microbiol* 81, 4947–4954. doi: 10.1128/AEM.00458-15/SUPPL_FILE/ZAM999116413SO1.PDF.

Chen, Y., Li, K., Liu, T., Li, R., Fu, G., Wan, Y., et al. (2021). Analysis of difference in microbial community and physicochemical indices between surface and central parts of Chinese special-flavor *Baijiu daqu*. *Front Microbiol* 11, 3340. doi: 10.3389/FMICB.2020.592421/BIBTEX.

Chen, Z., Wu, Q., Wang, L., Chen, S., Lin, L., Wang, H., et al. (2020). Identification and quantification of surfactin, a nonvolatile lipopeptide in Moutai liquor. *Int J Food Prop* 23, 189–198. doi: 10.1080/10942912.2020.1716791/SUPPL_FILE/LJFP_A_1716791_SM3052.DOCX.

Chetrariu, A., and Dabija, A. (2021). Spent grain from malt Whisky: assessment of the phenolic compounds. *Molecules 2021, Vol. 26, Page 3236* 26, 3236. doi: 10.3390/MOLECULES26113236.

Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S. J., and Moir, A. (2002). Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endoscope germination. *Microbiology (N Y)* 148, 2383–2392. doi: 10.1099/00221287-148-8-2383/CITE/REFWORKS.

Cho, K. M., Math, R. K., Hong, S. Y., Asraful Islam, S. M., Mandanna, D. K., Cho, J. J., et al. (2009). Iturin produced by *Bacillus pumilus* HY1 from Korean soybean sauce (*kanjang*) inhibits growth of aflatoxin producing fungi. *Food Control* 20, 402–406. doi: 10.1016/J.FOODCONT.2008.07.010.

Cochrane, S. A., and Vederas, J. C. (2016). Lipopeptides from *Bacillus* and *Paenibacillus* spp.: a gold mine of antibiotic candidates. *Med Res Rev* 36, 4–31. doi: 10.1002/MED.21321.

Coorevits, A., de Jonghe, V., Vandroemme, J., van Landschoot, A., Heyndrickx, M., and de Vos, P. (2010). "How can the type of dairy farming influence the bacterial flora in milk?," in

Organic farming and peanut crops, eds. D. C. Grossman and T. L. Barrios (New York: Nova), 123–136.

Corsetti, A., Gobbetti, M., De Marco, B., Balestrieri, F., Paoletti, F., Russi, L., et al. (2000). Combined effect of sourdough lactic acid bacteria and additives bread firmness and staling. *J Agric Food Chem* 48, 3044–3051. doi: 10.1021/jf990853e.

Curtis, R. I. (2009). Umami and the foods of classical antiquity. *Am J Clin Nutr* 90, 712S-718S. doi: 10.3945/AJCN.2009.27462C.

Dakwa, S., Sakyi-Dawson, E., Diako, C., Annan, N. T., and Amoa-Awua, W. K. (2005). Effect of boiling and roasting on the fermentation of soybeans into dawadawa (soy-dawadawa). *Int J Food Microbiol* 104, 69–82. doi: 10.1016/J.IJFOODMICRO.2005.02.006.

Danilova, I., and Sharipova, M. (2020). The practical potential of bacilli and their enzymes for industrial production. *Front Microbiol* 11, 1782. doi: 10.3389/FMICB.2020.01782/BIBTEX.

de Jonghe, V., Coorevits, A., de Block, J., van Coillie, E., Grijspeerdt, K., Herman, L., et al. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *Int J Food Microbiol* 136, 318–325. doi: 10.1016/J.IJFOODMICRO.2009.11.007.

Delbrück, A. I., Zhang, Y., Heydenreich, R., and Mathys, A. (2021a). *Bacillus* spore germination at moderate high pressure: a review on underlying mechanisms, influencing factors, and its comparison with nutrient germination. *Compr Rev Food Sci Food Saf* 20, 4159–4181. doi: 10.1111/1541-4337.12789.

Delbrück, A. I., Zhang, Y., Hug, V., Trunet, C., and Mathys, A. (2021b). Isolation, stability, and characteristics of high-pressure superdormant *Bacillus subtilis* spores. *Int J Food Microbiol* 343, 109088. doi: 10.1016/J.IJFOODMICRO.2021.109088.

Deleu, M., Paquot, M., and Nylander, T. (2005). Fengycin interaction with lipid monolayers at the air–aqueous interface—implications for the effect of fengycin on biological membranes. *J Colloid Interface Sci* 283, 358–365. doi: 10.1016/J.JCIS.2004.09.036.

den Besten, H. M. W., Wells-Bennik, M. H. J., and Zwietering, M. H. (2018). Natural diversity in heat resistance of bacteria and bacterial spores: impact on food safety and quality. *Annu Rev Food Sci Technol* 9, 383–410. doi: 10.1146/annurev-food-030117-012808.

Deng, Y., Huang, D., Han, B., Ning, X., Yu, D., Guo, H., et al. (2021). Correlation: between autochthonous microbial diversity and volatile metabolites during the fermentation of Nongxiang Daqu. *Front Microbiol* 12, 2117. doi: 10.3389/FMICB.2021.688981/BIBTEX.

di Stefano, M., Miceli, E., Gotti, S., Missanelli, A., Mazzocchi, S., and Corazza, G. R. (2007). The effect of oral α-galactosidase on intestinal gas production and gas-related symptoms. *Dig Dis Sci* 52, 78–83. doi: 10.1007/S10620-006-9296-9/FIGURES/3.

Digaitiene, A., Hansen, Å. S., Juodeikiene, G., Eidukonyte, D., and Josephsen, J. (2012). Lactic acid bacteria isolated from rye sourdoughs produce bacteriocin-like inhibitory substances active against *Bacillus subtilis* and fungi. *J Appl Microbiol* 112, 742. doi: 10.1111/j.1365-2672.2012.05249.x.

Doona, C. J., Feeherry, F. E., Setlow, B., Wang, S., Li, W., Nichols, F. C., et al. (2016). Effects of high-pressure treatment on spores of *Clostridium* species. *Appl Environ Microbiol* 82, 5287–5297. doi: 10.1128/AEM.01363-16.

Du, F., Zhang, X., Gu, H., Song, J., and Gao, X. (2019). Dynamic changes in the bacterial community during the fermentation of traditional Chinese fish sauce (TCFS) and their

correlation with TCFS quality. *Microorganisms* 7. doi: 10.3390/MICROORGANISMS7090371.

Dunlap, C. A., Schisler, D. A., Price, N. P., and Vaughn, S. F. (2011). Cyclic lipopeptide profile of three *Bacillus subtilis* strains; antagonists of *Fusarium* head blight. *The Journal of Microbiology 2011 49:4* 49, 603–609. doi: 10.1007/S12275-011-1044-Y.

EFSA Panel on Biological Hazards (BIOHAZ) (2016). Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs. *EFSA Journal* 14. doi: 10.2903/J.EFSA.2016.4524.

Ehling-Schulz, M., Frenzel, E., and Gohar, M. (2015). Food-bacteria interplay: pathometabolism of emetic *Bacillus cereus*. *Front Microbiol* 6, 704. doi: 10.3389/FMICB.2015.00704/BIBTEX.

Ehling-Schulz, M., Koehler, T. M., and Lereclus, D. (2019). The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. *Microbiol Spectr* 7. doi: 10.1128/MICROBIOLSPEC.GPP3-0032-2018.

Ehling-Schulz, M., and Messelhäusser, U. (2014). "The genus *Bacillus*," in *DNA Methods in Food Safety: Molecular Typing of Foodborne and Waterborne Bacterial Pathogens*, eds. O. A. Oyarzabal and S. Kathariou (John Wiley & Sons, Ltd), 163–184. doi: 10.1002/9781118278666.CH8.

Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Märtlbauer, E., et al. (2005). Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Appl Environ Microbiol* 71, 105–113. doi: 10.1128/AEM.71.1.105-113.2005.

Elshaghabee, F. M. F., Rokana, N., Gulhane, R. D., Sharma, C., and Panwar, H. (2017). *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Front Microbiol* 8. doi: 10.3389/fmicb.2017.01490.

Fan, B., Chen, X. H., Budiharjo, A., Bleiss, W., Vater, J., and Borriss, R. (2011). Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. *J Biotechnol* 151, 303–311. doi: 10.1016/j.jbiotec.2010.12.022.

Fangio, M. F., Roura, S. I., and Fritz, R. (2010). Isolation and identification of *Bacillus* spp. and related genera from different starchy foods. *J Food Sci* 75, 218–221. doi: 10.1111/j.1750-3841.2010.01566.x.

Farkas, D. F. (2016). "A short history of research and development efforts leading to the commercialization of high-pressure processing of food," in *High Pressure Processing of Food* (Springer, New York, NY), 19–36. doi: 10.1007/978-1-4939-3234-4_2.

Feng, Z., Gao, W., Ren, D., Chen, X., and Li, J. (2013). Evaluation of bacterial flora during the ripening of Kedong sufu, a typical Chinese traditional bacteria-fermented soybean product. *J Sci Food Agric* 93, 1471–1478. doi: 10.1002/JSFA.5918.

Ferreira, S. da C., Nakasone, A. K., do Nascimento, S. M. C., de Oliveira, D. A., Siqueira, A. S., Cunha, E. F. M., et al. (2021). Isolation and characterization of cassava root endophytic bacteria with the ability to promote plant growth and control the *in vitro* and *in vivo* growth of *Phytopythium* sp. *Physiol Mol Plant Pathol* 116, 101709. doi: 10.1016/J.PMPP.2021.101709.

Food and Agricultural Organization of the United Nations and World Health Organization (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. *World Health Organization*.

Gänzle, M. (2014). Enzymatic and bacterial conversions during sourdough fermentation. *Food Microbiol* 37, 2–10. doi: 10.1016/j.fm.2013.04.007.

Gänzle, M. (2019). "Fermented foods," in *Food Microbiology: Fundamentals and Frontiers*, eds. M. P. Michael P. Doyle, F. Diez-Gonzalez, and C. Hill (wiley), 855–900. doi: 10.1128/9781555819972.ch33.

Gänzle, M. (2022). The periodic table of fermented foods: limitations and opportunities. *Appl Microbiol Biotechnol* 106, 2815–2826. doi: 10.1007/S00253-022-11909-Y.

Gänzle, M., Höltzel, A., Walter, J., Jung, G., and Hammes, W. P. (2000). Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. *Appl Environ Microbiol* 66, 4325–4333. doi: 10.1128/AEM.66.10.4325-4333.2000.

Gänzle, M., and Vogel, R. F. (2003). Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. *Int J Food Microbiol* 80, 31–45. doi: 10.1016/S0168-1605(02)00146-0.

Glasset, B., Herbin, S., Guillier, L., Cadel-Six, S., Vignaud, M., Grout, J., et al. (2016). *Bacillus cereus*-induced food-borne outbreaks in France, 2007 to 2014: epidemiology and genetic characterisation. *Euro surveillance* 21, 1–11. doi: 10.2807/1560-7917.

Go, J., Kim, J. E., Kwak, M. H., Koh, E. K., Song, S. H., Sung, J. E., et al. (2016). Neuroprotective effects of fermented soybean products (*Cheonggukjang*) manufactured by mixed culture of

Bacillus subtilis MC31 and *Lactobacillus sakei* 383 on trimethyltin-induced cognitive defects mice. *Nutr Neurosci* 19, 247–259. doi: 10.1179/1476830515Y.0000000025.

Gordillo, A., and Maldonado, M. C. (2012). "Purification of peptides from *Bacillus* strains with biological activity," in *Chromatography and Its Applications* (InTech), 201–225. doi: 10.5772/36906.

Griffiths, M. W., and Schraft, H. (2017). "*Bacillus cereus* food poisoning," in *Foodborne Diseases* (Academic Press), 395–405. doi: 10.1016/B978-0-12-385007-2.00020-6.

Gu, F., Chen, Y., Fang, Y., Wu, G., and Tan, L. (2015). Contribution of *Bacillus* isolates to the flavor profiles of vanilla beans assessed through aroma analysis and chemometrics. *Molecules 2015, Vol. 20, Pages 18422-18436* 20, 18422–18436. doi: 10.3390/MOLECULES201018422.

Hansen, A., and Schieberle, P. (2005). Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. *Trends Food Sci Technol* 16, 85–94. doi: 10.1016/J.TIFS.2004.03.007.

Harwood, C. R., Mouillon, J. M., Pohl, S., and Arnau, J. (2018). Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. *FEMS Microbiol Rev* 42, 721–738. doi: 10.1093/FEMSRE/FUY028.

Hayden, R. T., Gu, Z., Ingersoll, J., Abdul-Ali, D., Shi, L., Pounds, S., et al. (2013). Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. *J Clin Microbiol* 51, 540–546. doi: 10.1128/JCM.02620-12.

Hayrapetyan, H., Boekhorst, J., Jong, A. de, Kuipers, O. P., Groot, M. N. N., and Abee, T. (2016). Draft whole-genome sequences of 11 *Bacillus cereus* food isolates. *Genome Announc* 4, 1–2. doi: 10.1128/genomeA.00485-16.

He, G., Huang, J., Wu, C., Jin, Y., and Zhou, R. (2020). Bioturbation effect of fortified Daqu on microbial community and flavor metabolite in Chinese strong-flavor liquor brewing microecosystem. *Food Research International* 129, 108851. doi: 10.1016/J.FOODRES.2019.108851.

He, G., Huang, J., Zhou, R., Wu, C., and Jin, Y. (2019). Effect of fortified *daqu* on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Front Microbiol* 10, 56. doi: 10.3389/FMICB.2019.00056/BIBTEX.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 11, 506–514. doi: 10.1038/NRGASTRO.2014.66.

Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., et al. (2011). High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem* 83, 8604–8610. doi: 10.1021/ac202028g.

Hindson, C. M., Chevillet, J. R., Briggs, H. A., Gallichotte, E. N., Ruf, I. K., Hindson, B. J., et al. (2013). Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods* 10, 1003–1008. doi: 10.1038/nmeth.2633.

Hitosugi, M., Hamada, K., and Misaka, K. (2015). Effects of *Bacillus subtilis* var. *natto* products on symptoms caused by blood flow disturbance in female patients with lifestyle diseases. *Int J Gen Med* 8, 41. doi: 10.2147/IJGM.S76588.

Hofstetter, S., Gebhardt, D., Ho, L., Gänzle, M., and McMullen, L. M. (2013a). Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. *Food Microbiol* 34, 46–51. doi: 10.1016/J.FM.2012.11.001.

Hofstetter, S., Winter, R., McMullen, L. M., and Gänzle, G. (2013b). *In situ* determination of *Clostridium* endospore membrane fluidity during pressure-assisted thermal processing in combination with nisin or reutericyclin. *Appl Environ Microbiol* 79, 2103–2106. doi: 10.1128/AEM.03755-12.

Holdsworth, S. D., and Simpson, R. (2016). "High-pressure thermal sterilization (HPTS) and ohmic heating (OH) applied to thermal food processing," in *Thermal Processing of Packaged Foods* (Cham: Springer International), 457–466. doi: 10.1007/978-3-319-24904-9_22.

Hsueh, Y. H., Huang, K. Y., Kunene, S. C., and Lee, T. Y. (2017). Poly-γ-glutamic acid synthesis, gene regulation, phylogenetic relationships, and role in fermentation. *International Journal of Molecular Sciences 2017, Vol. 18, Page 2644* 18, 2644. doi: 10.3390/IJMS18122644.

Hu, Y., Ge, C., Yuan, W., Zhu, R., Zhang, W., Du, L., et al. (2010). Characterization of fermented black soybean natto inoculated with *Bacillus natto* during fermentation. *J Sci Food Agric* 90, 1194–1202. doi: 10.1002/jsfa.3947.

Huang, T.-P., Tzeng, D. D.-S., Wong, A. C. L., Chen, C.-H., Lu, K.-M., Lee, Y.-H., et al. (2012). DNA polymorphisms and biocontrol of *Bacillus antagonistic* to citrus bacterial canker with

indication of the interference of phyllosphere biofilms. *PLoS One* 7, 1–11. doi: 10.1371/journal.pone.0042124.

Huang, Y., Yi, Z., Jin, Y., Huang, M., He, K., Liu, D., et al. (2017). Metatranscriptomics reveals the functions and enzyme profiles of the microbial community in Chinese Nong-flavor liquor starter. *Front Microbiol* 8, 1747. doi: 10.3389/FMICB.2017.01747/BIBTEX.

Im, S. M., Yu, N. H., Joen, H. W., Kim, S. O., Park, H. W., Park, A. R., et al. (2020). Biological control of tomato bacterial wilt by oxydifficidin and difficidin-producing *Bacillus methylotrophicus* DR-08. *Pestic Biochem Physiol* 163, 130–137. doi: 10.1016/J.PESTBP.2019.11.007.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., et al. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature* 423, 87–91. doi: 10.1038/nature01582.

Jamal, Q., Monkhung, S., Munir, S., Cho, J. Y., Moon, J. H., Khattak, B. U., et al. (2019). Identification of cyclo(L-Pro-D-Tyr) from *Bacillus amyloliquefaciens* Y1 exhibiting antifungal activity against *Fusarium graminearum* to control crown rot in wheat. *Appl Ecol Environ Res* 17, 6299–6314. doi: 10.15666/aeer/1703_62996314.

Jang, C. H., Oh, J., Lim, J. S., Kim, H. J., and Kim, J. S. (2021). Fermented soy products: beneficial potential in neurodegenerative diseases. *Foods* 10, 636. doi: 10.3390/FOODS10030636.

Jasim, B., Sreelakshmi, K. S., Mathew, J., and Radhakrishnan, E. K. (2016). Surfactin, iturin, and fengycin biosynthesis by endophytic *Bacillus* sp. from *Bacopa monnieri*. *Microb Ecol* 72, 106–119. doi: 10.1007/S00248-016-0753-5.

Jeyaram, K., Mohendro Singh, W., Premarani, T., Devi, A. R., Chanu, K. S., Talukdar, N. C., et al. (2008). Molecular identification of dominant microflora associated with 'Hawaijar' – a traditional fermented soybean (*Glycine max* (L.)) food of Manipur, India. *Int J Food Microbiol* 122, 259–268. doi: 10.1016/J.IJFOODMICRO.2007.12.026.

Jimenez, M. E., O'Donovan, C. M., Ullivarri, M. F. de, and Cotter, P. D. (2022). Microorganisms present in artisanal fermented food from South America. *Front Microbiol* 0, 3332. doi: 10.3389/FMICB.2022.941866.

Jin, F., Cheng, X., Shi, Y., and Zhang, C. (1990). Isolation of new thermophilic aerobic bacteria which produce thermostable α-amylase. *J Gen Appl Microbiol* 36, 415–424. doi: 10.2323/JGAM.36.415.

Jin, G., Zhu, Y., and Xu, Y. (2017). Mystery behind Chinese liquor fermentation. *Trends Food Sci Technol* 63, 18–28. doi: 10.1016/J.TIFS.2017.02.016.

Jo, Y. J., Cho, I. H., Song, C. K., Shin, H. W., and Kim, Y. S. (2011). Comparison of fermented soybean paste (*Doenjang*) prepared by different methods based on profiling of volatile compounds. *J Food Sci* 76, C368–C379. doi: 10.1111/J.1750-3841.2011.02068.X.

Juck, D., Charles, T., Whyte, L. G., and Greer, C. W. (2000). Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. *FEMS Microbiol Ecol* 33, 241–249. doi: 10.1111/J.1574-6941.2000.TB00746.X.

Kada, S., Ishikawa, A., Ohshima, Y., and Yoshida, K. I. (2013). Alkaline serine protease AprE plays an essential role in poly-γ-glutamate production during natto fermentation. *Biosci Biotechnol Biochem* 77, 120965. doi: 10.1271/BBB.120965.

Kada, S., Yabusaki, M., Kaga, T., Ashida, H., and Yoshida, K. I. (2008). Identification of two major ammonia-releasing reactions involved in secondary natto fermentation. *Biosci Biotechnol Biochem* 72, 0806060936–0806060936. doi: 10.1271/BBB.80129.

Kanaan, J., Murray, J., Higgins, R., Nana, M., DeMarco, A. M., Korza, G., et al. (2022). Resistance properties and the role of the inner membrane and coat of *Bacillus subtilis* spores with extreme wet heat resistance. *J Appl Microbiol* 132, 2157–2166. doi: 10.1111/JAM.15345.

Katina, K., Sauri, M., Alakomi, H. L., and Mattila-Sandholm, T. (2002). Potential of lactic acid bacteria to inhibit rope spoilage in wheat sourdough bread. *LWT - Food Science and Technology* 35, 38–45. doi: 10.1006/fstl.2001.0808.

Kaur, B., and Chakraborty, D. (2013). Biotechnological and molecular approaches for vanillin production: a review. *Appl Biochem Biotechnol* 169, 1353–1372. doi: 10.1007/S12010-012-0066-1/FIGURES/3.

Keishing, S., and Banu, A. T. (2013). Hawaijar-a fermented soya of Manipur, India: review. *IOSR J Environ Sci Toxicol Food Technol* 4, 29–33. doi: 10.9790/2402-0422933.

Khan, N., Martínez-Hidalgo, P., Ice, T. A., Maymon, M., Humm, E. A., Nejat, N., et al. (2018). Antifungal activity of *Bacillus* species against fusarium and analysis of the potential mechanisms used in biocontrol. *Front Microbiol* 9, 2363. doi: 10.3389/FMICB.2018.02363/BIBTEX.

Kim, H. E., Han, S. Y., and Kim, Y. S. (2010). Quality characteristics of *gochujang meju* prepared with different fermentation tools and inoculation time of *Aspergillus oryzae*. *Food Science and Biotechnology 2010 19:6* 19, 1579–1585. doi: 10.1007/S10068-010-0224-6.

Kim, J.-G. (2007). Anti-aflatoxigenic activity of some bacteria related with fermentation. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* 1, 322–328.

Konuray, G., and Erginkaya, Z. (2018). Potential use of *Bacillus coagulans* in the food industry. *Foods 2018, Vol. 7, Page 92* 7, 92. doi: 10.3390/FOODS7060092.

Kort, R., O'Brien, A. C., Stokkum, I. H. M. van, Oomes, S. J. C. M. J. C. M., Crielaard, W., Hellingwerf, K. J. J., et al. (2005). Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. *Appl Environ Microbiol* 71, 3556–3564. doi: 10.1128/AEM.71.7.3556.

Koumoutsi, A., Chen, X. H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., et al. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J Bacteriol* 186, 1084. doi: 10.1128/JB.186.4.1084-1096.2004.

Kourkouta, L., Koukourikos, K., and Tsaloglidou (2017). Bread and health. *Journal of Pharmacy and Pharmacology* 5, 821–826. doi: 10.17265/2328-2150/2017.11.005.

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., et al. (2020). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). *EFSA Journal* 18. doi: 10.2903/J.EFSA.2020.5966.

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., et al. (2022). Update of the list of QPS-recommended biological agents intentionally added to

food or feed as notified to EFSA 15: suitability of taxonomic units notified to EFSA until September 2021. *EFSA Journal* 20, e07045. doi: 10.2903/J.EFSA.2022.7045.

Krawczyk, A. O., Berendsen, E. M., de Jong, A., Boekhorst, J., Wells-Bennik, M. H. J., Kuipers, O. P., et al. (2016). A transposon present in specific strains of *Bacillus subtilis* negatively affects nutrient- and dodecylamine-induced spore germination. *Environ Microbiol* 18, 4830–4846. doi: 10.1111/1462-2920.13386.

Krawczyk, A. O., de Jong, A., Omony, J., Holsappel, S., Wells-Bennik, M. H. J., Kuipers, O. P., et al. (2017). Spore heat activation requirements and germination responses correlate with sequences of germinant receptors and with the presence of a specific *spoVA*^{2mob} operon in foodborne strains of *Bacillus subtilis*. *Appl Environ Microbiol* 83. doi: 10.1128/AEM.03122-16/FORMAT/EPUB.

Kumari, S., and Sarkar, P. K. (2016). *Bacillus cereus* hazard and control in industrial dairy processing environment. *Food Control* 69, 20–29. Available at: http://dx.doi.org/10.1016/j.foodcont.2016.04.012.

la Duc, M. T., Satomi, M., Agata, N., and Venkateswaran, K. (2004). *gyrB* as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. *J Microbiol Methods* 56, 383–394. doi: 10.1016/J.MIMET.2003.11.004.

Ledenbach, L. H., and Marshall, R. T. (2009). "Microbiological spoilage of dairy products," in *Compendium of the Microbiological Spoilage of Foods and Beverages. Food Microbiology and Food Safety*, eds. W. , Sperber and M. Doyle (Springer, New York, NY), 41–67. doi: 10.1007/978-1-4419-0826-1_2.

Lee, J. H., Nam, S. H., Seo, W. T., Yun, H. D., Hong, S. Y., Kim, M. K., et al. (2012). The production of surfactin during the fermentation of *cheonggukjang* by potential probiotic *Bacillus subtilis* CSY191 and the resultant growth suppression of MCF-7 human breast cancer cells. *Food Chem* 131, 1347–1354. doi: 10.1016/J.FOODCHEM.2011.09.133.

Lee, J. Y., Shim, J. M., Yao, Z., Liu, X., Lee, K. W., Kim, H. J., et al. (2016a). Antimicrobial activity of *Bacillus amyloliquefaciens* EMD17 isolated from *Cheonggukjang* and potential use as a starter for fermented soy foods. *Food Science and Biotechnology 2016 25:2* 25, 525–532. doi: 10.1007/S10068-016-0073-Z.

Lee, M. H., Lee, J., Nam, Y. do, Lee, J. S., Seo, M. J., and Yi, S. H. (2016b). Characterization of antimicrobial lipopeptides produced by *Bacillus* sp. LM7 isolated from *chungkookjang*, a Korean traditional fermented soybean food. *Int J Food Microbiol* 221, 12–18. doi: 10.1016/J.IJFOODMICRO.2015.12.010.

Li, C., Xu, T., Liu, X. W., Wang, X., and Xia, T. (2021a). The expression of β-glucosidase during natto fermentation increased the active isoflavone content. *Food Biosci* 43, 101286. doi: 10.1016/J.FBIO.2021.101286.

Li, P., Aflakpui, F. W. K., Yu, H., Luo, L., and Lin, W.-T. (2015a). Characterization of activity and microbial diversity of typical types of Daqu for traditional Chinese vinegar. *Ann Microbiol* 65, 2019–2027.

Li, P., Liang, H., Lin, W.-T., Feng, F., and Luo, L. (2015b). Microbiota dynamics associated with environmental conditions and potential roles of cellulolytic communities in traditional Chinese cereal starter solid-state fermentation. *Appl Environ Microbiol* 81, 5144–5156. doi: 10.1128/AEM.01325-15.

Li, P., Lin, W., Liu, X., Wang, X., Gan, X., Luo, L., et al. (2017). Effect of bioaugmented inoculation on microbiota dynamics during solid-state fermentation of Daqu starter using autochthonous of *Bacillus*, *Pediococcus*, *Wickerhamomyces* and *Saccharomycopsis*. *Food Microbiol* 61, 83–92. doi: 10.1016/J.FM.2016.09.004.

Li, P., Lin, W., Liu, X., Wang, X., and Luo, L. (2016). Environmental factors affecting microbiota dynamics during traditional solid-state fermentation of Chinese daqu starter. *Front Microbiol* 7, 1237. doi: 10.3389/fmicb.2016.01237.

Li, R. Y., Zheng, X. W., Zhang, X., Yan, Z., Wang, X. Y., and Han, B. Z. (2018). Characterization of bacteria and yeasts isolated from traditional fermentation starter (Fen-Daqu) through a 1 H NMR-based metabolomics approach. *Food Microbiol* 76, 11–20. doi: 10.1016/J.FM.2018.03.015.

Li, T., Li, L., Du, F., Sun, L., Shi, J., Long, M., et al. (2021b). Activity and mechanism of action of antifungal peptides from microorganisms: a review. *Molecules 2021, Vol. 26, Page 3438* 26, 3438. doi: 10.3390/MOLECULES26113438.

Li, W., and Olivier, M. (2013). Current analysis platforms and methods for detecting copy number variation. *Physiol Genomics* 45, 1–16. doi: 10.1152/physiolgenomics.00082.2012.

Li, Y., Davis, A., Korza, G., Zhang, P., Li, Y., Setlow, B., et al. (2012). Role of a spoVA protein in dipicolinic acid uptake into developing spores of *Bacillus subtilis*. *J Bacteriol* 194, 1875–1884. doi: 10.1128/JB.00062-12.

Li, Z., Bai, Z., Wang, D., Zhang, W., Zhang, M., Lin, F., et al. (2014). Cultivable bacterial diversity and amylase production in three typical Daqus of Chinese spirits. *Int J Food Sci Technol* 49, 776–786. doi: 10.1111/IJFS.12365.

Li, Z., Schottroff, F., Simpson, D. J., and Gänzle, M. (2019). The copy number of the *spoVA*^{2mob} operon determines pressure resistance of *Bacillus* endospores. *Appl Environ Microbiol* 85, e01596-19. doi: 10.1128/aem.01596-19.

Li, Z., Siepmann, F. B., Rojas Tovar, L. E., Chen, X., and Gänzle, M. (2020). Effect of copy number of the *spoVA*^{2mob} operon, sourdough and reutericyclin on ropy bread spoilage caused by *Bacillus* spp. *Food Microbiol* 91, 103507. doi: 10.1016/j.fm.2020.103507.

Liang, J., Li, D., Shi, R., Wang, J., Guo, S., Ma, Y., et al. (2019). Effects of microbial community succession on volatile profiles and biogenic amine during sufu fermentation. *LWT* 114, 108379. doi: 10.1016/J.LWT.2019.108379.

Lin, X. B., Lohans, C. T., Duar, R., Zheng, J., Vederas, J. C., Walter, J., et al. (2015). Genetic determinants of reutericyclin biosynthesis in *Lactobacillus reuteri*. *Appl Environ Microbiol* 81, 2032–41. doi: 10.1128/AEM.03691-14.

Liu, D., and Tong, C. (2017). Bacterial community diversity of traditional fermented vegetables in China. *LWT* 86, 40–48. doi: 10.1016/J.LWT.2017.07.040.

Liu, J., Chen, J., Fan, Y., Huang, X., and Han, B. (2018). Biochemical characterisation and dominance of different hydrolases in different types of Daqu – a Chinese industrial fermentation starter. *J Sci Food Agric* 98, 113–121. doi: 10.1002/JSFA.8445.

Liu, J., Wu, Q., Wang, P., Lin, J., Huang, L., and Xu, Y. (2017). Synergistic effect in core microbiota associated with sulfur metabolism in spontaneous Chinese liquor fermentation. *Appl Environ Microbiol* 83, 1475–1492. doi: 10.1128/AEM.01475-17/FORMAT/EPUB.

Logan, N. A., and Rodríguez-Díaz, M. (2006). "*Bacillus* spp. and related genera," in *Principles and Practice of Clinical Bacteriology: Second Edition*, eds. S. H. Gillespie and P. M. Hawkey (John Wiley & Sons, Ltd), 139–158. doi: 10.1002/9780470017968.CH9.

Lücking, G., Stoeckel, M., Atamer, Z., Hinrichs, J., and Ehling-Schulz, M. (2013). Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. *Int J Food Microbiol* 166, 270–279. doi: 10.1016/j.ijfoodmicro.2013.07.004.

Luo, Y., Korza, G., DeMarco, A. M., Kuipers, O. P., Li, Y. Q., and Setlow, P. (2021). Properties of spores of *Bacillus subtilis* with or without a transposon that decreases spore germination and increases spore wet heat resistance. *J Appl Microbiol* 131, 2918–2928. doi: 10.1111/JAM.15163.

Luu, S., Cruz-Mora, J., Setlow, B., Feeherry, F. E., Doona, C. J., and Setlow, P. (2015). The effects of heat activation on *Bacillus* spore germination, with nutrients or under high pressure, with or without various germination proteins. *Appl Environ Microbiol* 81, 2927–2938. doi: 10.1128/AEM.00193-15/SUPPL_FILE/ZAM999116199SO1.PDF.

Lyngwi, N. A., and Joshi, S. R. (2014). "Economically important *Bacillus* and related genera: a mini review," in *Biology of Useful Plants and Microbes*, ed. A. Sen (Narosa Publishing House, New Delhi, India), 33–43.

Maget-Dana, R., Thimon, L., Peypoux, F., and Ptak, M. (1992). Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. *Biochimie* 74, 1047–1051. doi: 10.1016/0300-9084(92)90002-V.

Magno-Pérez-Bryan, M. C., Martínez-García, P. M., Hierrezuelo, J., Rodríguez-Palenzuela, P., Arrebola, E., Ramos, C., et al. (2015). Comparative genomics within the *Bacillus* genus reveal the singularities of two robust *Bacillus amyloliquefaciens* biocontrol strains. *Mol Plant Microbe Interact* 28, 1102–1116.

Maksong, S., Yemor, T., Yanmanee, S., Arreola, J., Vahidpour, F., Wagner, T., et al. (2017). The bacterial endospore stain on Schaeffer Fulton using variation of methylene blue solution. *J Phys Conf Ser* 812, 012066. doi: 10.1088/1742-6596/812/1/012066.

Man Cho, K., Hwan Lee, J., Dae Yun, H., Yong Ahn, B., Kim, H., and Taek Seo, W. (2011). Changes of phytochemical constituents (isoflavones, flavanols, and phenolic acids) during *cheonggukjang* soybeans fermentation using potential probiotics *Bacillus subtilis* CS90. *Journal of Food Composition and Analysis* 24, 402–410. doi: 10.1016/j.jfca.2010.12.015.

Mandic-Mulec, I., Stefanic, P., and van Elsas, J. D. (2016). "Ecology of *Bacillaceae*," in *The Bacterial Spore: From Molecules to Systems*, eds. A. Driks and P. Eichenberger (John Wiley & Sons, Ltd), 59–85. doi: 10.1128/9781555819323.CH3.

Mante, E. S., Sakyi-Dawson, E., and Amoa-Awua, W. K. (2003). Antimicrobial interactions of microbial species involved in the fermentation of cassava dough into agbelima with particular reference to the inhibitory effect of lactic acid bacteria on enteric pathogens. *Int J Food Microbiol* 89, 41–50. doi: 10.1016/S0168-1605(03)00103-X.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., et al. (2017). Health benefits of fermented foods: microbiota and beyond. *Curr Opin Biotechnol* 44, 94–102. doi: 10.1016/J.COPBIO.2016.11.010.

Marco, M. L., Sanders, M. E., Gänzle, M., Arrieta, M. C., Cotter, P. D., de Vuyst, L., et al. (2021). The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on fermented foods. *Nat Rev Gastroenterol Hepatol* 18, 196–208. doi: 10.1038/s41575-020-00390-5.

Margosch, D., Ehrmann, M. A., Buckow, R., Heinz, V., Vogel, R. F., and Gänzle, M. (2006). High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. *Appl Environ Microbiol* 72, 3476–3481. doi: 10.1128/AEM.72.5.3476-3481.2006.

Margosch, D., Ehrmann, M. A., Gaenzle, M., and Vogel, R. F. (2004a). Comparison of pressure and heat resistance of *Clostridium botulinum* and other endospores in mashed carrots. *J Food Prot* 67, 2530–2537. doi: 10.4315/0362-028X-67.11.2530.

Margosch, D., Gänzle, M., Ehrmann, M. A., and Vogel, R. F. (2004b). Pressure inactivation of *Bacillus* endospores. *Appl Environ Microbiol* 70, 7321–7328. doi: 10.1128/AEM.70.12.7321-7328.2004.

Matser, A. M., Krebbers, B., Van Den Berg, R. W., and Bartels, P. V (2004). Advantages of high pressure sterilisation on quality of food products. *Trends Food Sci Technol* 15, 79–85. doi: 10.1016/j.tifs.2003.08.005.

Mbozo, A. B. V., Kobawila, S. C., Anyogu, A., Awamaria, B., Louembe, D., Sutherland, J. P., et al. (2017). Investigation of the diversity and safety of the predominant *Bacillus pumilus sensu lato* and other *Bacillus* species involved in the alkaline fermentation of cassava leaves for the production of Ntoba Mbodi. *Food Control* 82, 154–162. doi: 10.1016/J.FOODCONT.2017.06.018.

Meena, K. R., and Kanwar, S. S. (2015). Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. *Biomed Res Int* 2015. doi: 10.1155/2015/473050.

Merenstein, D. J., Sanders, M. E., and Tancredi, D. J. (2020). Probiotics as a Tx resource in primary care. *J Fam Pract* 69, E1–E10.

Miotke, L., Lau, B. T., Rumma, R. T., and Ji, H. P. (2014). High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR. *Anal Chem* 86, 2618–2624. doi: 10.1021/ac403843j.

Montanhini, M. T. M., Montanhini, R. N., Pinto, and Bersot, L. S. (2013). Effect of temperature on the lipolytic and proteolytic activity of *Bacillus cereus* isolated from dairy products. *Int Food Res J* 20, 1417–1420.

Morisset, D., Štebih, D., Milavec, M., Gruden, K., and Žel, J. (2013). Quantitative analysis of food and feed samples with droplet digital PCR. *PLoS One* 8, 1–9. doi: 10.1371/journal.pone.0062583.

Moyne, A.-L., Cleveland, T. E., and Tuzun, S. (2004). Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. *FEMS Microbiol Lett* 234, 43–49. doi: 10.1111/J.1574-6968.2004.TB09511.X.

Müller, M. R. A., Wolfrum, G., Stolz, P., Ehrmann, M. A., and Vogel, R. F. (2001). Monitoring the growth of Lactobacillus species during a rye flour fermentation. *Food Microbiol* 18, 217–227.

Murugan, K., Sekar, K., and Al-Sohaibani, S. (2014). Detoxification of cyanides in cassava flour by linamarase of *Bacillus subtilis* KM05 isolated from cassava peel. *Afr J Biotechnol* 11, 7232–7237. doi: 10.4314/ajb.v11i28.

Nagao, T., Adachi, K., Sakai, M., Nishijima, M., and Sano, H. (2001). Novel macrolactins as antibiotic lactones from a marine bacterium. *J Antibiot (Tokyo)* 54, 333–339. doi: 10.7164/ANTIBIOTICS.54.333.

Needham, R., Williams, J., Beales, N., Voysey, P., and Magan, N. (2005). Early detection and differentiation of spoilage of bakery products. *Sens Actuators B Chem* 106, 20–23. doi: 10.1016/j.snb.2004.05.032.

Nerandzic, M. M., and Donskey, C. J. (2010). Triggering germination represents a novel strategy to enhance killing of *Clostridium difficile* spores. *PLoS One* 5, e12285. doi: 10.1371/JOURNAL.PONE.0012285.

Nicholson, W. L. (2002). Roles of *Bacillus* endospores in the environment. *Cellular and Molecular Life Sciences* 59, 410–416. doi: 10.1007/s00018-002-8433-7.

Nithya, V., and Halami, P. M. (2013). Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources. *Ann Microbiol* 63, 129–137. doi: 10.1007/S13213-012-0453-4/FIGURES/4.

Obilie, E. M., Tano-Debrah, K., and Amoa-Awua, W. K. (2003). Microbial modification of the texture of grated cassava during fermentation into akyeke. *Int J Food Microbiol* 89, 275–280. doi: 10.1016/S0168-1605(03)00294-0.

Odoux, E., Escoute, J., and Verdeil, J. L. (2006). The relation between glucovanillin, β-d-glucosidase activity and cellular compartmentation during the senescence, freezing and traditional curing of vanilla beans. *Annals of Applied Biology* 149, 43–52. doi: 10.1111/J.1744-7348.2006.00071.X.

Ogawa, Y., Yamaguchi, F., Yuasa, K., and Tahara, Y. (1997). Efficient production of γ-polyglutamic acid by *Bacillus subtilis* (*natto*) in jar fermenters. *Biosci Biotechnol Biochem* 61, 1684–1687. doi: 10.1271/BBB.61.1684.

Ongena, M., and Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol* 16, 115–125. doi: 10.1016/J.TIM.2007.12.009.

Orabi, H., El-Fakharany, E., Abdelkhalek, E., and Sidkey, N. (2019). Production, optimization, purification, characterization, and anti-cancer application of extracellular L-glutaminase produced from the marine bacterial isolate. *Prep Biochem Biotechnol* 50, 408–418. doi: 10.1080/10826068.2019.1703193.

Owusu-Kwarteng, J., Parkouda, C., Adewumi, G. A., Ouoba, L. I. I., and Jespersen, L. (2020). Technologically relevant *Bacillus* species and microbial safety of West African traditional alkaline fermented seed condiments. *Critical Reviews in Food Science and Nutrition* 62, 871–888. doi: 10.1080/10408398.2020.1830026.

Pagán, R., and Mackey, B. (2000). Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: differences between exponential-and stationary-phase cells and variation among strains. *Appl Environ Microbiol* 66, 2829–34. doi: 10.1128/AEM.66.7.2829-2834.2000.

Paidhungat, M., Setlow, B., Daniels, W. B., Hoover, D., Papafragkou, E., and Setlow, P. (2002). Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. *Appl Environ Microbiol* 68, 3172–3175. doi: 10.1128/AEM.68.6.3172-3175.2002.

Paidhungat, M., Setlow, B., Driks, A., and Setlow, P. (2000). Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. *J Bacteriol* 182, 5505–5512. doi:

10.1128/JB.182.19.5505-5512.2000/ASSET/84938F89-EA87-4FD0-9821-7389D6B07B8E/ASSETS/GRAPHIC/JB1900589006.JPEG.

Paidhungat, M., and Setlow, P. (2000). Role of Ger proteins in nutrient and nonnutrient triggering of spore germination in *Bacillus subtilis*. *J Bacteriol* 182, 2513–2519. doi: 10.1128/JB.182.9.2513-2519.2000/ASSET/3B7E80A2-09EA-4C4E-9E75-239F8A1C2E98/ASSETS/GRAPHIC/JB0901679004.JPEG.

Park, J. N., Ishida, K., Watanabe, T., Endoh, K. I., Watanabe, K., Murakami, M., et al. (2002). Taste effects of oligopeptides in a Vietnamese fish sauce. *Fisheries science* 68, 921–928. doi: 10.1046/J.1444-2906.2002.00511.X.

Parkouda, C., Nielsen, D. S., Azokpota, P., Ivette Iréne Ouoba, L., Amoa-Awua, W. K., Thorsen, L., et al. (2009). The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia. *Crit Rev Microbiol* 35, 139–156. doi: 10.1080/10408410902793056.

Pasolli, E., de Filippis, F., Mauriello, I. E., Cumbo, F., Walsh, A. M., Leech, J., et al. (2020). Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. *Nature Communications 2020 11:1* 11, 1–12. doi: 10.1038/s41467-020-16438-8.

Pepe, O., Blaiotta, G., Moschetti, G., Greco, T., and Villani, F. (2003). Rope-producing strains of *Bacillus* spp. from wheat bread and strategy for their control by lactic acid bacteria. *Appl Environ Microbiol* 69, 2321–2329. doi: 10.1128/aem.69.4.2321-2329.2003.

Pérez Silva, A., Gunata, Z., Lepoutre, J. P., and Odoux, E. (2011). New insight on the genesis and fate of odor-active compounds in vanilla beans (*Vanilla planifolia* G. Jackson) during

traditional curing. *Food Research International* 44, 2930–2937. doi: 10.1016/J.FOODRES.2011.06.048.

Perez-Valdespino, A., Li, Y., Setlow, B., Ghosh, S., Pan, D., Korza, G., et al. (2014). Function of the SpoVAEa and SpoVAF proteins of *Bacillus subtilis* spores. *J Bacteriol* 196, 2077–2088. doi: 10.1128/JB.01546-14/SUPPL_FILE/ZJB999093167SO1.PDF.

Phengnuam, T., and Suntornsuk, W. (2013). Detoxification and anti-nutrients reduction of *Jatropha curcas* seed cake by *Bacillus* fermentation. *J Biosci Bioeng* 115, 168–172. doi: 10.1016/J.JBIOSC.2012.08.017.

Pinheiro, L. B., Coleman, V. A., Hindson, C. M., Herrmann, J., Hindson, B. J., Bhat, S., et al. (2012). Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal Chem* 84, 1003–1011. doi: 10.1021/ac202578x.

Postollec, F., Mathot, A. G., Bernard, M., Divanac'h, M. L., Pavan, S., and Sohier, D. (2012). Tracking spore-forming bacteria in food: from natural biodiversity to selection by processes. *Int J Food Microbiol* 158, 1–8. doi: 10.1016/J.IJFOODMICRO.2012.03.004.

Poutanen, K., Flander, L., and Katina, K. (2009). Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiol* 26, 693–699. doi: 10.1016/J.FM.2009.07.011.

Qin, D., Hara, Y., Raboy, V., and Saneoka, H. (2020). Characteristics and quality of Japanese traditional fermented soybean (natto) from a low-phytate line. *Plant Foods for Human Nutrition* 75, 651–655. doi: 10.1007/S11130-020-00865-5.

Quan, J., and Tian, J. (2014). "Circular polymerase extension cloning," in *DNA Cloning and Assembly Methods*, eds. S. Valla and R. Lale (Totowa, NJ: Humana Press Inc.), 103–117. doi: 10.1007/978-1-62703-764-8_8.

Quattrini, M., Liang, N., Fortina, M. G., Xiang, S., Curtis, J. M., and Gänzle, M. (2018). Exploiting synergies of sourdough and antifungal organic acids to delay fungal spoilage of bread. *Int J Food Microbiol*. doi: 10.1016/j.ijfoodmicro.2018.09.007.

Raboy, V. (2020). *Low phytic acid* crops: observations based on four decades of research. *Plants 2020, Vol. 9, Page 140* 9, 140. doi: 10.3390/PLANTS9020140.

Ramos, C. L., Sousa, E. S. O. de, Ribeiro, J., Almeida, T. M. M., Santos, C. C. A. do A., Abegg, M. A., et al. (2015). Microbiological and chemical characteristics of *tarubá*, an indigenous beverage produced from solid cassava fermentation. *Food Microbiol* 49, 182–188. doi: 10.1016/J.FM.2015.02.005.

Raso, A., and Biassoni, R. (2014). "Twenty years of qPCR: a mature technology?," in *Quantitative real-time PCR. methods in molecular biology (methods and protocols)*, eds. R. Biassoni and A. Raso (New York: Humana Press), 1–3. doi: 10.1007/978-1-4939-0733-5_1.

Redzepi, R., and Zilber, D. (2018). *The Noma guide to fermentation: foundations of flavor*. New York, New York: Artisan Books.

Reginensi, S., González, M. J., Gorgoroso, F., Reginensi, S. M., Olivera, J. A., and Bermúdez, J. (2013). Polyphasic identification of closely related *Bacillus subtilis* and *Bacillus amyloliquefaciens* isolates from dairy farms and milk powder. *Journal of Microbiology, Biotechnology and Food Sciences* 2, 2326–2331.

Reineke, K., Mathys, A., Heinz, V., and Knorr, D. (2013a). Mechanisms of endospore inactivation under high pressure. *Trends Microbiol* 21, 296–304. doi: 10.1016/J.TIM.2013.03.001.

Reineke, K., Schlumbach, K., Baier, D., Mathys, A., and Knorr, D. (2013b). The release of dipicolinic acid — the rate-limiting step of *Bacillus* endospore inactivation during the high

pressure thermal sterilization process. *Int J Food Microbiol* 162, 55–63. doi: 10.1016/J.IJFOODMICRO.2012.12.010.

Robinson, R. J., Fraaije, B. A., Clark, I. M., Jackson, R. W., Hirsch, P. R., and Mauchline, T. H. (2016). Endophytic bacterial community composition in wheat (*Triticum aestivum*) is determined by plant tissue type, developmental stage and soil nutrient availability. *Plant Soil* 405, 381–396. doi: 10.1007/s11104-015-2495-4.

Röcken, W., and Spicher, G. (1993). Fadenziehende Bakterien-Vorkommen, Bedeutung Gegenmaßnahmen. *Getreide Mehl Brot* 47, 30–35.

Rodríguez, A., Rodríguez, M., Luque, M. I., Justesen, A. F., and Córdoba, J. J. (2012). A comparative study of DNA extraction methods to be used in real-time PCR based quantification of ochratoxin A-producing molds in food products. *Food Control* 25, 666–672. doi: 10.1016/J.FOODCONT.2011.12.010.

Röling, W. F. M., Kerler, J., Braster, M., Apriyantono, A., Stam, H., and van Verseveld, H. W. (2001). Microorganisms with a taste for vanilla: microbial ecology of traditional Indonesian vanilla curing. *Appl Environ Microbiol* 67, 1995. doi: 10.1128/AEM.67.5.1995-2003.2001.

Roongsawang, N., Washio, K., and Morikawa, M. (2010). Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. *International Journal of Molecular Sciences 2011, Vol. 12, Pages 141-172* 12, 141–172. doi: 10.3390/IJMS12010141.

Rosenquist, H., and Hansen, Å. (1998). The antimicrobial effect of organic acids, sour dough and nisin against *Bacillus subtilis* and *B. licheniformis* isolated from wheat bread. *J Appl Microbiol* 85, 621–631. doi: 10.1046/j.1365-2672.1998.853540.x.

Sadeghi, A. (2008). The secrets of sourdough; a review of miraculous potentials of sourdough in bread shelf life. *Biotechnology* 7, 413–417. doi: 10.3923/biotech.2008.413.417.

Sakandar, H. A., Hussain, R., Farid Khan, Q., and Zhang, H. (2020). Functional microbiota in Chinese traditional *Baijiu* and *Mijiu Qu* (starters): a review. *Food Research International* 138, 109830. doi: 10.1016/J.FOODRES.2020.109830.

Salvador, E. M., Steenkamp, V., and McCrindle, C. M. E. (2014). Production, consumption and nutritional value of cassava (*Manihot esculenta*, Crantz) in Mozambique: an overview. *Journal of Agricultural Biotechnology and Sustainable Development* 6, 29–38. doi: 10.5897/JABSD2014.0224.

Saranraj, P., and Geetha, M. (2012). Microbial spoilage of bakery products and its control by preservatives. *International Journal of Pharmaceutical & Biological Archives* 3, 38–48.

Sathish, T., and Prakasham, R. S. (2010). Isolation and identification of L-glutaminase an antileukemic enzyme producing mico-organism from Godavari river bank soils in Andhra Pradesh. *International Research Journal of Phamacy* 1, 367–373.

Schirawski, J., and Perlin, M. H. (2018). Plant–microbe interaction 2017—the good, the bad and the diverse. *International Journal of Molecular Sciences 2018, Vol. 19, Page 1374* 19, 1374. doi: 10.3390/IJMS19051374.

Schoeni, J. L., and Lee Wong, A. C. (2005). *Bacillus cereus* food poisoning and its toxins. *J Food Prot* 68, 636–648. doi: 10.4315/0362-028X-68.3.636.

Senthilkumar, P. K., Uma, C., and Saranraj, P. (2012). Amylase production by *Bacillus* sp. using cassava as substrate. *International Journal of Pharmaceutical & Biological Archives* 3, 274–280.

Setlow, B., Cabrera-Hernandez, A., Cabrera-Martinez, R. M., and Setlow, P. (2004). Identification of aryl-phospho-beta-D-glucosidases in *Bacillus subtilis*. *Arch Microbiol* 181, 60–67. doi: 10.1007/S00203-003-0628-2.

Setlow, P. (2003). Spore germination. *Curr Opin Microbiol* 6, 550–556. doi: 10.1016/j.mib.2003.10.001.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* 101, 514–525. doi: 10.1111/j.1365-2672.2005.02736.x.

Setlow, P. (2014). Germination of spores of *Bacillus* species: what we know and do not know. *J Bacteriol* 196, 1297–1305. doi: 10.1128/JB.01455-13/FORMAT/EPUB.

Setlow, P., and Johnson, E. A. (2019). "Spores and their significance," in *Food Microbiology: Fundamentals and Frontiers* (John Wiley & Sons, Ltd), 23–63. doi: 10.1128/9781555819972.CH2.

Sevenich, R., and Mathys, A. (2018). Continuous versus discontinuous ultra-high-pressure systems for food sterilization with focus on ultra-high-pressure homogenization and high-pressure thermal sterilization: a review. *Compr Rev Food Sci Food Saf* 17, 646–662. doi: 10.1111/1541-4337.12348.

Sewalt, V., Shanahan, D., Gregg, L., la Marta, J., and Carrillo, R. (2016). The Generally Recognized as Safe (GRAS) process for industrial microbial enzymes. *Industrial Biotechnology* 12, 295–302. doi: 10.1089/ind.2016.0011.

Shahzad, R., Waqas, M., Khan, A. L., Asaf, S., Khan, M. A., Kang, S.-M., et al. (2016). Seed-borne endophytic *Bacillus amyloliquefaciens* RWL-1 produces gibberellins and regulates

endogenous phytohormones of *Oryza sativa*. *Plant Physiology and Biochemistry* 106, 236–243. doi: 10.1016/j.plaphy.2016.05.006.

Shobharani, P., and Halami, P. M. (2014). Cellular fatty acid profile and H^+-ATPase activity to assess acid tolerance of *Bacillus* sp. for potential probiotic functional attributes. *Appl Microbiol Biotechnol* 98, 9045–9058. doi: 10.1007/S00253-014-5981-3/TABLES/4.

Shrestha, A. K., Dahal, N. R., and Ndungutse, V. (2013). *Bacillus* fermentation of soybean: a review. *Journal of Food Science and Technology Nepal* 6, 1–9. doi: 10.3126/jfstn.v6i0.8252.

Skovgaard, N. (2004). *Fundamental food microbiology*. Third. Elsevier Science doi: 10.1016/J.IJFOODMICRO.2004.08.005.

Slivinski, C. T., Mallmann, E., de Araújo, J. M., Mitchell, D. A., and Krieger, N. (2012). Production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation using a medium based on okara with sugarcane bagasse as a bulking agent. *Process Biochemistry* 47, 1848–1855. doi: 10.1016/J.PROCBIO.2012.06.014.

Smith, T., Williamson, C. H. D., Hill, K., Sahl, J., and Keim, P. (2018). Botulinum neurotoxin-producing bacteria. Isn't it time that we called a species a species? *mBio* 9, e01469-18. doi: 10.1128/mBio.01469-18.

Sorokulova, I. B., Reva, O. N., Smirnov, V. V., Pinchuk, I. V., Lapa, S. V., and Urdaci, M. C. (2003). Genetic diversity and involvement in bread spoilage of *Bacillus* strains isolated from flour and ropy bread. *Lett Appl Microbiol* 37, 169–173. doi: 10.1046/j.1472-765X.2003.01372.x.

Srichuwong, S., Sunarti, T. C., Mishima, T., Isono, N., and Hisamatsu, M. (2005). Starches from different botanical sources I: contribution of amylopectin fine structure to thermal properties

and enzyme digestibility. *Carbohydr Polym* 60, 529–538. doi: 10.1016/J.CARBPOL.2005.03.004.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol Microbiol* 56, 845–857. doi: 10.1111/J.1365-2958.2005.04587.X.

Stewart, K. A. v., Yi, X., Ghosh, S., and Setlow, P. (2012). Germination protein levels and rates of germination of spores of *Bacillus subtilis* with overexpressed or deleted genes encoding germination proteins. *J Bacteriol* 194, 3156–3164. doi: 10.1128/JB.00405-12/ASSET/AF4C1829-33CD-43E3-9430-8D1CC6E392C5/ASSETS/GRAPHIC/ZJB9990915450007.JPEG.

Strain, M. C., Lada, S. M., Luong, T., Rought, S. E., Gianella, S., Terry, V. H., et al. (2013). Highly precise measurement of HIV DNA by droplet digital PCR. *PLoS One* 8, 1–8. doi: 10.1371/journal.pone.0055943.

Su, Y., Liu, C., Fang, H., and Zhang, D. (2020). *Bacillus subtilis*: a universal cell factory for industry, agriculture, biomaterials and medicine. *Microbial Cell Factories 2020 19:1* 19, 1–12. doi: 10.1186/S12934-020-01436-8.

Szymańska, S., Płociniczak, T., Piotrowska-Seget, Z., and Hrynkiewicz, K. (2016). Endophytic and rhizosphere bacteria associated with the roots of the halophyte *Salicornia europaea* L. - community structure and metabolic potential. *Microbiol Res* 192, 37–51. doi: 10.1016/j.micres.2016.05.012.

Tamang, J. P. (2015). Naturally fermented ethnic soybean foods of India. *Journal of Ethnic Foods* 2, 8–17. doi: 10.1016/J.JEF.2015.02.003.

Tang, X., Kudo, Y., Baker, J. L., Labonte, S., Jordan, P. A., McKinnie, S. M. K., et al. (2020). Cariogenic *Streptococcus mutans* produces tetramic acid strain-specific antibiotics that impair commensal colonization. *ACS Infect Dis* 6, 563–571. doi: 10.1021/ACSINFECDIS.9B00365/ASSET/IMAGES/LARGE/ID9B00365_0003.JPEG.

Tao, Y., Bie, X. mei, Lv, F. xia, Zhao, H. zhen, and Lu, Z. xin (2011). Antifungal activity and mechanism of fengycin in the presence and absence of commercial surfactin against *Rhizopus stolonifer*. *The Journal of Microbiology 2011 49:1* 49, 146–150. doi: 10.1007/S12275-011-0171-9.

Tilak, K. V. B. R., and Reddy, B. S. (2006). *Bacillus cereus* and *B. circulans* – novel inoculants for crops. *Curr Sci* 90, 642–644. doi: 10.2307/24089111.

Tovar-Rojo, F., Chander, M., Setlow, B., and Setlow, P. (2002). The products of the spoVA operon are involved in dipicolinic acid uptake into developing spores of *Bacillus subtilis*. *J Bacteriol* 184, 584–587. doi: 10.1128/jb.184.2.584-587.2002.

Toyokawa, Y., Takahara, H., Reungsang, A., Fukuta, M., Hachimine, Y., Tachibana, S., et al. (2010). Purification and characterization of a halotolerant serine proteinase from thermotolerant *Bacillus licheniformis* RKK-04 isolated from Thai fish sauce. *Appl Microbiol Biotechnol* 86, 1867–1875. doi: 10.1007/S00253-009-2434-5/FIGURES/6.

Udomsil, N., Rodtong, S., Choi, Y. J., Hua, Y., and Yongsawatdigul, J. (2011). Use of *Tetragenococcus halophilus* as a starter culture for flavor improvement in fish sauce fermentation. *J Agric Food Chem* 59, 8401–8408. doi: 10.1021/JF201953V.

Uehara, H., Yoneda, Y., Yamane, K., and Maruo, B. (1974). Regulation of neutral protease productivity in *Bacillus subtilis*: transformation of high protease productivity. *J Bacteriol* 119, 82. doi: 10.1128/JB.119.1.82-91.1974.

Vaičiulytė-Funk, L., Žvirdauskienė, R., Šalomskienė, J., and Šarkinas, A. (2015). The effect of wheat bread contamination by the *Bacillus* genus bacteria on the quality and safety of bread. *Zemdirbyste-Agriculture* 102, 351–358. doi: 10.13080/z-a.2015.102.045.

Valerio, F., de Bellis, P., di Biase, M., Lonigro, S. L. L., Giussani, B., Visconti, A., et al. (2012). Diversity of spore-forming bacteria and identification of *Bacillus amyloliquefaciens* as a species frequently associated with the ropy spoilage of bread. *Int J Food Microbiol* 156, 278–285. doi: 10.1016/j.ijfoodmicro.2012.04.005.

Valerio, F., De Bellis, P., Lonigro, S. L., Visconti, A., and Lavermicocca, P. (2008). Use of *Lactobacillus plantarum* fermentation products in bread-making to prevent *Bacillus subtilis* ropy spoilage. *Int J Food Microbiol* 122, 328–332. doi: 10.1016/j.ijfoodmicro.2008.01.005.

Valerio, F., di Biase, M., Huchet, V., Desriac, N., Lonigro, S. L., Lavermicocca, P., et al. (2015). Comparison of three *Bacillus amyloliquefaciens* strains growth behaviour and evaluation of the spoilage risk during bread shelf-life. *Food Microbiol* 45, 2–9. doi: 10.1016/J.FM.2014.07.006.

Vanittanakom, N., Loeffler, W., Koch, U., and Jung, G. (1986). Fengycin-a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J Antibiot (Tokyo)* 39, 888–901. doi: 10.7164/ANTIBIOTICS.39.888.

Vassilev, B., Fifani, B., Steels, S., Helmus, C., Delacuvellerie, A., Deracinois, B., et al. (2022). Coculture of *Trichoderma harzianum* and *Bacillus velezensis* based on metabolic cross-

feeding modulates lipopeptide production. *Microorganisms 2022, Vol. 10, Page 1059* 10, 1059. doi: 10.3390/MICROORGANISMS10051059.

Velásquez, J., Schuurman-Wolters, G., Birkner, J. P., Abee, T., and Poolman, B. (2014). *Bacillus subtilis* spore protein SpoVAC functions as a mechanosensitive channel. *Mol Microbiol* 92, 813–823. doi: 10.1111/mmi.12591.

Venkatesh, N., Greco, C., Drott, M. T., Koss, M. J., Ludwikoski, I., Keller, N. M., et al. (2022). Bacterial hitchhikers derive benefits from fungal housing. *Current Biology* 32, 1523-1533.e6. doi: 10.1016/J.CUB.2022.02.017.

Vepachedu, V. R., Hirneisen, K., Hoover, D. G., and Setlow, P. (2007). Studies of the release of small molecules during pressure germination of spores of *Bacillus subtilis*. *Lett Appl Microbiol* 45, 342–348. doi: 10.1111/j.1472-765X.2007.02204.x.

Vepachedu, V. R., and Setlow, P. (2004). Analysis of the germination of spores of *Bacillus subtilis* with temperature sensitive *spo* mutations in the *spoVA* operon. *FEMS Microbiol Lett* 239, 71–77. doi: 10.1016/j.femsle.2004.08.022.

Vepachedu, V. R., and Setlow, P. (2007). Role of SpoVA proteins in release of dipicolinic acid during germination of *Bacillus subtilis* spores triggered by dodecylamine or lysozyme. *J Bacteriol* 189, 1565–72. doi: 10.1128/JB.01613-06.

Vessey, J. K., and Buss, T. J. (2002). *Bacillus cereus* UW85 inoculation effects on growth, nodulation, and N accumulation in grain legumes: Controlled-environment studies. *Canadian Journal of Plant Science* 82, 283–290.

Voysey, P. A., and Hammond, J. C. (1993). “Reduced-additive breadmaking technology,” in *Technology of Reduced-Additive Foods* (Boston, MA: Springer US), 80–94. doi: 10.1007/978-1-4615-2115-0_4.

Wakayama, M., Yamagata, T., Kamemura, A., Bootim, N., Yano, S., Tachiki, T., et al. (2005). Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. *J Ind Microbiol Biotechnol* 32, 383–390. doi: 10.1007/S10295-005-0257-7/FIGURES/7.

Wang, C. L., Shi, D. J., and Gong, G. L. (2008). Microorganisms in Daqu: a starter culture of Chinese Maotai-flavor liquor. *World J Microbiol Biotechnol* 24, 2183–2190. doi: 10.1007/S11274-008-9728-0/TABLES/5.

Wang, L., and Li Wang, C. (2022). Research trends in Jiang-flavor baijiu fermentation: from fermentation microecology to environmental ecology. *J Food Sci* 87, 1362–1374. doi: 10.1111/1750-3841.16092.

Wang, P., Wu, Q., Jiang, X., Wang, Z., Tang, J., and Xu, Y. (2017). *Bacillus licheniformis* affects the microbial community and metabolic profile in the spontaneous fermentation of Daqu starter for Chinese liquor making. *Int J Food Microbiol* 250, 59–67. doi: 10.1016/J.IJFOODMICRO.2017.03.010.

Wang, S., Faeder, J. R., Setlow, P., and Li, Y. Q. (2015). Memory of germinant stimuli in bacterial spores. *mBio* 6. doi: 10.1128/MBIO.01859-15.

Wang, Y., Ametaj, B. N., Ambrose, D. J., and Gänzle, M. G. (2013). Characterisation of the bacterial microbiota of the vagina of dairy cows and isolation of pediocin-producing *Pediococcus acidilactici*. *BMC Microbiol* 13, 1–11. doi: 10.1186/1471-2180-13-19.

Wang, Z., Li, P., Luo, L., Simpson, D. J., and Gänzle, M. (2018). Daqu fermentation selects for heat-resistant *Enterobacteriaceae* and bacilli. *Appl Environ Microbiol* 84, e01483-18. doi: 10.1128/AEM.01483-18.

Wastyk, H. C., Fragiadakis, G. K., Perelman, D., Dahan, D., Merrill, B. D., Yu, F. B., et al. (2021). Gut-microbiota-targeted diets modulate human immune status. *Cell* 184, 4137-4153.e14. doi: 10.1016/J.CELL.2021.06.019.

Wei, Q. K., Chen, T. R., and Chen, J. T. (2008). Use of *Bacillus subtilis* to enrich isoflavone aglycones in fermented natto. *J Sci Food Agric* 88, 1007–1011. doi: 10.1002/JSFA.3181.

Wu, Q., Zhang, R., Peng, S., and Xu, Y. (2015). Transcriptional characteristics associated with lichenysin biosynthesis in *Bacillus licheniformis* from Chinese Maotai-flavor liquor making. *J Agric Food Chem* 63, 888–893. doi: 10.1021/JF5036806/SUPPL_FILE/JF5036806_SI_002.XLS.

Wu, X., Jiang, Q., Wang, Z., Xu, Y., Chen, W., Sun, J., et al. (2021). Diversity, enzyme production and antibacterial activity of *Bacillus* strains isolated from sesame-flavored liquor Daqu. *Arch Microbiol* 203, 5831–5839. doi: 10.1007/S00203-021-02552-8/FIGURES/4.

Wuytack, E. Y., Soons, J., Poschet, F., and Michiels, C. W. (2000). Comparative study of pressure- and nutrient-induced germination of *Bacillus subtilis* spores. *Appl Environ Microbiol* 66, 257–61. doi: 10.1128/aem.66.1.257-261.2000.

Xiao, C., Lu, Z. M., Zhang, X. J., Wang, S. T., Ao, L., Shen, C. H., et al. (2017). Bio-heat is a key environmental driver shaping the microbial community of medium-temperature Daqu. *Appl Environ Microbiol* 83, 1550–1567. doi: 10.1128/AEM.01550-17/FORMAT/EPUB.

Xie, M., Lv, F., Ma, G., Farooq, A., Li, H., Du, Y., et al. (2020). High throughput sequencing of the bacterial composition and dynamic succession in Daqu for Chinese sesame flavour liquor. *Journal of the Institute of Brewing* 126, 98–104. doi: 10.1002/JIB.592.

Xu, Y., Zhao, J., Liu, X., Zhang, C., Zhao, Z., Li, X., et al. (2022). Flavor mystery of Chinese traditional fermented baijiu: the great contribution of ester compounds. *Food Chem* 369, 130920. doi: 10.1016/J.FOODCHEM.2021.130920.

Xue, G. P., Johnson, J. S., and Dalrymple, B. P. (1999). High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. *J Microbiol Methods* 34, 183–191. doi: 10.1016/S0167-7012(98)00087-6.

Yan, B., Sadiq, F. A., Cai, Y., Fan, D., Chen, W., Zhang, H., et al. (2019). Microbial diversity in traditional type I sourdough and jiaozi and its influence on volatiles in Chinese steamed bread. *LWT* 101, 764–773. doi: 10.1016/J.LWT.2018.12.004.

Yan, Z., Zheng, X. W., Chen, J. Y., Han, J. S., and Han, B. Z. (2013). Effect of different *Bacillus* strains on the profile of organic acids in a liquid culture of *Daqu*. *Journal of the Institute of Brewing* 119, 78–83. doi: 10.1002/JIB.58.

Yang, H., Li, X., Li, X., Yu, H., and Shen, Z. (2015). Identification of lipopeptide isoforms by MALDI-TOF-MS/MS based on the simultaneous purification of iturin, fengycin, and surfactin by RP-HPLC. *Anal Bioanal Chem* 407, 2529–2542. doi: 10.1007/S00216-015-8486-8/TABLES/1.

Yang, J. G., Dou, X., Han, P. J., Bai, F. Y., Zhou, J., Zhang, S. Y., et al. (2017). Microbial diversity in Daqu during production of Luzhou flavored liquor. *Journal of the American Society of Brewing Chemists* 75, 136–144. doi: 10.1094/ASBCJ-2017-2879-01.

Yang, R., Paparini, A., Monis, P., and Ryan, U. (2014). Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples. *Int J Parasitol* 44, 1105–1113. doi: 10.1016/j.ijpara.2014.08.004.

Yang, W. W., and Ponce, A. (2009). Rapid endospore viability assay of *Clostridium sporogenes* spores. *Int J Food Microbiol* 133, 213–216. doi: 10.1016/J.IJFOODMICRO.2009.04.024.

Ye, M., Liu, X., and Zhao, L. (2013). Production of a novel salt-tolerant L-glutaminase from *Bacillus amyloliquefaciens* using agro-industrial residues and its application in Chinese soy sauce fermentation. *Biotechnology* 12, 25–35. doi: 10.3923/BIOTECH.2013.25.35.

Yogesh, D., and Halami, P. M. (2015). A fibrin degrading serine metallo protease of *Bacillus circulans* with α-chain specificity. *Food Biosci* 11, 72–78. doi: 10.1016/J.FBIO.2015.04.007.

Yongsawatdigul, J., Rodtong, S., and Raksakulthai, N. (2007). Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. *J Food Sci* 72, M382–M390. doi: 10.1111/J.1750-3841.2007.00532.X.

Yuan, J., Zhao, M., Li, R., Huang, Q., Rensing, C., Raza, W., et al. (2016). Antibacterial compounds-macrolactin alters the soil bacterial community and abundance of the gene encoding PKS. *Front Microbiol* 7. doi: 10.3389/FMICB.2016.01904/FULL.

Zare, F., Dow, M., Monteleone, N., Hosny, A., and Nabavi, S. (2017). An evaluation of copy number variation detection tools for cancer using whole exome sequencing data. *BMC Bioinformatics* 18, 1–13. doi: 10.1186/s12859-017-1705-x.

Zeigler, D. R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., et al. (2008). The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *J Bacteriol* 190, 6983–6995. doi: 10.1128/JB.00722-08.

Zhang, B., Xu, L., Ding, J., Wang, M., Ge, R., Zhao, H., et al. (2022). Natural antimicrobial lipopeptides secreted by *Bacillus* spp. and their application in food preservation, a critical review. *Trends Food Sci Technol* 127, 26–37. doi: 10.1016/J.TIFS.2022.06.009.

Zhang, C., Brandt, M. J., Schwab, C., and Gänzle, M. (2010). Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiol* 27, 390–395. doi: 10.1016/j.fm.2009.11.019.

Zhang, R., Wu, Q., Xu, Y., and Qian, M. C. (2014). Isolation, identification, and quantification of lichenysin, a novel nonvolatile compound in Chinese distilled spirits. *J Food Sci* 79, C1907–C1915. doi: 10.1111/1750-3841.12650.

Zhang, Y., and Mathys, A. (2019). Superdormant spores as a hurdle for gentle germination-inactivation based spore control strategies. *Front Microbiol* 10, 3163. doi: 10.3389/FMICB.2018.03163/BIBTEX.

Zhang, Y. Z., Chen, W. F., Li, M., Sui, X. H., Liu, H. C., Zhang, X. X., et al. (2012). *Bacillus endoradicis* sp. nov., an endophytic bacterium isolated from soybean root. *Int J Syst Evol Microbiol* 62, 359–363. doi: 10.1099/IJS.0.028936-0/CITE/REFWORKS.

Zheng, J., Zhao, X., Lin, X. B., and Gänzle, M. (2016). Comparative genomics *Lactobacillus reuteri* from sourdough reveals adaptation of an intestinal symbiont to food fermentations. *Sci Rep* 5, 18234. doi: 10.1038/srep18234.

Zheng, X. W., and Han, B. Z. (2016). *Baijiu* (白酒), Chinese liquor: history, classification and manufacture. *Journal of Ethnic Foods* 3, 19–25. doi: 10.1016/J.JEF.2016.03.001.

Zheng, X. W., Tabrizi, M. R., Robert Nout, M. J., and Han, B. Z. (2011). Daqu-a traditional Chinese liquor fermentation starter. *Journal of the Institute of Brewing* 117, 82–90. doi: 10.1002/J.2050-0416.2011.TB00447.X.

Zheng, X. W., Yan, Z., Nout, M. J. R., Smid, E. J., Zwietering, M. H., Boekhout, T., et al. (2014). Microbiota dynamics related to environmental conditions during the fermentative production of Fen-Daqu, a Chinese industrial fermentation starter. *Int J Food Microbiol* 182–183, 57–62. doi: 10.1016/J.IJFOODMICRO.2014.05.008.

Zheng, Y., Zheng, X. W., Han, B. Z., Han, J. S., Nout, M. J. R., and Chen, J. Y. (2013). Monitoring the ecology of *Bacillus* during *Daqu* incubation, a fermentation starter, using culture-dependent and culture-independent methods. *J Microbiol Biotechnol* 23, 614–622. Available at: http://dx.doi.org/10.4014/jmb.1211.11065.

Zhi, Y., Wu, Q., Du, H., and Xu, Y. (2016). Biocontrol of geosmin-producing *Streptomyces* spp. by two *Bacillus* strains from Chinese liquor. *Int J Food Microbiol* 231, 1–9. doi: 10.1016/J.IJFOODMICRO.2016.04.021.

Zhou, T., Meng, C., and He, P. (2018). Soy isoflavones and their effects on xenobiotic metabolism. *Curr Drug Metab* 20, 46–53. doi: 10.2174/1389200219666180427170213.

Zong, C., Lu, S., Chapman, A. R., and Xie, X. S. (2012). Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. *Science (1979)* 338, 1622–1626. doi: 10.1126/science.1229164.