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Thermophilic spore formers in powdered dairy products: Source tracking, population dynamics and genomic characterisation of persisting strains

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ABSTRACT

Besides not being harmful to humans, thermophilic spore formers determine the microbial product quality of milk powders. Low-spore powder is highly demanded, and the compliance with critical spore limits of 10^2 - 10^3 spores/g in powders challenges dairy manufacturers. The focus of this work was the microbiological analysis of population dynamics of contaminants in powder productions to track the contamination route of thermophilic spores and assess critical entry points. In order to do so, the most abundant species were characterised.

Initially, a method to quantify thermophilic spores was developed. Pasteurisation at 80 °C for 10 min was determined as to be the optimal temperature-time combination. Treatment at higher temperature and longer time significantly reduced the spore population in process samples. Moreover, the optimal heat resistance of spores was shown to depend on the growth phase and thus on the maturity level of the spore.

For analysis of thermophilic population dynamics during powder processing, German powder plants were sampled extensively. Surprisingly, no relation between the thermophilic spore count, the microbiota of bulk tank milk and the final powder was found. Instead, growth of residual thermophilic bacteria during the first processing steps and early production time led to high spore counts in powder (up to 10^5 spores/g). Compared to other spore formers, obligate thermophilic species of *Anoxybacillus* and *Geobacillus* were highly abundant in powder but not in bulk tank milk. However, *A. flavithermus* strains from milk and powder are phylogenetically distant compared to strains of other habitats.

Using the developed, discriminative RAPD-PCR strain typing method, individual *A. flavithermus* strains were detected in the same production process for up to 24 months. Moreover, process isolates expressed a specialised phenotype which is characterised by fast proliferation, excellent spore yield and enhanced spore resistance. Probably, this combination of characters is advantageous for the long-term survival in production plants when compared to bulk tank milk isolates. At the genome level, the comparative analysis of phenotype and genome sequence indicated the association of sporulation genes, metabolic genes and genetic information processing genes with enhanced fitness in production plants.

A. flavithermus strains of the plant microbiota might originate from the microevolutionary adaptation of bulk tank milk strains during propagation in the production site. This hypothesis was tested using a laboratory-scale evolutionary experiment where sensitive bulk tank isolates were exposed to heat / NaOH stress for 29 cycles. However, the experiment did not lead to an adaptation to plant specific stress conditions. Sampling of specialised strains from raw milk which were already adapted to plant stress conditions seems to be a likely alternative explanation. These strains were then isolated from powder as persisters. The occasional isolation of potentially suitable candidates from bulk tank milk in this work supports this hypothesis.

ZUSAMMENFASSUNG

Thermophile Sporenbildner sind ausschlaggebend für die mikrobiologische Produktqualität von Milchpulver, obgleich sie nicht mit Krankheitsverläufen beim Menschen assoziiert sind. Die Nachfrage an Pulver mit geringem Sporengehalt ist außerordentlich hoch und die Einhaltung kritischer Sporenwerte von 10^2 − 10^3 Sporen/g ist häufig eine große Herausforderung für milchverarbeitende Betriebe. Um den Kontaminationsweg thermophiler Sporenbildner nachzuverfolgen und deren kritische Eintragspunkte zu bestimmen, stand die umfassende Analyse der Populationsdynamik der Kontaminanten in Pulverproduktionen im Fokus dieser Arbeit. Zudem wurden die am häufigsten vorkommenden Spezies charakterisiert.

Zunächst wurde eine Methode zur Quantifizierung von thermophilen Sporen in Milchpulver entwickelt. Die Pasteurisierung bei 80 °C für 10 min erwies sich als die optimale Temperatur – Zeit Kombination. Eine höhere Temperatur und längere Zeiten führten zu einer signifikanten Reduktion der Sporenpopulation in Prozessproben. Es zeigte sich, dass die optimale Hitzeresistenz der Spore von der Wachstumsphase und damit vom Reifungsgrad abhängt.

Zur Analyse der Dynamik thermophiler Sporenbildner innerhalb der Pulverproduktion wurden deutsche Produktionsstätten umfassend beprobt. Ein Zusammenhang zwischen der thermophilen Sporenzahl und Mikrobiota von Rohmilch und Endprodukt zeigte sich nicht. Vielmehr führte das Wachstum thermophiler und in der Anlage residierender Bakterien auf den ersten Prozessstufen und zu frühen Produktionszeiten zu hohen Sporenzahlen in Pulver (bis zu 10^5 Sporen/g). Obligat thermophile Bazillen, v.a. *Anoxybacillus* Spezies und *Geobacillus* Spezies waren im Vergleich zu anderen Sporenbildnern in Pulvern, jedoch nicht in Rohmilch, sehr weit verbreitet. Allerdings sind *A. flavithermus* Stämme aus Milch und Milchpulver phylogenetisch von Stämmen aus anderen Habitaten abgegrenzt.

Mittels der entwickelten RAPD-PCR Stammtypisierungsmethode konnten individuelle *A. flavithermus* Stämme über einen Zeitraum von bis zu 24 Monaten im selben Produktionsprozess nachgewiesen werden. Zudem zeigten Prozessisolate einen spezialisierten Phänotyp, der durch schnelle Vermehrung, ausgezeichnete Sporulation und erhöhte Sporenresistenz gekennzeichnet ist. Dies ist im Vergleich zu Rohmilchisolaten für ein langfristiges Überleben in Produktionsanlagen vermutlich von

Vorteil. Auf genetischer Ebene deutete die vergleichende Analyse von Phänotyp und Genomsequenzen auf die Assoziation von Sporulationsgenen, metabolischen Genen und Genen für die Prozessierung genetischer Information mit einer erhöhten Fitness in Produktionsanlagen hin.

Der Ursprung von den in Produktionsanlagen florierenden *A. flavithermus* Stämmen könnte in der mikroevolutiven Anpassung von Rohmilchstämmen während der Propagation in den Anlagen liegen. Diese Hypothese wurde durch ein Laborevolutionsexperiment getestet, in dem sensitive Isolate aus Rohmilch über 29 Zyklen einem Hitze / NaOH – Stress unterworfen wurden. Dieses Experiment führte allerdings zu keiner Anpassung an die Stressbedingungen. Vielmehr deutet es sich zum jetzigen Zeitpunkt an, dass sich bereits spezialisierte Stämme aus Rohmilch in Anlagen festgesetzt haben. Diese wurden dann als Persistierer aus Milchpulver isoliert. Für diese Hypothese spricht, dass in dieser Arbeit auch in Rohmilch vereinzelt potentiell geeignete Stämme gefunden wurden.

ABBREVIATIONS

BF	bactofugation
BTM	bulk tank milk
cfu	colony forming unit
cgMLST	core genome multilocus sequence typing
CIP	Cleaning in place
DNA	desoxyribonucleic acid
DPA	dipicolinic acid
FTIR	Fourier-transform infrared spectroscopy
KEGG	Kyoto encyclopedia of genes and genomes
LPSN	List of Prokaryotic names with Standing in Nomenclature
Mb	megabase
MF	microfiltration
ML	maximum likelihood
MP	milk powder
NCBI	National Center for Biotechnology Information
panGWAS	pan-genome-wide association studies
PCR	polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature ~22 °C
SASP	small acid-soluble proteins
SC	thermophilic spore count
SM	skim milk
SMP	skim milk powder
TBE	Tris (hydroxymethyl) – aminomethane borate EDTA
TCC	total thermophilic cell count
TSA	tryptic soy agar
UHT	ultra-high temperature
WGS	whole genome sequencing
WMP	whole milk powder
WP	whey powder

Abbreviations of genera

A.	*Anoxybacillus*
B.	*Bacillus*
C.	*Clostridium*
G.	*Geobacillus*

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I GENERAL INTRODUCTION

1 Powdered dairy products

Milk products are a valuable part of the human diet. Raw milk contains many precious nutrients, including vitamins, minerals, proteins, fat and carbohydrates, while it is sterile when leaving the udder of the cow. At a neutral pH and high water content, this nutrient-rich environment is very suitable for microbial proliferation (Quigley et al., 2013) as soon as bacteria are entered during milking, transportation and processing, leading to spoilage and often to a reduced shelf-life. The complex raw milk microbiota may, amongst others, harbour Gram-positive *Bacillus* or *Microbacterium*, Gram-negative Pseudomonads or *Enterobacteriaceae* as well as lactic acid bacteria species (reviewed by Quigley et al., 2013). Refrigeration is applied to reduce microbial growth by providing a temperature which is not optimal for the proliferation of many spoilage organisms. Other measures to extend the shelf-life, to prevent foodborne disease and to reduce the spoilage potential address the inactivation of vegetative cells. Heat treatments like pasteurisation at 72-75 °C or ultra-high temperature (UHT) treatments (135-150 °C) are used (Kessler, 2002, Pearce et al., 2012). Moreover, the physical removal of bacteria using bactofugation (BF) or filtration strategies like microfiltration (MF) is applied, also in combination with a thermal treatment. Nevertheless, the storage of milk and dairy products is very challenging. Long-term storage is demanded and is mandatory for trading dairy products globally. To this purpose, drying of dairy products is widely used to massively deteriorate the conditions for microbial growth and improve trading conditions at the same time. Powdered dairy products have a reduced water activity (a_w < 0.26, Hill and Smythe, 2012), inadequate for bacterial growth, are of reduced weight and volume (up to a 10-fold concentration of the dry mass) and can be more easily stored and transported to any location. From 100 L milk, approximately 9 kg skim milk powder (SMP) or 13 kg whole milk powder (WMP) can be produced (Pearce, 2017). The less strict storage conditions allow this highly valuable good to reach third world countries and places of extreme environmental conditions without capabilities for storage and transport of refrigerated milk.

Dairy powders do not only comprise classical milk such as whole milk or skim milk powder (WMP, SMP), but also powders of processed products like buttermilk, whey or

yoghurt which have diverse applications. For example, they are supplements for other food products, e.g. confectionary, as recombined drinking milk which includes the highly relevant infant formula sector, in feed or as supplements for diets of particular needs (Pearce, 2017). European or German milk powders receive a high reputation and are of great value for exporting worldwide. Huge investments were realised in the last decades, and the production volume of dairy powder in Germany continuously increased from 876,000 t in 1990 to 1,016,000 t in 2019 whereby approximately 75 % were exported (MiV, 2020). The European Union was the largest exporter of SMP in 2019 (962,000 t). In the same year, New Zealand was number one and exporting 1,536,000 t WMP of which the majority was directly shipped to China (USDA/FAS, 2020). In spring 2020, China imported about 26 % of SMP and 40 % of whey powder from Europe which depicts the importance of the Chinese milk powder market (European Commission milk market observatory, as of 17/06/2020). The customer's and consumer's demands include not only an increased volume but also an excellent product quality to provide safe food without reducing the nutritious value.

1.1 Milk powder production process

The basic principle of milk powder production is the stepwise reduction of the water content from ~87 % in milk (Zhang et al., 2018) to 3-7 % in the dry product (Kessler, 2002). Since the spectrum of powders on the market is vast, details of the production strategy depend on the manufacturer and specification of the product. However, a few fundamental processing steps are applied in all processes (Figure I.1). The processing structure varies mainly in the first part of the process until the concentration starts (Pereira and Sant'Ana, 2018, Scott et al., 2007, Watterson et al., 2014, unpublished project data of FEI18356N and FEI19825N). After that, standardised and stored products (e.g. skim milk (SM) or whey) are concentrated stepwise in a similar manner: a pre-concentration step by evaporation or membrane filtration is often applied first to obtain concentrate (e.g. SM or whey concentrate). Subsequently, the drying process is composed of the two final concentration steps using evaporation, followed by spray drying. For optimised energy consumption, the evaporation is often conducted in multi-stage evaporators at temperatures between 40 – 70 °C (Moejes and van Boxtel, 2017, Zhang et al., 2018). For spray drying, the injection of hot air of 180 – 230 °C into the spray tower, where atomised concentrate droplets fall, removes the last water residues

to finalise the powder (Moejes and van Boxtel, 2017). Plate or tubular heat exchangers are used throughout the process to heat or cool the product to temperatures between 10 and 70 °C.

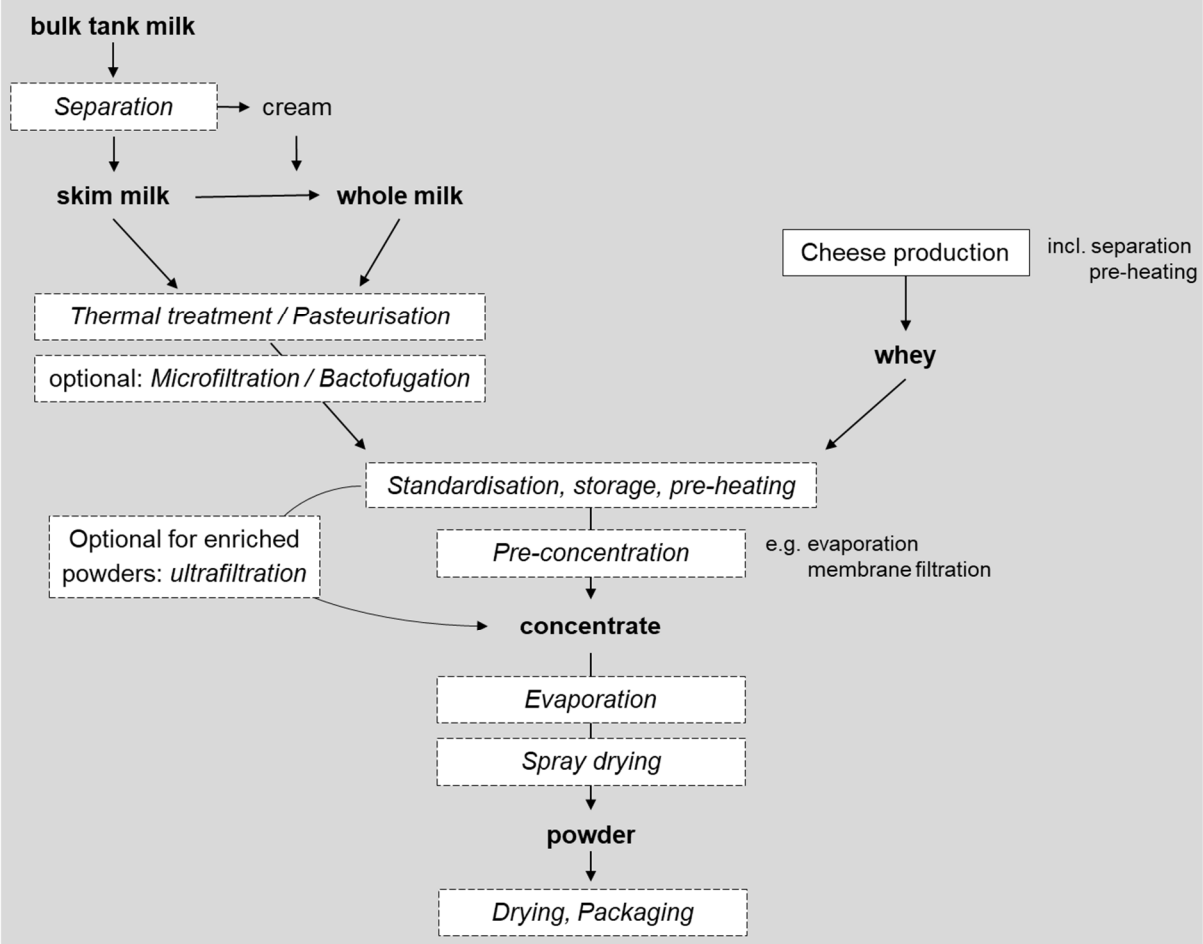


Figure I.1: Processing scheme of milk powders including the main processing steps, intermediate products and options for skim milk, whole milk, whey and enriched (e.g. whey protein concentrate) powder (Pereira and Sant'Ana, 2018, Scott et al., 2007, Watterson et al., 2014 and unpublished survey data of the project FEI18356N and FEI19825N).

The raw product of milk-based powers is non-processed raw milk which is stored in bulk tanks at the production site (Figure I.1). Hereof, skim milk is separated from cream using centrifugation. This separation can be carried out at hot temperatures around 50 °C or cold temperatures < 20 °C (Moejes and van Boxtel, 2017). The cream is then used for standardising the fat content of the product, if needed, or given to other processes like butter production. For whey-based powder (WP), the process initiates with whey that already underwent several processing steps through cheese production. Amongst others, the cheese milk is heat-treated (thermisation,

pasteurisation), standardised, including prior separation and bacterial cultures or coagulating rennet is added (Bylund, 2015). Two types of whey and consequently WP are generally distinguished: acid whey (pH < 5.6) from acid-coagulated cheese production like Greek-style yoghurt and sweet whey (pH > 5.6) from rennet coagulated cheese production (Guo and Wang, 2019, Ramos et al., 2016).

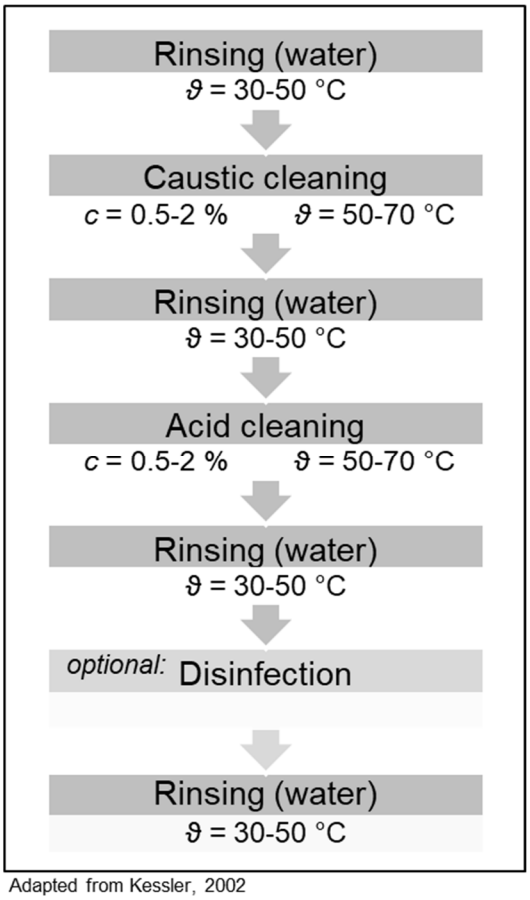
Moreover, the additional thermal treatment defines the specification of the product as low, medium or high heat powder which is treated at 70 – 80 °C, 90 – 100 °C or ≥ 120°C, respectively (Martin et al., 2007, Patel et al., 2007). The production of enriched powders implements membrane technologies such as ultrafiltration to increase the protein content, for example, for milk and whey protein concentrate powder (McHugh et al., 2017).

Pasteurisation is essential for food safety and decontaminates the product from pathogenic and other vegetative bacteria (Pearce et al., 2012). Both high- or low-temperature pasteurisation (72 –75 °C for 15 – 20 s or 62 – 65 °C for 30 min, respectively (Kessler, 2002) are applied. High-temperature treatment is often preferred due to the higher effect on the inactivation of bacteria. Physical separation techniques such as bactofugation (BF) or microfiltration (MF) are further options for microbial decontamination. The effectiveness of both methods highly depends on the operation parameters (e.g. temperature, pressure, type of membrane) and size, shape and composition of the cells. At a usual pore size of 1.4 µm, MF can remove between 2 and 6 log levels bacterial cells (Elwell and Barbano, 2006, Gésan-Guiziou, 2010, Schmidt et al., 2012, Tomasula et al., 2011). Smaller sized spores are removed by 2.0 – 4.5 log levels only (Gésan-Guiziou, 2010, Tomasula et al., 2011). In contrast, BF is less useful for decontamination. Denser spores are removed by 1 – 1.7 log levels, while cells of lower density are only reduced by 1 log level (density: 1.3 $g \cdot mL^{-1}$ and 1.1 $g \cdot mL^{-1}$, respectively, Gésan-Guiziou, 2010). Both methods, MF and BF, are useful in decontaminating the product but not able to remove indefinite amounts of bacterial spores and cells. Therefore, the bacterial load before the treatment is crucial for the effectiveness.

1.2 Cleaning of dairy production plants

The production of high quality and safe foods requires a clean manufacturing environment. Defined sanitation schedules are applied to maintain good plant hygiene, from delivery of raw materials through production, storage and packaging of the product. Clean processing equipment is of enormous impact as it is a matrix for the adherence of particles (fouling), organisms (formation of biofilms) and directly contacts the product.

Nowadays, the Cleaning in place methodology (CIP) is utilised to clean dairy plants without disassembling the equipment as introduced in the 1990s (Romney, 1990). The automation of continuously circulating cleaning solutions are cost and time effective and lead to a more directed cleaning effect without dismantling any part of the equipment (Chisti, 2014). CIP strategies were developed to keep a clean and hygienic production environment, including all subsystems of the desired object (e.g. piping systems). The process typically includes two main cleaning steps alternating with the circulation of water (Figure I.2). In the beginning, a pre-rinse removes residual product and soluble dirt while wetting the interior. The adjacent caustic cleaning addresses mainly organic deposits such as protein or fat to be lifted from the equipment's surface (Chisti, 2014, Tamime, 2008). Sodium hydroxide is a very widely used alkaline detergent. Intermediate rinsing intends to remove all remaining detergent and solubilised dirt from the production site to prepare the next cleaning step as well as to reduce the amount of circulating dirt in the site. As a second cleaning step, the acidic wash clears inorganic deposits (e.g. minerals, milk dust) which were not affected by the caustic wash and



Adapted from Kessler, 2002

Figure I.2: Main steps of Cleaning in Place (CIP) as applied in food industry.

neutralises residual alkaline cleaners (Chisti, 2014, Tamime, 2008). Widely used acids like nitric acid are very effective but also attack the elastic part of the equipment, e.g. valve seals or gaskets. After the next intermediate rinse, disinfection may follow but is not always included in daily CIP schedules. Nowadays, disinfection is more likely performed using sanitisers based on hydrogen peroxide (e.g. peracetic acid) rather than chlorine (Chisti, 2014, Tamime, 2008). Disinfection addresses the removal and inactivation of microorganisms. Residual organic matter in the production plant decreases the effect, which is optimal when biofilms, fouling deposits or residual detergents are removed before.

An option for better efficiency of CIP is a pre-cleaning step at the very beginning that flushes the equipment to remove a significant part of product residues before starting the circulation. Furthermore, the supplementation of additives (e.g. surfactants, emulsifiers, proteolytic active substances) can increase the cleaning capacity (Bremer et al., 2006, Parkar et al., 2004). The re-use of rinsing water as well as cleaning solution is very cost-efficient and environmentally friendly. However, it also brings the risk of post-CIP or particularly post-disinfection cross-contamination if for example, removed but not fully inactivated spores are spread (Tamime, 2008). The accumulated pollution in recirculating solutions over time can impair the cleaning success (Merin et al., 2002).

1.3 Microbiological quality of milk powder

The regulations of the European Commission determine the food safety of milk powder in Europe. For safe milk powders, the presence of the potentially harmful pathogens *Salmonella*, coagulase-positive *Staphylococcus*, *Enterobacteriaceae* (especially *Cronobacter sakazakii*) and presumptive *Bacillus cereus* is restricted or prohibited, in particular for infant formula and dried dietary foods for medical needs (European Commission, version 08/03/2020).

Besides safety regulations, the level of non-pathogenic endospores mainly determines the product quality, which is also an indicator of poor hygiene (Burgess et al., 2010). Endospores withstand the extreme conditions in powders and their outgrowth after reconstitution of the product, also when used as an additive for other products may lead to premature spoilage. With this, accompanying toxin production by *Bacillus* species can be critical for food safety (De Jonghe et al., 2010, Ehling-Schulz and

Messelhausser, 2013, Logan, 2012). The production of microbial enzymes during growth may induce textural defects and off-flavours, e.g. flat-sour spoilage and bitterness, leading to reduced organoleptic properties (Heyndrickx and Scheldeman, 2002, Kalogridou-Vassiliadou, 1992, Lucking et al., 2013).

Product specifications differentiate between limits for aerobic mesophilic and thermophilic spore counts among the spore population. With this, the thermophilic spore count has emerged as the most crucial parameter which challenges manufactures every day. Governmental regulations of the US Dairy Export Council demand < 500 cfu·g^{-1} mesophilic and < 1·10^{4} cfu·g^{-1} thermophilic spores, respectively (Watterson et al., 2014). Other legislations limit the overall aerobic spore count to < 10^{4} cfu·g^{-1} in Ireland or < 10^{3} cfu·g^{-1} in China, for example (FSAI, 2014, Sadiq et al., 2018, Yuan et al., 2012). Additionally, customers often claim for even less spore content. The broad range between < 10 and > 10^{5} cfu·g^{-1} of thermophilic spores in powder and in tendency lower mesophilic spore counts up to 10^{4} cfu·g^{-1} depicts the difficulty to meet the given limits (Buehner et al., 2015, Hill and Smythe, 2012, Kent et al., 2016, Sadiq et al., 2016b, Scott et al., 2007, Watterson et al., 2014, Yuan et al., 2012).

The discussion about spore limits intensifies as the spore level highly depends on the test method used (Kent et al., 2016, Sadiq et al., 2016b). Especially the recovery of mesophilic spores was shown to reduce by increasing heating time and temperature. The quantification of mesophilic spores is fortunately standardised and utilises the pasteurisation at 80 °C for 10 min (Frank and Yousef, 2004, VDLUFA, 1985). Also, highly heat-resistant spores are standardised and enumerated after ISO/TS 27265:2009 and heating at 106 °C for 30 min. In contrast, Chinese regulations require heat treatment at 100 °C for 30 min (Ministry of Agriculture of the People's Republic of China, 2007, based on NEN 6809:1999). Instead, the quantification of thermophilic spores is not harmonised, and methodologies are often applied upon customer’s specifications. These include standards for mesophilic as well as highly heat-resistant spores. The diversity in testing parameters for thermophilic spores, together with the strict limits complicate global trading, transparency of product quality and makes it less comparable.

The three primary contaminants of the thermophilic milk powder microbiota are *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* and *Bacillus licheniformis*. Other less prevalent spore formers include isolates of *Aeribacillus*, *Aneurinibacillus*, *Brevibacillus*, *Paenibacillus*, *Ureibacillus* and other *Bacillus* species as well as *Laceyella* and *Thermoactinomyces* which express a fungi-like morphology on agar plates (summarised by Sadiq et al., 2018). The abundance of each species highly depends on the analysed product and *A. flavithermus* as well as *B. licheniformis* were found to dominate the spore microbiota in samples around the globe (Burgess et al., 2010, Kent et al., 2016, Miller et al., 2015, Pereira and Sant'Ana, 2018, Ronimus et al., 2003, Sadiq et al., 2018, Yuan et al., 2012). While *B. licheniformis* is also highly abundant in non-processed bulk tank milk (BTM), obligately thermophilic species such as *A. flavithermus* and *G. stearothermophilus* were detected only very infrequently in BTM (Miller et al., 2015, Scott et al., 2007). It is assumed that the contamination by those spore formers may instead result from the production environment (Hill and Smythe, 2012, Martinez et al., 2017, Murphy et al., 1999, Scott et al., 2007).

2 Thermophilic spore forming bacteria

Known bacteria are classified using defined criteria of bacterial taxonomy based on genetics as well as on morphological, physiological and biochemical properties. Factors that influence the proliferation are often studied in detail since bacterial growth is essential for the analysis of all phenotypic criteria. The environment, including nutrients, temperature, pH, water and oxygen availability and the presence of competitors defines the ability for growth. In the end, each parameter expresses a specific optimal value for the best growth rate or protein yield, if intended. Microbial growth can be controlled as long as the conditions are kept within the range of tolerance where cells are not inactivated. However, performance decelerates when moving towards the limits.

The categorisation according to the temperature growth range defines four groups: psychrophiles, mesophiles, thermophiles and hyperthermophiles (in ascending order, Figure I.3, Madigan et al. (2019). Psychrophilic microorganisms may grow between 0 ─ 20 °C, and the optimal growth temperature is < 15 °C. For psychrotolerants, the

range extends up to 40 °C. The mesophiles are of highest relevance as the temperature range covers ambient as well as body temperature and includes most pathogenic species. Microorganisms with an optimal growth temperature higher than 45 °C or 80 °C are categorised as thermophiles and hyperthermophiles, respectively. While some *Archeae* are known to proliferate at temperatures > 100 °C, no bacterial species was identified as such yet (Madigan et al., 2019). In principle, the central temperature range is specific to each group, but there is always a particular span of overlap between two groups and the borders merge. So-called obligate bacterial species are exclusively categorised into one group, for example, *Anoxybacillus* and *Geobacillus* species as obligate thermophiles (Coorevits et al., 2012, Nazina et al., 2001, Pikuta et al., 2000). However, species of extended ranges beyond the classical categorisation exist as well. For example, mesophilic *B. licheniformis* optimally grows at 30 °C, but the growth range extends up to 60 °C (Vos et al., 2009, Warth, 1978). A temperature between 55 and 60 °C is optimal for thermophilic growth and exceeds the mesophilic range whereby *B. licheniformis* is also categorised as facultative thermophilic.

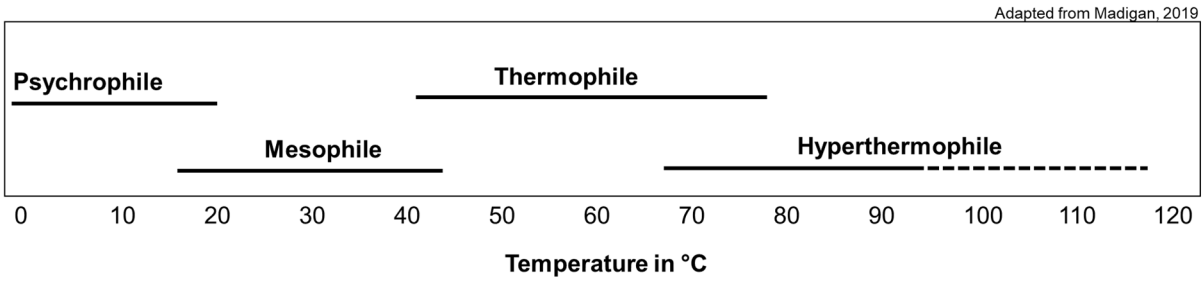


Figure I.3: Temperature ranges of four bacterial groups: psychrophilic, mesophilic, thermophilic and hyperthermophilic bacteria (Madigan et al., 2019).

The temperature growth range similarly describes the conditions of the natural habitat of isolates. Thermophilic bacteria are omnipresent in environments where elevated temperatures prevail, e.g. in geothermal areas or hot sediments (Madigan et al., 2019). Commercial applications of enzymes of thermophiles are widely used as they catalyse reactions at elevated temperatures which is often advantageous. The most famous example is the discovery of the *Taq* polymerase, isolated from thermophilic *Thermus aquaticus* in 1969 (Brock and Freeze, 1969). In the food sector, thermophilic bacteria capable of endospore formation are significant contaminants of various heat-treated and long shelf-life products such as canned and dried foods (Andre et al., 2017).

2.1 Endospore structure and spore formation

The phylum *Firmicutes* includes the anaerobic genus of *Clostridium* and the aerobic family of *Bacillaceae* as endospore formers. Even though oxygen tolerance separates both taxa, the basic spore structure and the mechanism of spore formation is evolutionarily conserved (de Hoon et al., 2010, Galperin et al., 2012). The research was mainly applied to mesophilic *B. subtilis* as the model organism among the spore formers that are ubiquitous in various environments. The diversity of ecological habitats is enormous and includes extreme temperature and pH areas like hot springs or arctic sediments, soil samples as well as the mammalian intestine (Hong et al., 2009, Vos et al., 2009). Some spore formers are of pathogenic relevance causing severe disease like anthrax (*B. anthracis*) or foodborne diseases (*B. cereus, C. difficile*). In contrast, food spoilage is a concern of many non-pathogenic spore formers (e.g. thermophilic spore formers).

Endospores, equivalent to spores, are “metabolically dormant cells composed of a partially dehydrated central core (containing the genome) surrounded by several concentrically arranged protective layers” (McKenney et al., 2013). The structural complexity renders them highly resistant towards unfavourable conditions such as extreme temperatures, radiation, desiccation, depletion of nutrients and more (Setlow, 2014). Enduring spores secure the long-term survival of the organism during extreme conditions. Comparable to the temperature growth range, endospores are categorised as mesophilic, thermophilic and highly heat-resistant spores according to their heat sensitivity (Lucking et al., 2013). The low water content of only 25 ─ 50 % of the wet weight of the spore (Setlow, 2007) does not allow for a detectable metabolic activity during the dormant state of the spore (Sunde et al., 2009).

The structure of endospores was determined to consist of seven basic layers using emerging microscopic techniques such as electron microscopy during many decades of spore research (Figure I.4). The structural details of spores and spore formation in this chapter, if not indicated differently, are based on the three publications by Leggett et al. (2012), McKenney et al. (2013) and Setlow (2007). The innermost part, the spore core contains the essential goods: the genome, RNA, ribosomes and most enzymes that are protected by the surrounding layers. Ca^{2+}-dipicolinic acid (DPA) replaces most of the water in the dehydrated core, and the DNA is saturated with α/β-type small acid-

soluble proteins (SASP) to protect from enzymatic cleavage attacks. Both small molecules, DPA and SASP, are unique for spores and substantially contribute to the spore resistance.

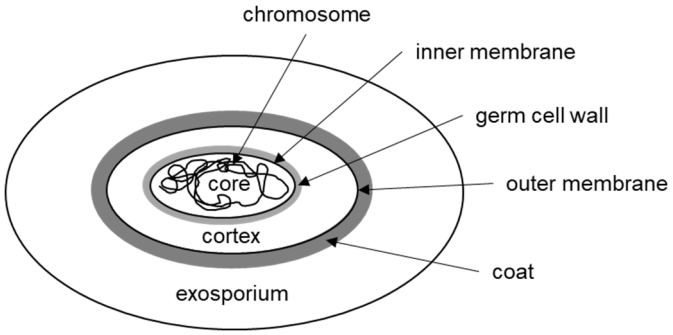


Figure I.4: The basic structure of a bacterial spore, adapted from Setlow (2007).

Around the core, the inner membrane is a strong permeability barrier which was found to be mainly impermeable during the dormancy of the spore. It develops as the cell plasma membrane during germination. The adjacent germ cell wall and cortex are composed of peptidoglycan similar to those of vegetative cells. Both structures are essential for the integrity of the inner membrane, and the bacterial cell wall develops from the germ cell wall during outgrowth. The outer membrane and the overlying spore coat protect the cortex. The spore coat is very rich in various and mostly spore-specific proteins that are sometimes even organised in sublayers. These proteins function in the protection from environmental stress (e.g. from lytic enzymes, reactive chemicals), also from other microbes and in the regulation of germination, e.g. by transmitting germinants such as sugars or amino acids. As the outermost layer of the spores, the exosporium defines the surface. It is not part of the spores of all spore forming species, may vary in size and layer structure. For example, the exosporium is present in *Geobacillus* species and *B. anthracis*, while absent in *B. subtilis* (Seale et al., 2010, Stewart, 2015). As the specific function is not known yet, the exosporium seems to not be essential for the spore resistance and function. Overall, the degree of conservation decreases towards the surface of the spore while the spore core and the inner membrane are highly conserved among different spore forming species.

Endospores are formed during sporulation which is part of the natural growth cycle of spore forming bacteria, in addition to the vegetative cycle of proliferation (Figure I.5). It is widely believed that spore formation, as a survival mechanism, is initiated by adverse conditions like nutrient depletion, e.g. at the onset of stationary phase in

bacterial cultivations, and environmental stress like heat or desiccation (Sonenshein, 2000). However, the exact molecular mechanisms can still not be entitled.

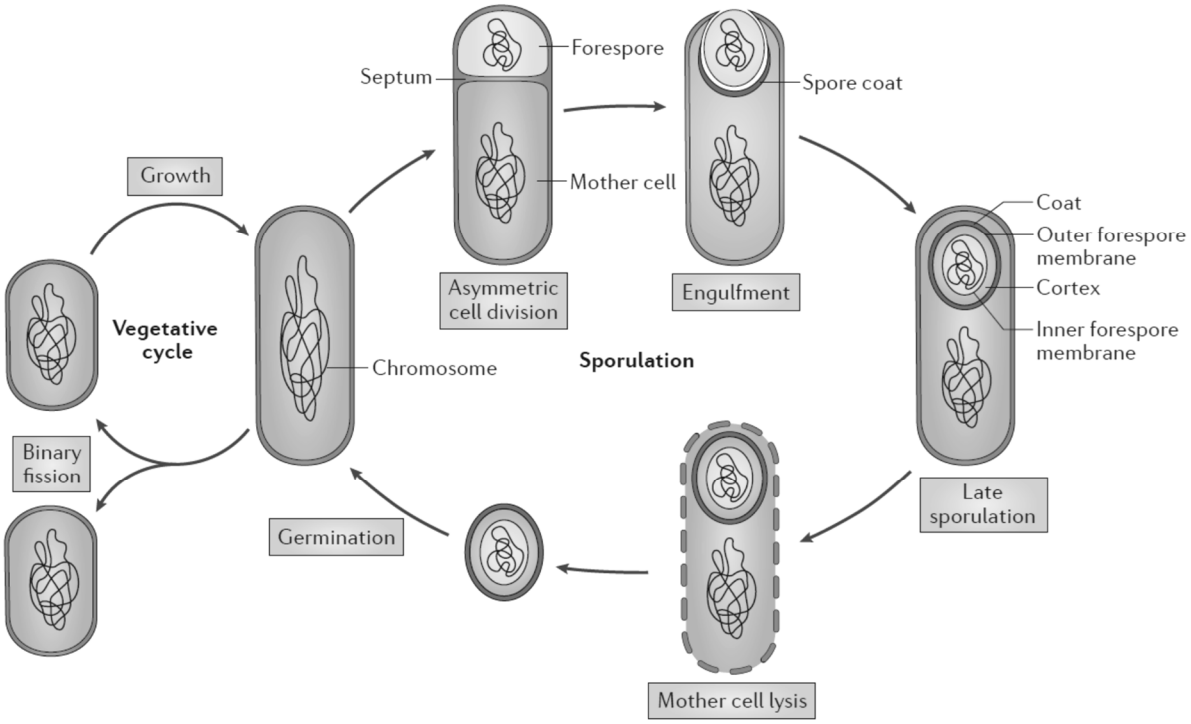


Figure I.5: The sporulation and germination cycle of *B. subtilis* (McKenney et al., 2013).

It is well studied that the phosphorylation of the transcription factor *spo0A* initiates and controls multiple sporulation processes and is essential for the onset of spore formation. Therefore, *spo0A* is known as the master regulator of sporulation (Burbulys et al., 1991, Piggot and Hilbert, 2004). When sporulation is induced, asymmetric cell division of the sporulating mother cell forms the forespore, which will develop as the mature spore (Figure I.5). During adjacent engulfment, the forespore is equipped with two membranes, and the spore coat proteins start to localise at the outer surface simultaneously. Through late sporulation, the spore is fully maturated, and size and water content are reduced. The assembly of peptidoglycan in the cortex between the inner and outer membrane initiates the dehydration and decrease in volume. Additionally, coat layers are packed, vast amounts of DPA are incorporated, and the exosporium is assembled in the end. Finally, cell lysis leads to the release of the mature spore into the environment.

In dormancy, spores are protected from the environment and can survive long-term up to several years or even more. An extraordinary example for long-term survival was isolated from an extinct bee of buried Dominican amber which is believed to be preserved for 24 to 40 million years. The isolate was most closely related to *B. sphaericus* in 1995 (Cano and Borucki, 1995). Moreover, spore isolates of milk powder, produced in the first roller drying factory in New Zealand, taken to Antarctica for an expedition in 1907 and stored there until sampling in 2002, could be revived as well (Ronimus et al., 2006).

Appropriate conditions of nutrients, availability of water or precise stress treatments like heat shocks can resume vegetative growth of spores. Spore germination is initiated by species-specific germinants that interact with germinant receptors on the spore inner membrane, e.g. amino acids, sugars (Setlow, 2003). In response, DPA and other cations are released in exchange to water (partial dehydration of the core), and the hydrolysis of the cortex peptidoglycan allows for the expansion of the core. During the following outgrowth, the spore returns to the state of a vegetative cell. The metabolism is initiated, spore specific small molecules (e.g. SASPs) are hydrolysed, spore specific layers are degraded, and the water content as well as the size increase. The vegetative cell can then proliferate following the vegetative or sporulation cycle, depending on the environmental triggers.

The bacterial environment does not only influence the fate for sporulation or vegetative growth but also affects the developing spore resistance (reviewed by Bressuire-Isoard et al, 2018). Sporulation temperature, medium and pH have a known effect on the resistance. While the optimal conditions preferably lead to an increased spore yield (Nguyen Thi Minh et al., 2011), extreme conditions lead to enhanced resistance. For example, the reduction of the nutrient concentration for sporulation of *Geobacillus* species led to the increased heat resistance of formed spores, expressed in the increase of $D_{121°C}$ values from 1.3 to 5.4 min (Guizelini et al., 2012). The analysis of *B. subtilis* showed the dependency of heat resistance on the maturation state of the spore, and the acquisition of the maximum wet heat resistance during late sporulation (Sanchez-Salas et al., 2011). This underlines the substantial role of the spore structure in spore resistance (Leggett et al., 2012, Setlow, 2014).

2.2 The genus *Anoxybacillus* and the dairy relevant species *A. flavithermus*

The Gram-positive and thermophilic genus *Anoxybacillus* belongs to the taxonomic family of spore forming *Bacillaceae*. *Anoxybacilli* were first described to be obligate anaerobic bacteria (Pikuta et al., 2000). This is expressed in the name: "*anoxy*", short for greek "*an oxygenium*", means without oxygen. Later, the revision of the genus adapted *Anoxibacilli* to be both, aero-tolerant and facultative anaerobes (Pikuta et al., 2003) and multiple aerobic species were added from time to time. Cells of *Anoxybacilli* are rod-shaped and form spherical spores terminally or sub-terminally (Figure I.6). They are ubiquitous in various habitats, in particular where elevated temperatures prevail.

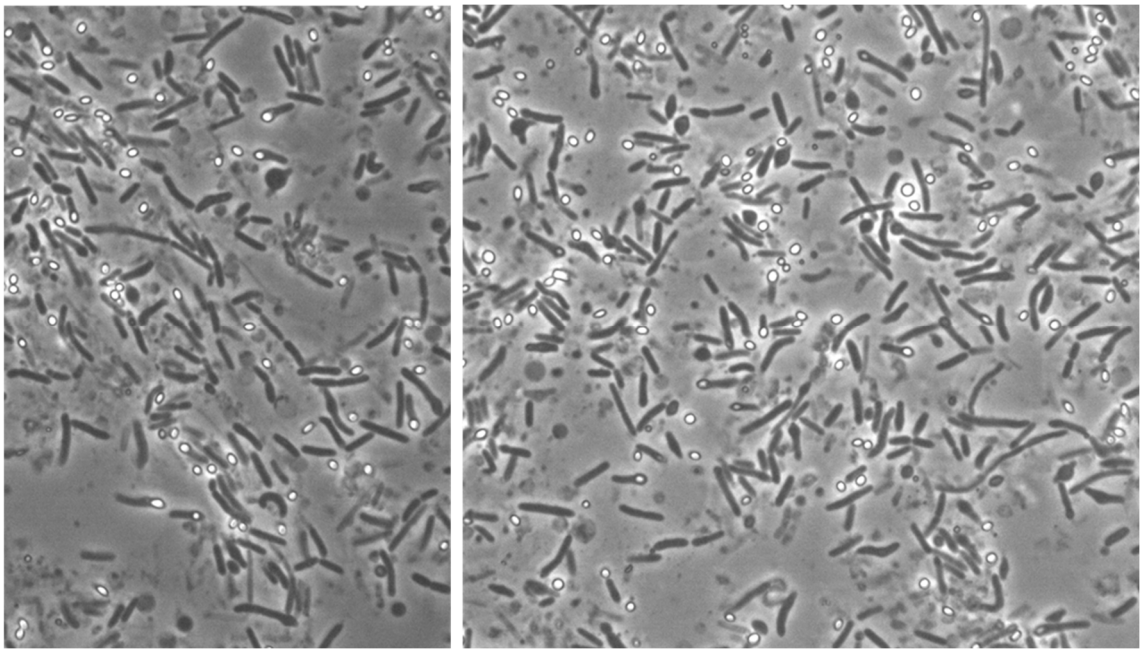


Figure I.6: Light microscopic image of *A. flavithermus.*

In July 2020, the List of Prokaryotic names with Standing in Nomenclature (LPSN, Parte, 2018) comprises 23 validly published *Anoxybacillus* species. The type species *A. pushchinoensis* was isolated from manure (Pikuta et al., 2000). The most recently described species is *A. geothermalis*, originating from mineral deposits of a geothermal station (Filippidou et al., 2016). The species *A. flavithermus* as a contaminant of multiple products such as milk powders and concentrated milk is an issue in the dairy environment (Burgess et al., 2010, Ronimus et al., 2003, Sadiq et al., 2018, Yuan et al., 2012). *A. flavithermus* was first named *Bacillus flavothermus* and reclassified into the genus of *Anoxybacillus* in 2000 (Heinen et al., 1982, Pikuta et al., 2000). The first

strain of a hot spring sample in New Zealand was isolated as a facultative aerobic bacterium of yellow-pigmented colonies, able to grow between 30 and 70 °C aerobically.

Currently, 13 genomes of *A. flavithermus* are published at the National Center for Biotechnology Information (NCBI). The origins of these isolates are dairy processes, wastewater, and environmental isolates of hot springs. Genomes are between 2.6 and 3.7 Mb in size, and the GC content ranges from 41.1 to 43.8 %. Interestingly, clustering of 12 of the published genomes based on the similarity of gene content led to the subdivision into groups of similar origin. In particular, the four dairy isolates were separated (Khalil et al., 2019). The genus *Geobacillus* is the closest phylogenetic relative to *Anoxybacillus* (Goh et al., 2014).

The high heat tolerance of *Anoxybacillus* proteins and their stability in alkaline conditions was attracting the attention for industrial applications. The potential of various enzymes for lignocellulose- (e.g. xylanases), starch-related applications (e.g. amylases), processes that could improve the generation of renewable energy, and others is high (summarised by Goh et al., 2013). However, this is a vast field which is more in the developmental stage at the moment but where the interest of different industries may increase in the future.

2.3 Importance for the milk powder processing industry

Thermophilic spore forming bacteria are a threat in the food industry, particularly in the production of concentrated and dried products. Their natural properties like the thermophilicity as well as endospore formation are beneficial to proliferate and survive at various points in the production plant and the product (Figure I.7, Goh et al., 2013, Hill and Smythe, 2012).

All parts of the production process that are operated at temperatures covering the thermophilic growth range (40 ─ 70 °C) are potentially weak points for thermophilic growth accompanied by spore formation. Processing steps like cream separation as well as heating steps (using heat exchangers) are even operated at optimal thermophilic temperatures between 50 ─ 60 °C as perfect conditions for optimal spore yield and growth rate. Evaporators, including their preheaters, may also be subjected to thermophilic growth which was observed previously (Scott et al., 2007). Similarly,

thermophilic growth was observed on concentration steps during cheese manufacture (Kable et al., 2019).

Once thermophilic spores are formed and entered in the product stream, it is very challenging to remove the spore load. Spores tolerate heat sanitation conditions and more extreme temperatures during the final evaporation and spray drying (Sadiq et al., 2016b, Wells-Bennik et al., 2018). Moreover, the resistance to cleaning agents was established (Wedel et al., 2019). If nutrient-rich milk fouling layers are formed through the passage of milk on heated processing equipment (Visser and Jeurnink, 1997), they serve as a suitable environment for thermophilic proliferation as well. Furthermore, milk fouling impairs the elimination of spores by CIP (Hinton et al., 2002, Wedel et al., 2020).

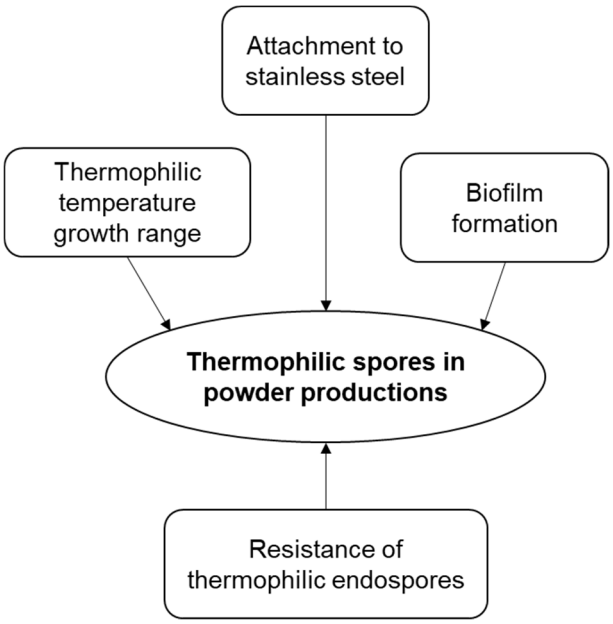


Figure I.7: Properties of thermophilic spore formers that support their presence in dairy powder.

The unique structure of endospores, in particular the overall charge and hydrophobicity, enable their attachment to processing equipment (Palmer et al., 2010). The attachment on stainless steel is further enhanced if the production surface is not clean but covered with milk protein residues which allows the spores to attach and remain in the production plant. Besides, the attachment to the equipment's surface was shown to increase the spore resistance (Simmonds 2003). Once thermophilic spore formers are attached to the equipment's surface, they can form biofilms (Burgess et al., 2009, Coleri et al., 2017, Zhao et al., 2013). For thermophilic *G. stearothermophilus* and *A. flavithermus*, the biofilm formation on stainless steel was more efficient than on polystyrene surfaces and better in production-like milk, compared to laboratory medium (Sadiq et al., 2017). Remarkably, *A. flavithermus* biofilms were composed of up to 50 % spores within eight hours (Burgess et al., 2009). Therefore, biofilms are a potential reservoir of spores during the next production runs,

especially if they are not removed efficiently during plant cleaning (Parkar et al., 2004, Zou and Liu, 2018). Remaining and protected residues can lead to the recontamination of the next production batch and the spread within the production environment.

3 Methods applied: an overview

The detailed methods of this work are described in the material and methods sections of the results chapter (II). In brief, the methods are categorised as following and applied in various parts of this work as indicated.

- **Microbiology of thermophilic spore forming bacteria (part 1-3)**
  - Classical cultivation of bacteria on solid and in liquid media and optimisation of the cultivation conditions.
  - Characterisation of growth and sporulation, also dependent on the growth temperature using classical cultivation methods.
  - Quantification of the total thermophilic cell (TCC) and spore count (SC) using classical plating of dilutions on agar plates. The SC is quantified after heating to 80 °C for 10 min in order to inactivate vegetative cells.
  - Preparation of spore suspensions for analysis of the sensitivity of fully maturated spores in comparison to spores in processed samples.
  - Inactivation experiments using a thermal treatment: analysis of the sensitivity of bacterial cells and spores to heat (80 ─ 98 °C for 0 ─ 30 min) and cleaning solution (1 % NaOH, 65 °C, 10 min). Subsequently, the surviving population is quantified by plating.
- **Identification of isolates to describe the biodiversity in processed samples (part 1-3)**
  - Assessment of the species identity using Fourier-transform infrared (FTIR) spectroscopy which is based on the phenotype.
  - Molecular biological sequencing of the partial 16s rRNA gene was applied to complement the phenotypic identification of species using FTIR spectroscopy.
  - Random Amplified Polymorphic DNA (RAPD) PCR was used to differentiate isolates at strain level by generating isolate-specific amplification patterns.

Before, the RAPD method for differentiation of *A. flavithermus* and *G. stearothermophilus* strains was developed and validated.

- **Industrial samples (part 1-2)**
  - Collection of samples from industrial powder processes which included raw, intermediate and end products of milk- and whey-based products. Detailed process analyses involved samples of different processing time and various intermediate processing steps of one production run.
  - Analysis of samples for their thermophilic TCC and SC. The composition of the thermophilic spore microbiota was determined by the isolation and identification of a representative amount of colonies. RAPD typing was then used to compare same-species isolates for their recurrence in the product of one process over time.
- **Generation of bacterial whole genome sequences (part 3)**
  - Preparation of genomic DNA for whole genome sequencing (WGS) including DNA extraction and preparation of the library for sequencing.
  - WGS using the Next Generation Sequencing platform of Illumina MiSeq.
  - Processing of sequencing data: quality check, filtering, *de-novo* assembly and annotation of bacterial genome sequences to obtain draft genomes.
- **Bioinformatic analysis of bacterial genomes (part 3)**
  - Evaluation of evolutionary relations of genome sequences based on the multiple sequence alignment of 92 conserved core genes.
  - Phylogenomic analysis of genome sequences based on core genome multilocus sequence typing (cgMLST) scheme analysis.
  - Calculation of the pan genome and association of phenotypic traits based on the trait-specific presence or absence of genes in order to associate phenotypic characteristics with genes.
  - Functional characterisation of genes using the Kyoto encyclopedia of genes and genomes (KEGG) databases.
- **Statistics (part 1, 3)** to determine the level of significance of inactivation data and results of growth and sporulation analysis

4 Objectives of this work

The aim of this project is the comprehensive analysis of the microbiology of milk powder productions in order to develop strategies for a constant reduction of thermophilic spore counts. The origin of thermophilic spores in milk powders (MP), which includes the analysis of thermophilic spore forming bacteria as the significant contaminants of milk powder, is assessed. In particular, the species *A. flavithermus* is of interest. The project was divided into three parts (Figure I.8).

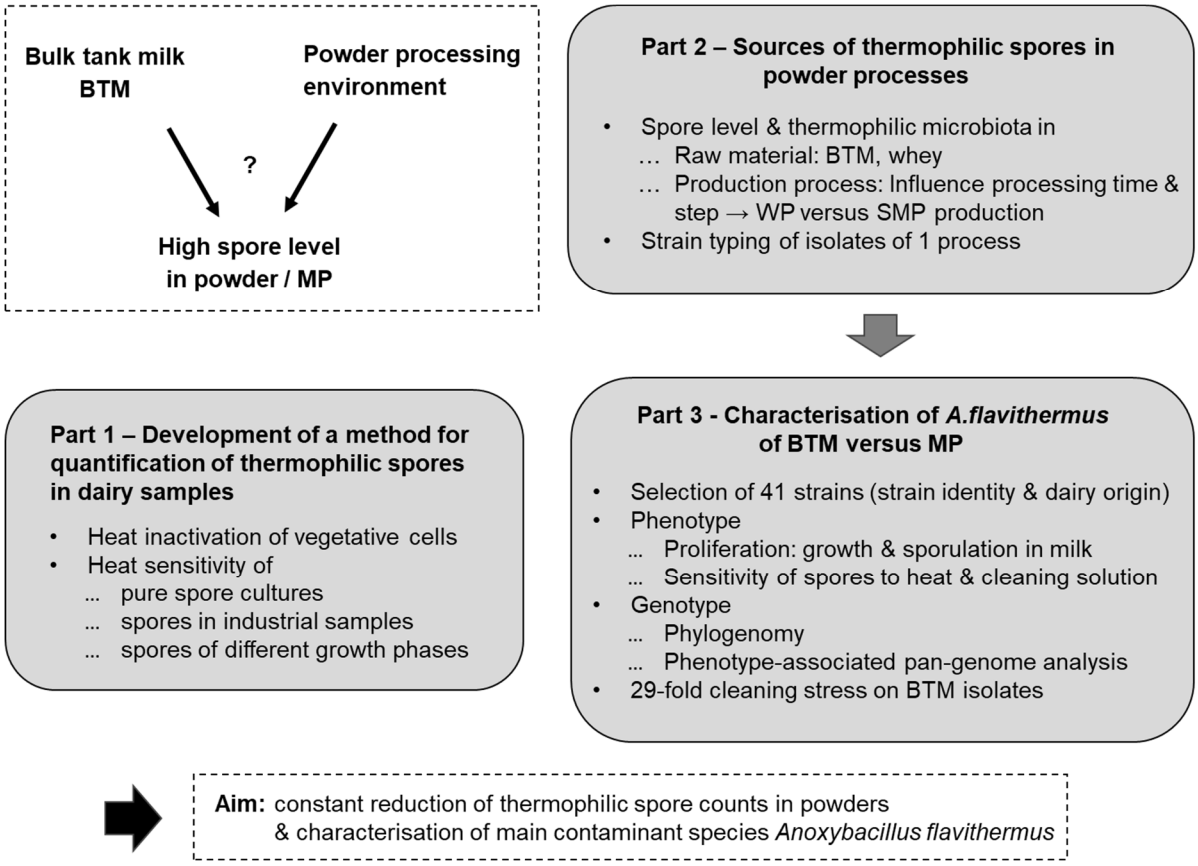


Figure I.8: Overview of the tasks of the three parts of this dissertation. They developed from the question on the origin of high amounts of thermophilic spores in powders (MP).

The quantification of thermophilic spores in dairy samples is not standardised at the moment. Therefore, the first part aims to develop the optimal temperature-time condition for the reliable quantification of thermophilic spores. This requires the inactivation of all vegetative cells while obtaining all spores of the prevalent contaminant species (*A. flavithermus* and *G. stearothermophilus*) and the applicability to industrial samples (e.g. whey and milk powder). The specified method is applied to

subsequent analyses of thermophilic spore count in dairy samples. The results will then not over- or underestimate the spore level but will be more harmonised and better comparable.

The analyses of German milk powder production processes during the second part focus on the contamination route of thermophilic spores. The raw materials, e.g. bulk tank milk or whey, and the recontamination due to the production environment are differentiated as possible origins of high spore levels in milk and whey powders. The assessment of the diversity of the thermophilic spore population of one process at strain level monitors the transmission of strains over time and from the raw materials to the final powders. Tracking the contamination route and assessing the spore level dynamics during production will identify critical processing steps and possibly persistence of thermophilic spores in powder plants. Once the critical steps are known, suggestions for directed optimisation strategies can be developed.

The third part comparatively analyses *A. flavithermus* isolates that originate from BTM and powder (including recurring strains) of different dairies. Parameters that are suspected of supporting their prevalence in powder are characterised phenotypically to identify determinants for their settlement. Associations of the phenotypic observations with the pan genome are used to screen for specific genomic features and functions which describe the phenotype based on whole genome sequences. Additionally, the phylogenomy among the dairy and non-dairy strains compares their evolutionary relations. Following the hypothesis of a microevolutionary adaptation of strains to the process conditions, a long-term adaptation experiment of sensitive strains which are exposed to plant cleaning conditions is conducted. The adaptation study, as well as the characterisation of isolates of different origin, will support the knowledge on entry and settlement of thermophilic spores in milk powder processes. The consideration of critical processing parameters will allow for getting back to a low-spore condition in the production plant, to prevent further increasing spore levels and the colonisation of the plant.

II RESULTS

The results of part 1 and 2 of this dissertation were published previously, and the original publications are added. The results of part 3 are presented as a drafted manuscript which is intended for publication. The personal contributions are as follows:

Part 1:

Dettling, A., Doll, E., Wedel, C., Hinrichs, J., Scherer, S., and Wenning, M. (2019). Accurate quantification of thermophilic spores in dairy powders. *Int Dairy J* 98:64-71.

Personal contribution: The study was designed by A. Dettling, E. Doll and M. Wenning. Pre-experiments were performed by E. Doll. A. Dettling conducted the final experiments and data analysis as published. The first draft of the manuscript was written by A. Dettling and edited by all other co-authors.

Part 2:

Dettling, A., Wedel C., Huptas C., Hinrichs, J., Scherer S. and Wenning, M. (2020). High counts of thermophilic spore formers in dairy powders originate from persisting strains in processing lines. *Int J Food Microbiol* 335

Personal contribution: The study was designed by A. Dettling, C. Wedel and M. Wenning. M. Wenning and C. Wedel collected the samples for the process analysis in dairy F. A. Dettling and M. Wenning supervised the collection of all other samples. All experiments and data analysis was conducted by A. Dettling, whereas C. Huptas conducted the calculations for phylogenomic UBCG clustering and cgMLST scheme analysis. A. Dettling wrote the first draft of the manuscript which was edited by all other co-authors.

Part 3:

Dettling, A., Huptas C., Ardern Z., Hinrichs J., Scherer S. and Wenning, M. Phenotypic and genomic characterisation of *Anoxybacillus flavithermus* strains originating from bulk tank milk versus powder production including persister strains. *Unpublished*.

Personal contributions: A. Dettling conducted the laboratory experiments and data analysis throughout the study. C. Huptas supervised the bioinformatic analyses and conducted the calculations for phylogenomic UBCG clustering and cgMLST scheme analysis. Z. Ardern advised on the evolution experiment. A. Dettling, M. Wenning and S. Scherer designed the study. A. Dettling wrote the first draft and all authors contributed to the writing of the manuscript.

1 Part 1: “Accurate quantification of thermophilic spores in dairy powders”

Summary

The quantity of thermophilic endospores determines the product quality of dairy powders. Unfortunately, the method for determination of the thermophilic spore count is not standardised yet. This questions the comparability and potential of over- or underestimation of the thermophilic spore load if different methods, including standards for mesophilic and highly heat-resistant spores, are applied. The analysis of the heat inactivation effect of different temperature-time combinations on vegetative cells, spores and processed samples aimed at finding reliable conditions. The focus was on obligate thermophilic bacilli. Ideally, the heating step should inactivate all vegetative cells while leaving the spores unaffected.

Spores of *A. flavithermus* and *G. stearothermophilus* were highly resistant towards heat at 80 − 95 °C when heating for up to 30 min, similarly when heating in milk or laboratory medium. In contrast, vegetative cells were very susceptible to heat at 80 °C, independent from the heating time and growth medium. The collected industrial samples of whey (n=17), WP (n=20) and SMP (n=23) of 13 different companies had initial thermophilic spore counts between 1.4 and 5.4 log cfu·mL^{-1}. While the combination 80 °C, 10 min did not affect the spore level, the intensification of the treatment (80 → 98 °C, 10 → 30 min) led to the significant reduction of the spore level by up to 1.3 log levels. This decrease is in discrepancy to the high resistance of pure spores. Between 85 − 93 % of the thermophilic spore microbiota of the industrial samples were composed of *A. flavithermus*. Mesophilic *B. licheniformis* was less prevalent and only isolated from 1/5th of samples. Further, thermophilic spore formers were investigated to develop their heat resistance dependent on the bacterial growth phase. At inoculum and during stationary phase, the heat sensitivity (80 − 95 °C, 10 min) was reduced, whereas newly formed spores were heat sensitive.

In conclusion, the spore population in industrial samples expressed a heterogeneity which does not reflect pure spore cultures, depicted in the increased heat inactivation effect. The analyses suggest that the full maturation of spores is necessary to acquire maximal heat resistance. As commercial powders contain a diverse spore population (species composition & maturation state), a temperature < 90 °C will provide more realistic test results. The proposal for reliable quantification of thermophilic spores is the application of 80 °C, 10 min similar to the standard for mesophilic spores.

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Accurate quantification of thermophilic spores in dairy powders

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ABSTRACT

Internationally, there are no official guidelines for the quantification of thermophilic spores in dairy products, which leads to variations in applied methodology. In this study, we assess the heat sensitivity of thermophilic spores, vegetative cells grown under laboratory conditions and spores in German dairy powders to determine appropriate heating conditions for accurate quantification of total thermophilic spores. The heat inactivation effect (80–95 °C) is limited for spores of *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* grown under laboratory conditions. However, for spores originating from whey, whey powder and skimmed milk powder (mostly identified as *A. flavithermus*), a different trend was observed; spore counts continuously reduced when heating time and temperature increased (80–98 °C, 10–30 min). The results indicate that data obtained using laboratory cultures cannot be extrapolated to commercial powders, and in this case, applying temperatures above 80 °C leads to an underestimation of spore counts in dairy powders.

1. Introduction

Powdered dairy products have high economic value and are well suited for global trading. They do not require strict storage conditions and have a long shelf life, as they are less prone to microbial growth due to low water activity ($a_w < 0.26$) (Hill & Smythe, 2012). Because vegetative cells are not able to survive the powder production process, the microbial load of milk powder is mostly due to heat stable endospores, as long as recontamination events are avoided (Reich et al., 2017).

Many previous studies have reported that endospores in dairy powders belong to the thermophilic bacilli group, and the microbiota is often dominated by the two non-pathogenic species *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* (Burgess, Lindsay, & Flint, 2010; Nazina et al., 2001; Pikuta et al., 2000; Ronimus et al., 2003; Sadiq, Flint, & He, 2018; Yuan et al., 2012). Both species are obligately thermophilic and believed to result from growth over the production process. Additionally, endospores of mesophilic bacilli are frequently isolated. *Bacillus licheniformis* is a mesophilic bacterium, with an extended temperature growth range up to 60 °C, and the third major spore-forming species found in powders worldwide (Burgess et al., 2010; Kent, Chauhan, Boor, Wiedmann, & Martin, 2016; Ronimus et al., 2003; Ruckert, Ronimus, & Morgan, 2004; Yuan et al., 2012).

The spore load of dairy powders has been reported to be highly variable between different batches of the same product, as well as between different product types, and covers between <10 cfu·g^{-1} and > 10^5 cfu·g^{-1} (Hill & Smythe, 2012; Kent et al., 2016). Besides the absence of pathogenic bacteria (e.g. *Staphylococcus aureus*), thermophilic spore populations have emerged as one of the most important parameters for production hygiene, and, as a result, stringent criteria have been set to limit spore content. In particular, specifications for acceptable thermophilic spore counts have been lowered, which poses a major challenge for manufacturers to fulfil. The US Dairy Export Council must adhere to a limit of 500 cfu·g^{-1} thermophilic spores (Watterson, Kent, Boor, Wiedmann, & Martin, 2014). However, in Ireland or China, the thermophilic count is not specified in detail, but rather the aerobic plate count is limited to <10^4 cfu·g^{-1} in Ireland (FSAI, 2014) and < 10^3 cfu·g^{-1} in China (Yuan et al., 2012). Global trading requires harmonised methods for quality control to ensure comparability of results between different countries, manufacturers and customers. Yet, unlike the

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0958-6946/

quantification of mesophilic and highly heat-resistant spores, there is no standardised methodology for the enumeration of thermophilic endospores, which leads to a large variation in methods applied (Burgess et al., 2010; Coorevits et al., 2008; McGuiggan, McCleery, Hannan, & Gilmour, 2002; Miller et al., 2015; Murphy, Lynch, & Kelly, 1999; Ruckert et al., 2004; Rueckert, Ronimus, & Morgan, 2005; Yuan et al., 2012).

Culture-based quantification of bacterial spores includes a heating step to inactivate vegetative cells. According to Reich et al. (2017), the inactivation temperature of vegetative thermophilic spore formers exceeds common pasteurisation temperatures, but lies well below 80–85 °C. The standard methodology for mesophilic spores of aerobic bacilli includes a heat treatment at 80 °C for 10 min, (VDLUFA M 7.17.2; Frank & Yousef, 2004), whereas highly heat-resistant spores are enumerated after heating for 30 min at 106 °C (ISO/TS 27265:2009). In China, regulations demand a heat treatment at 100 °C for 30 min, including the equilibration of the sample (PSPRC, 2007), based on NEN 6809:1999. These parameters, however, have been shown to influence spore level detection as well as species recovery after heating (Kent et al., 2016). By increasing temperature, the counts of both mesophilic and thermophilic spores decreased, although the effect was much more pronounced for mesophilic spores.

This study analyses the impact of different temperature–time combinations on the thermophilic spore count in dairy powders of German origin, focusing on obligate thermophilic bacilli. Different temperature–time combinations were analysed for their effect on vegetative cells and spores of thermophilic spore formers grown in laboratory conditions. In addition, processed samples of skimmed milk powder (SMP), whey powder (WP) and whey from German manufacturers were analysed for their microbial composition and heat sensitivity of thermophilic spores. This data ultimately contributes to the effort of finding the optimal temperature–time combination for reliably inactivating vegetative cells while leaving spores unaffected to prevent over- or underestimation of thermophilic spore counts in dairy powders.

2. Materials and methods

2.1. Processed dairy samples

Processed dairy samples (whey, WP and SMP) were obtained from 13 different companies. Altogether, the study included 17 samples of whey, 20 samples of WP and 23 different SMPs. Whey samples were used directly for thermal treatments, whereas the powdered dairy products had to be reconstituted. Therefore, the respective powders were dissolved homogeneously 1:10 in ¼ Ringer's solution (Merck).

2.2. Bacterial strain selection

All strains used in this study were originally isolated from dairy products like SMP or raw milk (see Table 1). Their identities were confirmed by 16S rDNA sequencing. Microorganisms were cryoconserved, stored at −80 °C and plated on tryptic-soy-agar plates (TSA, Roth) prior to use.

2.3. Preparation of spore and cell suspensions for heat experiments

2.3.1. Spore suspensions

Spore suspensions were prepared for three thermophilic strains: *A. flavithermus* G8748 and *G. stearothermophilus* EG1950 and EG1938. First, a culture in 7 mL tryptic soy broth (TSB, Merck) was inoculated and incubated at 55 °C for 24 h ± 30 min. Second, 200 µL culture was plated on sporulation media [TSA (Oxoid) where

Table 1
Thermophilic bacilli used in this study and their respective isolation sources.[a]

Species	Strain	Food source
Anoxybacillus flavithermus	G8748[2]	Milk powder
	EG1851	Skim milk powder
	EG3109	Skim milk powder
	F48[1]	Skim milk powder
	G10613	Skim milk powder
Geobacillus stearothermophilus	EG1938	Skim milk powder
	EG1950	Skim milk powder
	EG1951	Skim milk powder
	EG1956[2]	Skim milk powder
	EG3113	Skim milk powder
	G6286	Raw milk
	G8742[2]	Milk powder

[a] References indicated by superscripts 1 and 2 are Lucking et al. (2013) and Reich et al. (2017), respectively.

0.1% (v/v) 1 mM $FeSO_4 \cdot 7H_2O$, 1 M $Ca(NO_3)_2 \cdot 4H_2O$ and 0.1 M $MnCl_2 \cdot 4H_2O$ (Roth) were added, in accordance with Wedel et al., 2018], and incubated at 55 °C for 5–13 days.

Using microscopy, the degree of sporulation was evaluated. When the spore level was higher than the level of vegetative cells, at high spore levels, the culture was harvested. 5 mL phosphate buffer (2 mM KH_2PO_4, 8 mM K_2HPO_4, 4 °C) were used twice to carefully wash spores from the surface of the plate. To prevent aggregation of spores, the harvested spore suspension was stirred for 1–2 h in an ice bath. The next day, the pellet of the suspension was washed three times in 20 mL cold phosphate buffer. In between, the suspension was centrifuged at 4000 rpm and 4 °C for 7 min. Following the last washing step, the pellet was resuspended in 10 mL phosphate buffer and 10 mL 70% ethanol to inactivate remaining vegetative cells. After cool storage for 2–3 days, this washing step was repeated three times using cold phosphate buffer to remove the ethanol. The final spore suspension was stored at 4 °C. For thermal inactivation experiments, spore suspensions were diluted in TSB or milk (UHT-milk, 1.5% fat). The heat sensitivity of all strains was analysed in three replicates.

2.3.2. Vegetative cells

The heat sensitivity of vegetative cells of thermophilic bacilli was evaluated in TSB (Roth) and milk (UHT-milk, 1.5% fat) using suspensions from freshly grown day cultures. For the selected strains (*A. flavithermus* EG1851, F48, EG3109, G8748 and *G. stearothermophilus* EG1951, EG3113, G8742), a fresh dilution streak on TSA was used for the inoculation of an overnight (O/N) culture in 10 mL TSB. On the following day, 50 mL of TSB or milk were inoculated 1:500 using the O/N culture. After 6 h of incubation at 55 °C with shaking at 110 rpm, the culture was harvested to determine heat sensitivity. A centrifugation step (2000 × *g*, 15 min, room temperature = RT) was included to increase the initial cell count of the culture. The cell pellet was resuspended in 5 mL phosphate buffer or milk. For the thermal treatment, the resuspended cell culture was diluted 1:10 in phosphate buffer or milk, and the cell count before and after a distinct heat treatment was measured by plating on TSA. Each strain was analysed in at least three independent biological replicates.

2.4. Thermal treatment

The thermal treatment of spore suspensions, vegetative cells and processed samples was conducted in a temperature-adjusted water bath where the temperature was controlled continuously. Each treatment was conducted in a volume of 10 mL. A pilot tube was used for monitoring the temperature of the samples during each heating experiment and determining the start of the holding

time (time for equilibration < 4 min). Chinese regulations require the application of a heat treatment at 100 °C for 30 min (PSPRC, 2007). As 100 °C in an open water bath is achievable only at sea level, this study was conducted at the uppermost temperature of 98 °C. In this case, holding time began directly after placing the samples into the water bath; in contrast, for 80, 90 and 95 °C, the holding time began after equilibration of the samples to their respective temperatures.

Spore suspensions were analysed at 80, 90 and 95 °C for 0, 15 and 30 min. A heat treatment for 0 min represents the effect of equilibrating the sample without any holding time, and the test tubes were cooled directly after the respective temperature was reached. For dairy samples, a trial at 98 °C for 30 min was additionally analysed. Vegetative cells were analysed at 80 °C and a heating time of 0 and 30 min.

Processed samples were analysed once, whereas spore suspensions and vegetative cells were analysed in at least three independent replicates. Thermophilic cell counts were measured by plating on TSA and incubating the plates for 48 h at 55 °C.

2.5. Biodiversity of thermophilic spore formers

Reconstituted WP and SMP, as well as whey samples, were heated at 80 °C and kept for 10 min. Afterwards, the samples were cooled to RT, plated on TSA and incubated at 55 °C for 48 h. Subsequently, 25 pure colonies per sample were isolated randomly to allow for quantitative analysis of species composition. Pure colonies were identified using FTIR spectroscopy and selected isolates additionally by partial 16S rDNA sequencing. FTIR spectroscopy was conducted as described previously and after incubation of isolates at 55 °C for 24 h ± 0.5 h on TSA (Doll, Scherer, & Wenning, 2017; Oberreuter, Seiler, & Scherer, 2002; Wenning et al., 2014). Partial 16S rDNA sequencing was executed using primers 27f and 1492r, as described by Doll et al. (2017), and the EzTaxon server was used for identification (Yoon et al., 2017). For five samples of SMP, the biodiversity could not be determined because the number of pure isolates was not appropriate (less than 10 colonies). If spore counts were low, all colonies present were chosen (14 colonies at least).

2.6. Heat sensitivity of spores during growth at 55 °C

Two strains, *A. flavithermus* G10613 and *G. stearothermophilus* EG1956, were chosen to test the sensitivity of spores in regards to heat during growth and spore formation in liquid culture. A fresh dilution streak on TSA was used for the inoculation of an O/N culture (55 °C, 150 rpm, 10 mL 1.5% fat UHT-milk). On the next day, UHT-milk (1.5% fat) was inoculated 1:1000 using the O/N culture and incubated at 55 °C, shaking at 150 rpm. The culture was sampled directly after inoculation (= 0 h) to determine the inoculation level. Here the total thermophilic cell count and spore count after heat treatment at 80 °C and 10 min were measured by plating on TSA.

Further samples were taken every hour between 1 and 8 h for *A. flavithermus* G10613 and between 3 and 8 h for *G. stearothermophilus* EG1956. The last sampling was after 24 h of cultivation. For all samples, the total thermophilic cell count and spore counts after heating at 80, 90 and 95 °C for 10 min were determined by plating on TSA. All plates were incubated at 55 °C for 48 h. Thermal treatments were carried out in a thermal shaker while shaking at 600 rpm (T_{set} ± 1 °C). The temperature was controlled continuously and the heating time began when T_{set}−1 °C was reached. After the treatment, the samples were cooled directly to RT using tap water.

For *A. flavithermus* and *G. stearothermophilus*, seven and eight cultures were respectively grown and analysed independently.

3. Results and discussion

3.1. Heat resistance of thermophilic spores

Spore suspensions of three thermophilic test strains were prepared and tested for survival in milk and TSB after heating at temperatures between 80 and 95 °C. Initial spore counts prior to the heat treatment were between log 3.9 cfu mL^{-1} and log 5.7 cfu mL^{-1}. As expected, spores were largely unaffected by the heat treatment (Table 2). The maximum reduction for *A. flavithermus* G8748 as well as *G. stearothermophilus* EG1950 was 0.1 log in TSB and milk. The highest reduction was measured for *G. stearothermophilus* EG1938 at 90 °C for 30 min in TSB (log reduction = log 0.17 ± log 0.29). However, the decrease at 95 °C was less pronounced and an inactivation of EG1938 in milk could not be observed (Table 2). It is therefore assumed, that the differences observed may originate from methodological variance. Overall, thermophilic spores of *A. flavithermus* and *G. stearothermophilus* formed at laboratory conditions exhibit a high resistance to temperatures between 80 and 95 °C, as applied in this study.

The results are in line with previous studies that have investigated the heat sensitivity of thermophilic spores with a more intense heat treatment at 100 °C for 30 min (Sadiq et al., 2016; Wells-Bennik et al., 2018). Sadiq et al. (2016) focused on the heat resistance of thermophilic isolates from Chinese milk powders (incl. *G. stearothermophilus*) and observed 100% survival of spores after heat treatment of 100 °C for 30 min for all test strains. Similarly, the inactivation effect among 18 strains of *G. stearothermophilus* (of various origin) in the study of Wells-Bennik et al. (2018) was smaller than 0.2 log.

Only heat treatments of higher intensity at > 100 °C lead to a heat inactivation effect (Wedel et al., 2018; Yuan et al., 2012). $D_{121°C}$-values among the species *G. stearothermophilus* vary between different strains and are in the range of less than one to several minutes (e.g. Dogan, Weidendorfer, Müller-Merbach, Lembke, & Hinrichs, 2009; Guizelini, Vandenberghe, Sella, & Soccol, 2012; Rigaux, Denis, Albert, & Carlin, 2013; Wells-Bennik et al., 2018).

3.2. Heat sensitivity of thermophilic vegetative cells

Four strains of *A. flavithermus* and three strains of *G. stearothermophilus* were chosen to analyse the thermal inactivation behaviour of freshly grown vegetative cells at 80 °C. During a screening of thermophilic growth in milk and TSB (data not shown), some strains grew in TSB but not in milk. For other strains it was vice versa, and only one strain of the test set (EG3113) demonstrated sufficient growth in both TSB and milk (cell count higher than log 4 cfu mL^{-1} within 6 h).

In TSB, the cells of the *G. stearothermophilus* strain G8742 were completely eliminated after equilibrating to 80 °C (0 min) (Fig. 1A). For the other four test strains (*A. flavithermus* EG1851 and F48, *G. stearothermophilus* EG1951 and EG3113) the cell count was decreased by at least 2.8 log. The inactivation effect did not increase with prolonged heating time (30 min), but ended in a plateau of equal cell counts and consequently resulted in a similar log reduction for these strains (Fig. 1A). In milk, the findings were comparable, as vegetative cells were either fully eliminated after equilibration to 80 °C (EG3109) or reduced by more than four log levels with a similar reduction for both heating times (Fig. 1B). Strain EG3113 displayed better growth in milk and reached an initial cell count that was 1 log level higher. As the spore levels were equal in both milk and TSB, we may attribute this effect to a larger fraction of vegetative cells that lead to the observed higher inactivation of 1 log level. As discussed above, thermophilic spores grown in lab culture can withstand temperatures <100 °C. The plateau

Table 2
Reduction of spores of *A. flavithermus* G8748 and *G. stearothermophilus* EG1950, EG1938 in milk and tryptic soy broth (TSB).[a]

Temp. (°C)	Heating time (min)	*A. flavithermus* G8748		*G. stearothermophilus* EG1950		*G. stearothermophilus* EG1938	
		TSB (log cfu·mL^{-1})	Milk (log cfu·mL^{-1})	TSB (log cfu·mL^{-1})	Milk (log cfu·mL^{-1})	TSB (log cfu·mL^{-1})	Milk (log cfu·mL^{-1})
80	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	15	0.01 ± 0.03	−0.05 ± 0.08	0.03 ± 0.01	0.07 ± 0.04	0.03 ± 0.11	−0.03 ± 0.03
	30	0.04 ± 0.01	0.03 ± 0.03	<0.01 ± 0.06	0.05 ± 0.03	0.06 ± 0.06	−0.01 ± 0.05
90	0	−0.01 ± 0.03	−0.03 ± 0.07	<0.01 ± 0.04	0.03 ± 0.04	0.03 ± 0.25	0.05 ± 0.24
	15	0.04 ± 0.02	0.03 ± 0.05	0.06 ± 0.02	0.07 ± 0.06	0.12 ± 0.27	<0.01 ± 0.24
	30	0.03 ± 0.02	0.10 ± 0.10	0.06 ± 0.01	0.05 ± 0.05	0.17 ± 0.29	−0.03 ± 0.21
95	0	−0.01 ± 0.02	0.01 ± 0.08	0.02 ± 0.05	0.04 ± 0.04	−0.10 ± 0.12	−0.08 ± 0.08
	15	<0.01 ± 0.01	0.05 ± 0.05	0.04 ± 0.01	0.11 ± 0.06	0.03 ± 0.15	−0.09 ± 0.04
	30	0.06 ± 0.03	0.11 ± 0.02	0.08 ± 0.03	0.05 ± 0.05	0.06 ± 0.14	−0.07 ± 0.02

[a] Thermal treatments were applied at 80, 90 and 95 °C. Cell counts were measured after heating times of 0, 15 and 30 min. Log reduction of cell counts after distinct heat treatment were calculated in relation to cell counts of the treatment at 80 °C and 0 min [$\log(N_{80°C,\ 0\ min}) - \log(N_t)$]. Each data point results from three replicates.

observed for all strains therefore likely represents spores that are not affected by the heat treatment. This spore count could be the result of sporulation during cultivation or residual spores of the inoculum.

In this study vegetative cells of seven test strains of *A. flavithermus* and *G. stearothermophilus* were highly sensitive to heat treatments at 80 °C. Thermal treatments of lower intensity (pasteurisation conditions at 63–73 °C) have previously been demonstrated to not be sufficiently effective for inactivating all vegetative cells, especially those of heat-stable strains (Reich et al., 2017). Thus, a treatment at temperatures below 80 °C may be insufficient, but 80 °C fully inactivates vegetative cells and only spores remain viable.

3.3. Analysis of processed dairy samples

Many different temperature–time combinations are used to determine thermophilic spore counts, and it is unclear to what extent they are comparable. Hence, we elucidated how heat treatments of various intensities affect spores occurring in dairy products. In addition, it was evaluated how heat resistance determined using laboratory spore suspensions compares to that of real samples. For this purpose, 17 samples of whey, 20 WP, as well as 23 SMP were analysed. Heating conditions were chosen between 80 and 98 °C and heating times varied between 10, 20, or 30 min to test heat inactivation effects in more detail than previously conducted for spore suspensions.

As samples were obtained from 13 different German companies and production sites throughout different seasons, the sample pool was highly diverse. Consequently, a large variance in microbial counts between samples of the same product type was observed. Total thermophilic cell counts determined without any heat treating ranged from log 1.4–4.1 cfu·mL^{-1} for whey, log 2.9–4.5 cfu·mL^{-1} for WP and log 2.6–5.4 cfu·mL^{-1} for SMP. Treating the samples at 80 °C for 10 min led to a minimal decrease in numbers, log 0.2 ± 0.2 cfu·mL^{-1} (SMP, WP), on average. No reduction was observed for whey samples.

To get an impression of microbial composition, the thermophilic microbiota of all samples were analysed after heating at 80 °C for 10 min. To allow for a quantitative evaluation, colonies were selected randomly and not by morphology. With six species in total, low biodiversity was detected. Independent of the product type (whey, WP, SMP), *A. flavithermus* was the dominant species, representing 85–93% of the isolates on average (Table 3). Occasionally, there were samples with lower fractions of *A. flavithermus*, but in each of the three products, there were many samples in which it was the only species detected (Table 3). *B. licheniformis*, a mesophilic spore-forming bacterium able to grow at the optimal thermophilic

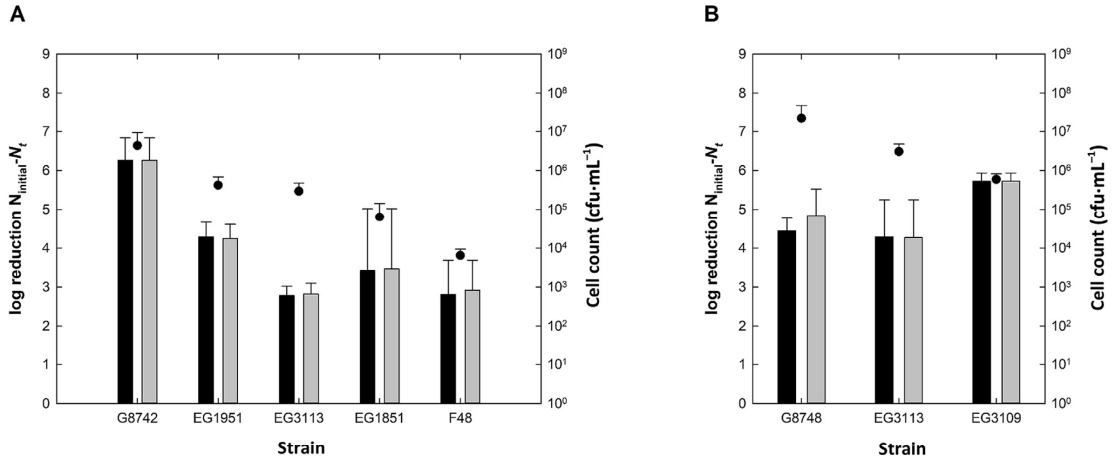

Fig. 1. Logarithmic reduction of cell counts for different strains of *A. flavithermus* (EG1851, F48, EG3109, G8748) and *G. stearothermophilus* (EG1951, EG3113, G8742). Cultures were grown for 6 h at 55 °C, 110 rpm, in tryptic soy broth (TSB, A) and milk (B). Cell counts were measured before the thermal treatment (initial cell count $N_{initial}$, ●) and after the treatment applied at 80 °C for 0 min (no holding time, ■) and 30 min (▒). The reduction [$\log(N_{initial}) - \log(N_t)$] was calculated. Each data point results from three independent biological replicates. Significant reduction for all strains ($p < 0.05$, paired t-test).

Table 3
Abundance of spore formers in processed dairy samples after heating at 80 °C for 10 min.[a]

Species	SMP (n = 18)			Whey (n = 17)			WP (n = 20)		
	average	range	X	average	range	X	average	range	X
Anoxybacillus flavithermus	85.4%	60–100%	18	92.7%	0–100%	16	92.2%	42–100%	20
Bacillus licheniformis	7.2%	0–30%	7	0.3%	0–5%	1	0.7%	0–5%	3
Geobacillus stearothermophilus	6.1%	0–35%	5	5.7%	0–89%	2	6.8%	0–58%	7
Aneurinibacillus thermoaerophilus	0.3%	0–5%	1	0.4%	0–7%	1			
Brevibacillus aydinogluensis	0.2%	0–4%	1				0.3%	0–5%	1
Paenibacillus cookii				0.3%	0–5%	1			
Not identified	0.8%	0–15%	1	0.6%	0–11%	1			

[a] Recovery and isolation at 55 °C. Abbreviations are: SMP = skimmed milk powder; WP = whey powder; n = number of samples; X = isolated from X samples per product type.

growth temperature of 55 °C, was the second highest representative among isolates from SMP, with fractions up to 30% in single samples and 7% on average (Table 3); for WP and whey samples, the abundance was even lower and <1% on average. Overall, *B. licheniformis* was isolated from only one fifth of all samples. The obligate thermophilic species *G. stearothermophilus* accounted for 6–7% of all isolates in samples of all product types and therefore represented the second highest fraction in whey and WP.

Other species like *Aneurinibacillus thermoaerophilus* and *Brevibacillus aydinogluensis* (both obligate thermophilic), as well as *Paenibacillus cookii* (mesophilic), were present in few samples in low abundance. Taken together, obligate thermophilic spores represented the major part of the microbiota of samples tested in this study; facultative thermophilic bacteria like *B. licheniformis* were of minor abundance. Prior analyses of milk powders have determined *A. flavithermus*, *B. licheniformis* and *G. stearothermophilus* as the most prominent representatives among the thermophilic microbiota at varying abundances, as summarised by Ruckert et al. (2004) and Pereira and Sant'Ana (2018) for different countries. Some studies primarily identified the facultative thermophilic *B. licheniformis* (e.g. Kent et al., 2016), whereas other studies are in accordance with our results and found a high prevalence of obligate thermophilic spores (e.g. Scott, Brooks, Rakonjac, Walker, & Flint, 2007). *B. licheniformis* is assumed to originate from raw milk, as its occurrence is ubiquitous in nature, whereas high counts of *Anoxybacillus* and *Geobacillus* are due to the production process itself (Miller et al., 2015; Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005; te Giffel, Wagendorp, Herrewegh, & Driehuis, 2002).

The spore inactivation was evaluated by calculating the log reduction [$\log(N_{80°C,10\ min}) - \log(N_t)$] of each thermal parameter related to the treatment at 80 °C for 10 min. Heat treatments at different temperatures resulted in an increasing reduction of spore counts upon intensifying the treatment, which indicates at least an elevated heat sensitivity of spores in commercial powder samples from German manufacturers (Fig. 2). The spore counts in whey and WP were more affected compared to SMP samples, while WP samples exhibited the highest sensitivity and reduction. Except for the SMP treatment at 80 °C for 20 min, all other heat treatments led to a statistically significant ($p < 0.05$) reduction in spore counts. At 80 °C, thermophilic spore counts were reduced only slightly by 0.1–0.3 log (Supplementary material Fig. 1A and B). Higher temperatures of 90 and 95 °C further decreased the spore counts, and highest median reductions were observed at 98 °C [0.9 log (SMP) < 1.1 log (whey) < 1.3 log (WP)]. By increasing heating time (10 < 20 < 30 min) and temperature (80 < 90 < 95 < 98 °C), the reduction of spore counts continuously increased (Fig. 2; Supplementary material Fig. 1) and the level of significance for this

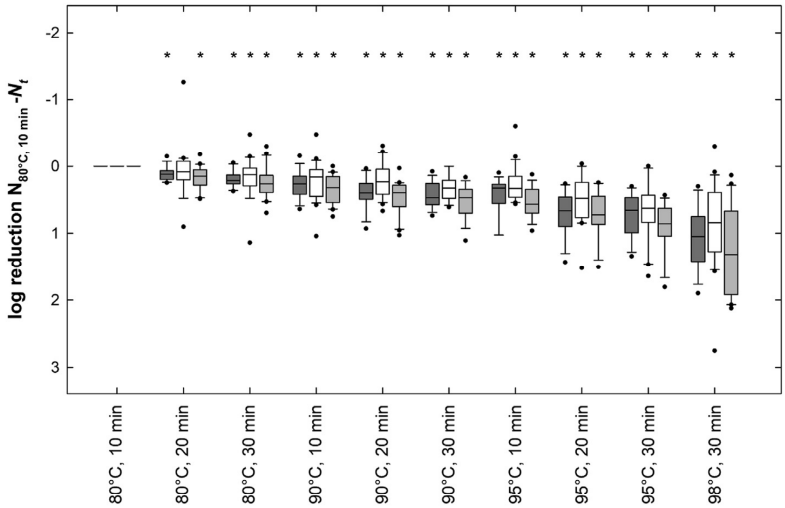

Fig. 2. Logarithmic reduction of thermophilic bacterial counts for processed dairy samples after heat treatments at 80, 90 and 95 °C for 10, 20 and 30 min excluding the time for equilibration and 98 °C for 30 min including equilibration of heating. Cell counts after heat treatments were related to the cell count at 80 °C, 10 min [$\log(N_{80°C,\ 10\ min}) - \log(N_t)$]. Analysis of whey (■; n = 17), whey powder (▒; n = 20), and skimmed milk powder (□; n = 23) is shown. A significant reduction ($p < 0.05$, paired t-test) compared with the 80 °C, 10 min treatment is marked with an asterisk (*).

effect increased by several orders of magnitude (e.g. $p < 1.9 \cdot 10^{-9}$ for WP at 95 °C for 10 min; Supplementary material Fig. 1D). There was a variance in reduction observed for SMP and WP, which was relatively low initially but increased for treatments at 95 °C (20 + 30 min) and largely increased at 98 °C (Fig. 2; Supplementary material Fig. 1C). Standard deviations for SMP and WP constantly ranged <0.3 log levels, but increased to approx. 0.6 log for the treatment at 98 °C (Supplementary material Fig. 1C). For whey, a continuous increase over intensified treatments was observed.

These findings do not correlate with those for spore suspensions and clearly indicate a difference in heat inactivation effects between thermophilic spores originating from dairy products in this study and laboratory spore suspensions. This discrepancy may be attributed to the presence of spores of mesophilic species, which have been shown to exhibit higher heat sensitivity than thermophilic spores (Andre, Zuber, & Remize, 2013; Kent et al., 2016). However, Kent et al. (2016) frequently isolated *B. licheniformis*, which is facultatively thermophilic, even after treatments at 106 °C. In addition, mesophilic spores were of low abundance (<10%) within the thermophilic microbiota of the present study. Thus, their selective inactivation cannot be causative for the decreasing spore counts observed. In addition, the reduction in SMP samples was lowest even though these samples contained the highest amounts of facultative thermophilic spores.

It is well known that the spore's characteristics, such as resistances or long-term survival, are influenced by many factors. Especially the environment (e.g. temperature, nutrients, osmolarity) of the sporulating mother cell influences the characteristics of the spores formed (Nguyen Thi Minh, Durand, Loison, Perrier-Cornet, & Gervais, 2011). Studies that have analysed the effect of temperature, pH, or nutrient availability on heat sensitivity of *G. stearothermophilus* strains found a strong dependency of heat resistance on optimal growth conditions. It was demonstrated that the heat resistance of *G. stearothermophilus* strains, as well as the spore yield, is highest at conditions providing the best growth (Durand, Planchon, Guinebretiere, Carlin, & Remize, 2015; Mtimet et al., 2015; Wells-Bennik et al., 2018). Such conditions are usually used for the preparation of laboratory spore suspensions, which hence result in spores of maximised heat resistance.

Thermophilic spores in dairy products display considerable heterogeneity. First, dairy products contain spores from different species, and different strains of each species may be present as well. Each species and strain displays different heat resistance (Durand et al., 2015; Lucking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013; Sadiq et al., 2016). In addition, the formation of spores most probably occurs under varying conditions. A part of the spores originates from the raw product (e.g. raw milk) and may be environmental contamination. Another part of the spore microbiota originates from growth over the production process, which consists of different steps with varying processing conditions (e.g. temperature or osmotic pressure). Furthermore, spores of the same strain in a dairy product may be at different stages of the maturation process (physical state and composition), particularly if they are the result of production line growth.

The diversity of spore populations in dairy products (concerning species composition or process parameters that lead to different amounts of mature spores and individual heat resistance of occurring strains) explains the differences observed across all samples. Over the production process, the spores are likely formed continuously, which results in a spore distribution with varying degrees of spore maturation regardless of individual heat resistance. This finding appears to be congruent with the observed continuous reduction of spore counts during inactivation experiments even at temperatures below 100 °C (Fig. 2). Lower temperatures (80 °C) inactivate vegetative cells. Then, not fully maturated and more heat-sensitive prespores or forespores could be inactivated and only fully mature spores survive the highest temperatures and longest holding times.

3.4. Heat sensitivity of thermophilic spores is dependent on growth phase

To investigate if and to what degree the heat sensitivity of spores varies between different phases of bacterial growth, two thermophilic isolates were cultivated in UHT-milk, and the effect of heat treatments varying in intensity (80, 90, 95 °C) was monitored. Growth and sporulation behaviour differed between both strains analysed. *A. flavithermus* G10613 (Fig. 3A) exhibited a faster growth rate and much higher degree of sporulation compared to *G. stearothermophilus* EG1956 (Fig. 3B) when cultivated at 55 °C. Proliferation of vegetative cells began in the early growth phase, which is expressed by rising total cell counts, but nearly constant spore counts.

The low reduction of spore counts observed may be due to germination of spores from the inoculum during early growth. Spore counts began to rise in late exponential or early stationary phase (3–4 h) until the spores reached a sporulation level near 30% in late stationary phase. In the case of *G. stearothermophilus* EG1956 (Fig. 3B), spore and total cell counts increased in parallel at nearly the same rate and relative spore levels remained comparably low (<1%).

The heat sensitivity of the spores was indeed influenced by growth phase (Fig. 3). Mature spores from the inoculum displayed the highest heat resistance, and the heat sensitivity of spores increased until it reached its apex and declined again during the stationary phase. Focusing on the magnitude of the heat inactivation effect, *G. stearothermophilus* (Fig. 3B) was more heat sensitive than *A. flavithermus* (Fig. 3A) during growth. After 7 h (early stationary phase), the inactivation effect was maximum and the spore count was reduced by log 1.8 ± 0.8 after heating to 95 °C for 10 min (right axis). This reduction was significantly different from all other time points ($p < 0.05$). A less intense heat treatment at 90 °C produced a less pronounced inactivation effect (log reduction $N_{80\ °C,\ 10\ min} - N_t = 0.8 \pm 1.0$). Spores of *A. flavithermus* also demonstrated the highest heat sensitivity in the early stationary phase, between 4 and 6 h of cultivation, but were more heat resistant that those of *G. stearothermophilus* (log reduction $N_{80\ °C,\ 10\ min} - N_t = 0.8 \pm 0.2$ at 95 °C, 10 min after 5 h).

These results demonstrate the effect of the growth phase on the heat sensitivity of spores regardless of detailed growth characteristics, degree of sporulation, or absolute heat sensitivity. Particularly, spores that are newly formed in the phase of rising spore counts are more affected by heat treatments and inactivated to a higher degree. The heat inactivation effect diminishes as a constant spore level is reached. Thus, young spores are more sensitive to heat, suggesting that full maturation is necessary for maximal heat resistance. This finding corroborates the study of Sanchez-Salas, Setlow, Zhang, Li, and Setlow (2011) in which spores of *Bacillus subtilis* were found to acquire their high wet heat resistance late in sporulation. The authors observed a direct positive correlation between the last steps in the maturation of the forespore (DPA uptake, final decrease in spore's water content) and increasing heat resistance. This discovery could help to explain the difference in heat sensitivity between laboratory spores and spores in commercial powders in this study. Although these experiments have been conducted in liquid culture and spores developing in the production line mostly originate from growth in a biofilm or fouling layer, it is likely, that the effects observed also apply for spores in real samples. The developing spore needs to undergo the different steps of maturation regardless of whether it is sessile or planktonic.

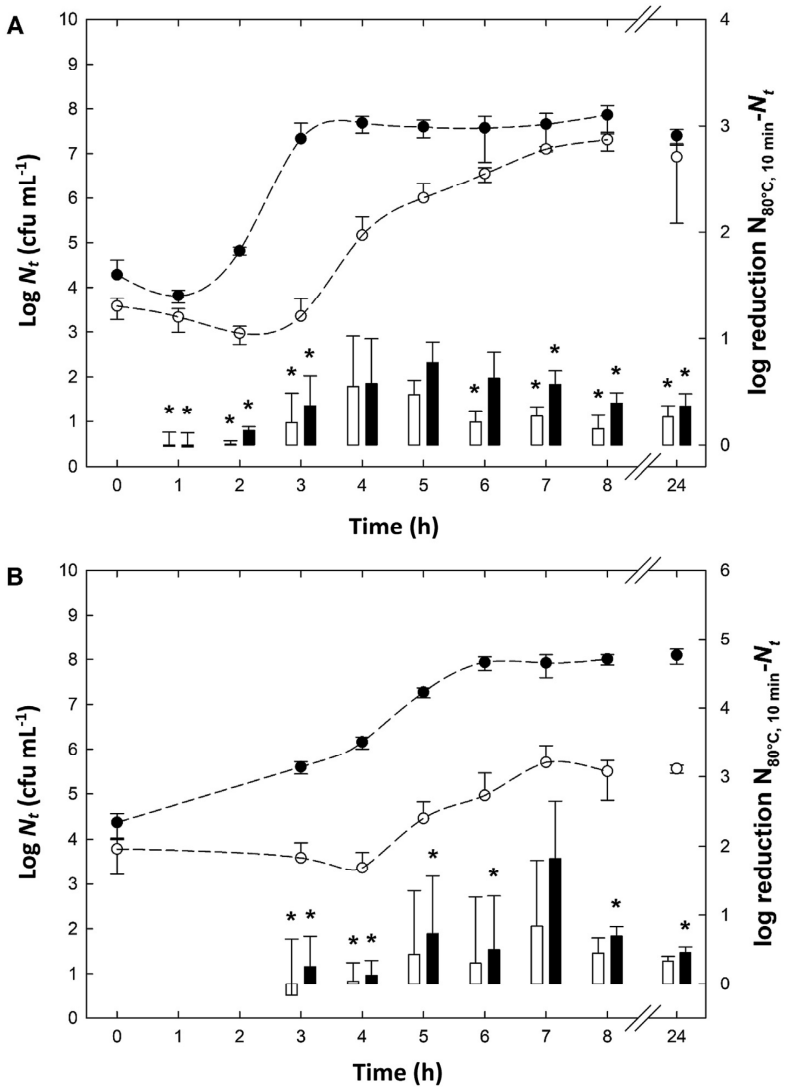

Fig. 3. Heat sensitivity of thermophilic spores dependent on the growth phase in milk. *A. flavithermus* G10613 (A, n = 7) and *G. stearothermophilus* EG1956 (B, n = 8) were cultivated for 24 h at 55 °C. Total cell counts (●) and spore counts (○; 80 °C, 10 min) were measured. Heat treatments at 90 °C (□) and 95 °C (■) for 10 min excluding the time for equilibration were applied to the samples, and the log reduction [$\log(N_{80°C, 10 min}) - \log(N_t)$] in relation to the spore count after the 80 °C, 10 min treatment was calculated. For data marked with (*), the reduction was significantly different ($p < 0.05$, paired t-test) from the reduction at 5 h (A) and 7 h (B).

The spore population in real samples is highly diverse and includes differently maturated spores, which leads to a certain variance in heat resistance. This variance results in a continuously increasing reduction of spore count as the temperature–time relation is increased, with relatively low standard deviations observed. However, the variance within one product type increases starting with the treatment at 95 °C for 20 min, and it is more pronounced for SMP and WP than for whey (Supplementary material Fig. 1C). This effect may be attributed to specific strain characteristics rather than to the maturation stage of the spores. Some samples exhibited very low spore reduction even at 98 °C for 30 min, whereas others' spores were diminished by almost 2 log levels. The critical conditions for selecting heat-resistant spores appear to be temperatures at or above 95 °C applied for 20 min or longer.

4. Conclusions

In the quantification of thermophilic spores, the heating step to inactivate all vegetative cells should ideally not affect the spores to prevent underestimation of spore counts. Temperatures above 90 °C, however, correspond to decreasing counts of thermophilic spores up to 1 log level or more in commercial powders used in this study (from German manufacturers), which cannot be attributed to a selective inactivation of mesophilic species. Instead, heat resistance of those spores may be lower compared with lab cultures because not all spores are fully maturated or have been formed under suboptimal conditions, which lead to less pronounced heat resistance. If the total thermophilic spore count is to be determined in commercial powders manufactured under similar conditions, temperatures <90 °C

therefore provide more realistic results than temperatures of 100 °C or higher. Based on the results obtained in the present study, we propose the application of 80 °C for 10 min to differentiate between spores and vegetative cells in similar powders, which is the same for mesophilic spores. In cases where highly heat-resistant spores are the object of investigation, temperatures of 100 °C or higher [e.g., according to ISO/TS 27265, IDF/RM 218:2009(E)] are suitable.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.07.003.

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Supplement

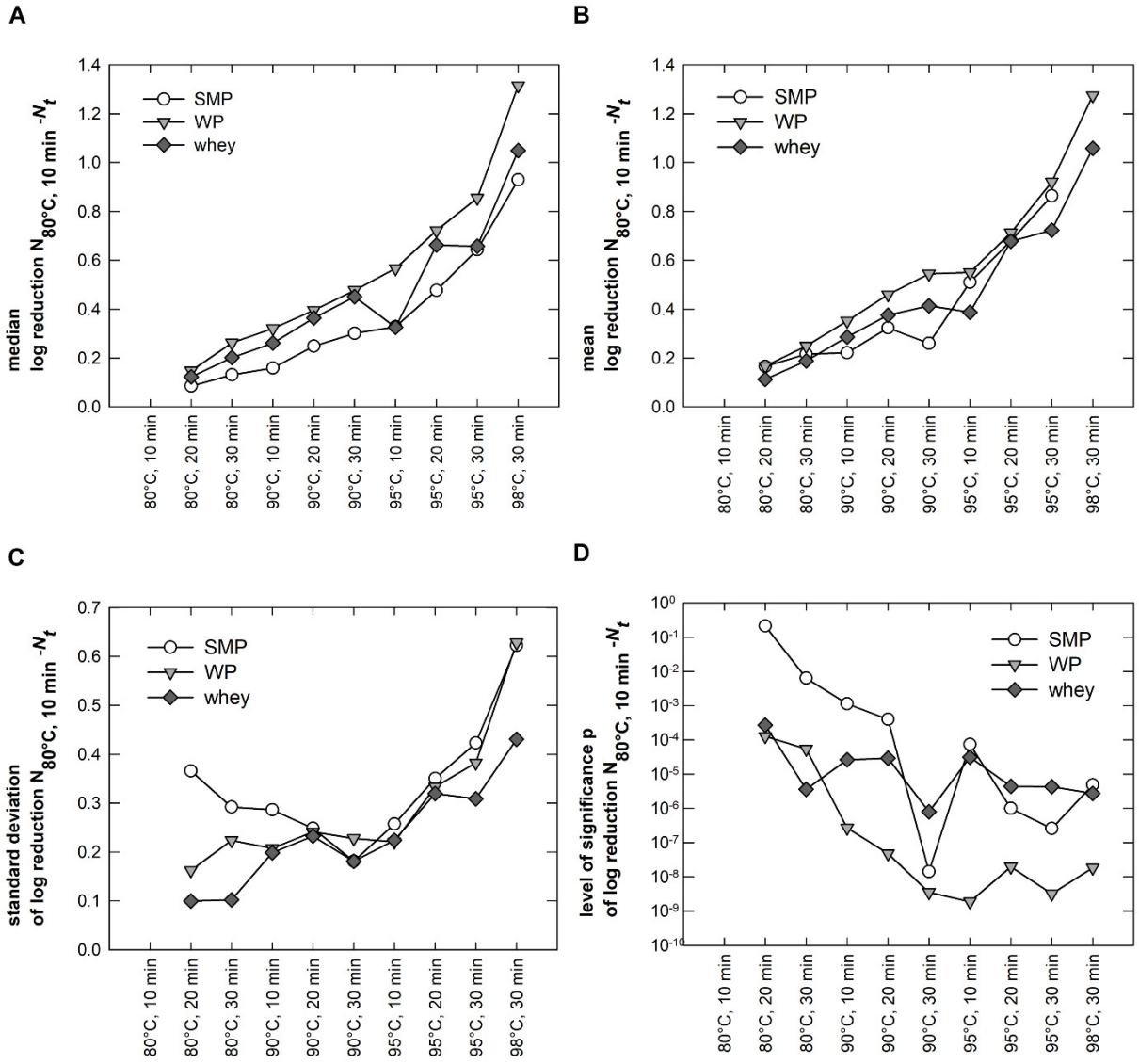

Fig. S1. Heat sensitivity of processed samples skimmed milk powder (○), whey powder (▼), and whey (◆) after heat treatments at 80, 90, and 95 °C for 10, 20, and 30 min excluding the time for equilibration and 98 °C for 30 min including equilibration of heating: A, median logarithmic reduction $\log(N_{80°C, 10 min}) - \log(N_t)$; B, mean logarithmic reduction $\log(N_{80°C, 10 min}) - \log(N_t)$ and respective (C) standard deviation; D, level of significance, compared with the 80 °C, 10 min treatment (paired t-test).

2 Part 2: “High counts of thermophilic spore formers in dairy powders originate from persisting strains in processing lines”

Summary

Thermophilic spore counts in milk powders are often elevated and exceed critical levels of 500 or 1000 cfu·g^{-1}, which leads to economic loss for producers. As data on detailed process analyses is limited, the study of the dynamics of microbial growth during powder processing and the impact of raw materials explores reasons for increasing thermophilic spore levels.

At first, the analysis of 33 BTM samples showed that the high spore load in powder is not related to the raw material and the prevalence of powder dominating obligate thermophiles was low. Instead, *B. licheniformis* marked more than 80 % of the BTM microbiota. The analysis of SMP production in three different dairies and dependent on processing time and step showed a significant onset of thermophilic growth and spore formation at skim milk level and from 6 − 8 h accordingly. The thermophilic microbiota changed simultaneously, and *B. licheniformis* was replaced by *A. flavithermus.* This is a strong indication that growth of *A. flavithermus* during production is responsible for high spore levels, already at the first processing steps before evaporation (e.g. initial heat treatments, cream separation) and early production hours. For WP production, thermophilic spores are highly abundant in whey feeding the production, and influence of the process was not observed. To further characterise the impact of the production environment, isolates of different production batches were tracked based on their strain identity due to RAPD typing. The relatedness of identical RAPD patterns was evaluated using phylogenomic treeing and cgMLST based on whole genome sequences to be very close. The analysis of eight manufacturers identified recurring *A. flavithermus* patterns in samples of one production process for up to 24 months. Interestingly, the profile of RAPD types was unique for each plant.

Based on the detailed process analyses, persistent thermophilic spores are widespread among German powder plants. As a consequence of incomplete elimination of reservoirs through plant cleaning, spores remain in the plant and recontaminate subsequent production batches. Options for decreasing thermophilic spore levels in powder should target the directed removal of existing reservoirs by improved cleaning and disinfection strategies, particularly at the very beginning of the production process.

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High counts of thermophilic spore formers in dairy powders originate from persisting strains in processing lines☆

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ABSTRACT

During the last decades, thermophilic spore counts became a very important quality parameter for manufacturers with regard to powdered dairy products. Low-spore count powders are highly demanded but challenging to produce when high production volume and long process times are intended. In this study a detailed monitoring of microbial levels in three skim milk powder plants was conducted. *Anoxybacillus flavithermus* was found to be primarily responsible for increased spore levels with increasing spore numbers being detected after 6–8 h already during initial processing steps. Simultaneously, the species composition shifted from a diverse bulk tank milk microbiota where different *Bacillus* species represented around 90% of the thermophilic bacteria to a dominance of *A. flavithermus* in the end product. The analysis of *A. flavithermus* isolates from different powder batches with RAPD PCR revealed recurring patterns in each of the eight German manufacturers sampled over several months. The high relatedness of isolates exhibiting identical RAPD patterns was exemplified by cgMLST based on whole genome sequences. We assume that *A. flavithermus* strains persisted in production plants and were not eliminated by cleaning. It is concluded that such persisting strains recurrently recontaminated subsequent powder productions. The data highlight that a targeted optimization of cleaning and disinfection procedures is the most promising measure to effectively reduce thermophilic spore counts in German dairy powders.

1. Introduction

Thermophilic spore forming bacteria are of greatest importance for dairy powders as the thermophilic spore count is a crucial parameter to assess microbiological product quality. Aerobic thermophilic spore formers are non-pathogenic, ubiquitous in nature and able to grow at temperatures of 50–70 °C which are typically applied during manufacturing of dairy powders. Additionally, these bacteria are able to form biofilms on stainless steel surfaces (Burgess et al., 2010; Coleri Cihan et al., 2017; Sadiq et al., 2017; Zhao et al., 2013) and survive unfavourable environmental conditions such as vacuum during spray drying or long-term storage due to endospore formation (Setlow, 2014).

Manufacturers face the challenge to fulfil very stringent criteria of 100–500 thermophilic spores per gram powder at maximum, which is demanded by many customers. However, the thermophilic spore load found in milk powder covers a broad range of < 1 log cfu·g^{-1} up to > 5 log cfu·g^{-1} (Dettling et al., 2019; Hill and Smythe, 2012; Kent et al., 2016), thereby quite often exceeding the customers' limits. Additionally, the load of thermophilic spore formers in bulk tank milk (BTM) is only < 2 log cfu·mL^{-1} (McGuiggan et al., 2002; Miller et al., 2015; Scott et al., 2007). The three main species found in powders are *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* and *Bacillus licheniformis*, the latter being a mesophilic

Abbreviations: BTM, bulk tank milk; SMP, skim milk powder; WMP, whole milk powder; WP, whey powder; WPC50, whey protein concentrate

☆ The DDBJ/ENA/GenBank accession numbers of the whole genome sequences of strains WS 5287, WS 5290, WS5292, WS 5490, WS 5493, WS 5492, WS 5491, WS 5279, WS 5291, WS 5446, WS 5281, WS 5364, WS 5497, WS 5496, WS 5495, WS 5494, WS 5294, WS 5285, WS 5286, WS 5448 and WS 5449 are JABJUV000000000, JABJUW000000000, JABJUY000000000, JABJVE000000000, JABJVH000000000, JABJVG000000000, JABJVF000000000, JABJUR000000000, JABJUX000000000, JABJVB000000000, JABJUS000000000, JABJVA000000000, JABJVL000000000, JABJVK000000000, JABJVJ000000000, JABJVI000000000, JABJUZ000000000, JABJUT000000000, JABJUU000000000, JABJVC000000000, JABJVD000000000.

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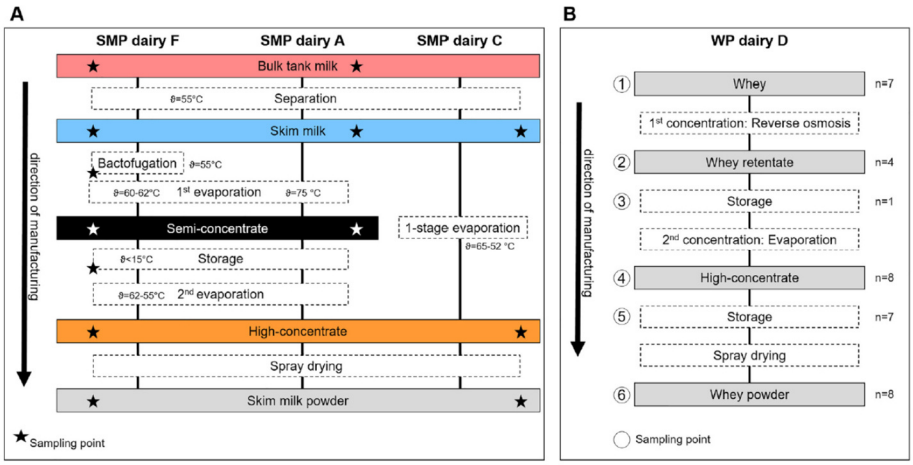

Fig. 1. (A) Schematic overview of the skim milk powder (SMP) production process of dairies F, A and C. Stars mark the respective sampling points. Operation temperatures (ϑ) of processing steps have been provided by the dairies. (B) Whey powder production process of dairy D. Numbers mark the six sampling points. n = number of samples per sampling point.

species, but able to grow at temperatures up to 60 °C (Vos et al., 2009; Warth, 1978). The abundance of each species highly depends on the production plant and varies between different studies. Both *B. licheniformis* and *A. flavithermus* were found to dominate thermophilic powder microbiota (Burgess et al., 2010; Dettling et al., 2019; Kent et al., 2016; Pereira and Sant'Ana, 2018; Ronimus et al., 2003; Sadiq et al., 2018; Yuan et al., 2012).

So far, only few studies analysed powder productions at different production steps in order to explore growth and, partly, the sporulation dynamics during processing. Murphy et al. (1999) showed that thermophilic growth occurred during preheat treatments of skim milk powder (SMP) production while sporulation during evaporation and in pre-heater sections of whole milk powder (WMP) production was described by Scott et al. (2007). To the contrary, physical concentration as the reason for increased spore load was found by Muir et al. (1986). Watterson et al. (2014) described concentration effects as the major but not the only contribution to elevated spore levels, particularly towards end of processing.

In a recent study we found *Anoxybacillus flavithermus* to be highly abundant in German skim milk and whey powders (Dettling et al., 2019). *Bacillus licheniformis* was detected in low numbers only, whereas the thermophilic spore load was very high, ranging between 3 and 5 log cfu·g^{-1} in the majority of samples.

The present work focuses on the dynamics of microbial growth during processing and the potential impact of raw materials such as bulk tank milk and whey as well as the production line on resulting spore counts in dairy powders. Process analyses were conducted to reveal steps critical for thermophilic growth in the powder production process. Genotyping of strains belonging to *A. flavithermus* and *G. stearothermophilus* by RAPD (Random Amplified Polymorphic DNA) PCR was used to track strains in different batches of dairy powders. Finally, the resolution of RAPD PCR for strain discrimination was cross-checked with core genome multilocus sequence typing (cgMLST) calculated from whole-genome sequences to confirm the validity of the results.

2. Material and methods

2.1. Determination of thermophilic spore level and biodiversity

Liquid samples (BTM, whey, milk concentrate) were used directly for analysis, whereas SMP and whey powder (WP) samples had to be reconstituted by dissolving homogeneously 1:10 in ¼ Ringer's solution (Merck) prior to analysis. Thermophilic spores were quantified after heating 1 mL or 10 mL of sample at 80 °C for 10 min followed by cooling to RT and plating on tryptic soy agar (TSA, Oxoid). Plates were incubated at 55 °C for 48 h. To determine the biodiversity of thermophilic spore formers, 5–50 colonies were randomly picked per sample beginning in the middle of one agar plate and moving helically to the outside regardless of colony shape. Depending on the type of analysis, 20 (process analyses samples) or 50 (BTM) isolates were collected, but for some samples there were less colonies available due to low thermophilic spore counts.

Each colony was identified using Fourier-transform infrared spectroscopy (FTIR) according to Wenning et al. (2014) after growing the cells at 55 °C for 24 h ± 0.5 h on TSA. Identification was achieved using an in-house reference database which was continuously extended by addition of new strains over the course of the project (comprising 448 spectra of 14 genera and 57 species in 2019). All new references were unequivocally identified by 16S rRNA gene sequencing in advance. Based on hierarchical cluster analysis of the FTIR spectra performed for each sample (Wenning and Scherer, 2013), representative isolates of each cluster were selected and additionally identified by 16S rRNA gene sequencing to confirm FTIR results. The primers 27f and 1492r were used for 16S rRNA sequencing according to von Neubeck et al. (2015).

2.2. Sampling of industrial powder productions

2.2.1. Bulk tank milk

Aerobic thermophilic spore counts of 33 BTM samples from 13 German dairies, sampled on the production site, were analysed over a period of two years (2015–2017). To cover a broad diversity of BTM, the samples were collected over all seasons of the year, 16 samples in 2015, 10 samples in 2016 and seven samples in 2017. Five dairies were only sampled once, two dairies twice, three dairies three times, two dairies four times and one dairy seven times. The biodiversity was determined for samples where at least 10 isolates could be obtained. Samples exhibiting very low aerobic thermophilic spore counts with less than 10 grown colonies were excluded from the analysis of biodiversity.

2.2.2. Process analyses of skim milk powder

This study covers the detailed analysis of three SMP production lines in the different dairies A, C and F (Fig. 1A). Each dairy used a characteristic production strategy, but the intermediate products skim milk, high-concentrate and the end product were part of all productions.

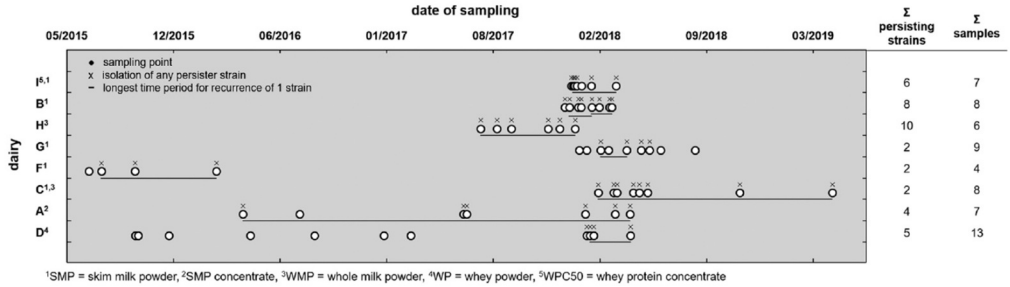

Fig. 2. Recurrence of strains with identical RAPD patterns in industrial samples of eight dairies (A–D, F–I). Circles mark the sampling points per dairy with respect to time. Crosses mark samples where at least one out of all (= Σ persisting strains) persisting strains was isolated. Black lines mark the time period of the longest time of recurrence. For dairy B, there were two strains following each other at an overlap in one sample in between. For dairy A, C and H persisting strains were recovered from 100% of samples.

Production F and A applied a two-stage evaporation strategy including an interruption of the process and storage of the semi-concentrate until starting the second evaporation. In contrast, production C followed a continuous evaporation strategy and the production was operated without interruptions and any intermediate storage throughout. Production F additionally included bactofugation prior to the first evaporation.

SMP production F was operated for 18 h for the first part of the production (bulk tank milk to semi-concentrate), 27 h for the second part (storage of semi-concentrate to powder) and samples were taken at seven different sampling points (Fig. 1A) in a 3 h time interval. The process analysis of SMP production A was carried out for 16 h and focused on the first part of the production (until semi-concentrate). Samples were taken at the beginning, after 8 h and at the end of the production run. SMP production C was analysed at three sampling points, namely skim milk, high-concentrate and SMP. Samples were taken at the beginning of the production and from 6 h to 18 h processing time with a 2 h time interval.

All samples were shipped to the laboratory for analysis. Except for powdered products, the samples were cooled using frozen cooling packs during transportation. The analysis included the quantification of thermophilic spores and the analysis of microbial composition by determination of the thermophilic biodiversity (3–20 isolates per sample) in order to monitor the dynamics during production. An exception was SMP production C where only samples at the end of processing were analysed for biodiversity. The dry matter content was provided by the dairy.

2.2.3. Process analysis of whey powder

The production strategy of the WP production process in dairy D was divided into six different process steps (Fig. 1B). Whey (step 1) was concentrated using reverse osmosis to obtain whey retentate (step 2). After storage (step 3), the 2nd concentration (step 4) was used to produce high-concentrate which was stored (step 5). At the end of the production process, spray drying produced the final WP (step 6). Samples at step 1 were taken from seven different storage tanks that fed the production. Step 2 was sampled at 2, 6, 8 and 12 h of processing time. Being a storage step, step 3 was only sampled once whereas samples of step 4 were taken every hour (0–8 h). Step 5 and 6 were sampled from 2 h in a time interval of 2 h until the production ended after 14 h or 16 h, respectively. The samples were analysed as described for SMP process analyses. 20 isolates per sample were identified to describe the biodiversity of thermophilic spore formers. The dry matter content was provided by the dairy.

2.2.4. Analysis for persisting spore formers

For determination of persistence of thermophilic spores in the powder manufacturing environment, samples from eight German dairies were analysed at different time points (dairy A–D, F–I, Fig. 2). The analysis started with dairies F, D and A in 2015 and 2016 and was subsequently extended with another five dairies sampled mainly in 2018. In this second sampling period, samples were collected in shorter intervals. Per dairy, samples were taken of one specific production line. For each dairy where the powdered product resulted from a continuous process with no interference from e.g. side streams, the powder (5× SMP, 2× WMP, 1× WP, 1× WPC50) was analysed. One dairy with a more complex production process that impaired the traceability of spores was sampled at concentrate level (1× SMP concentrate). The product type of the specific production line changed for two dairies during analysis (dairy C, I) whereas it was consistent for the others. The time intervals between different samples varied among different manufacturers but were chosen to cover at least two rounds of plant cleaning. Overall, this study covered a time period between 2 and 24 months depending on the dairy (Fig. 2). For evaluation of the persistence of thermophilic spores, 10 isolates per species (*A. flavithermus*, *G. stearothermophilus*) and sample were analysed for their strain identity using RAPD typing. *A. flavithermus* was isolated from all batches (concentrate or powder), whereas *G. stearothermophilus* was isolated only very infrequently in samples of three dairies (C, H, I). Strains were typed if isolates from at least two different batches were obtained.

2.3. RAPD typing

A. flavithermus and *G. stearothermophilus* isolates were analysed using RAPD (Random Amplified Polymorphic DNA) PCR to check for strain identities (Williams et al., 1990). Cell lysis of isolates was executed as described by von Neubeck et al. (2015) and the DNA concentration was adjusted to 50 ng·µL^{-1} for each cell lysate. The PCR was performed using the KAPA2G™ Robust HotStart PCR Kit (KAPABIOSYSTEMS) in a total volume of 25 µL. Each isolate was analysed in three independent reactions using one of three primers of 10–11 bp length per reaction (Table 1). Primer sequences were optimized to discriminate *A. flavithermus* and *G. stearothermophilus* isolates at the strain level (data not shown). The characteristic of primer OPZ-14 is its applicability for strain typing of both species. For each reaction 5 µL 5× Enhancer, 1.5 µL 5× Buffer A, 0.5 µL 10 mM dNTP Mix, 2 µL 50 pmol·µL^{-1} primer (Table 1), 11.4 µL sterile water and 0.1 µL 5 U·µL^{-1} KAPA2G Robust HotStart DNA Polymerase were mixed and 1 µL 50 ng·µL^{-1} template DNA was added. The reproducibility of different PCR runs was ensured by two control strains per species originating from other sources that were analysed in parallel in each run. For *A. flavithermus* strain WS 5452 and WS 5425 and for *G. stearothermophilus* strain WS 5454 and WS 5440 were used. All strains were originally isolated from dairy powders. Strain identifiers correspond to

the Weihenstephan Microbial Strain Collection (http://micbio.wzw.tum.de/cms/docs/stammsammlung_e.php).

Following an initial denaturation at 95 °C for 3 min, the amplification was performed in cycles of 94 °C for 15 s, annealing at varying temperatures for 15 s and elongation at 72 °C for 3 min. The number of cycles and the primer specific annealing temperatures are listed in Table 1. The reaction was stopped after a final elongation at 72 °C for 8 min.

PCR products were separated using 2% agarose gels in 0.5 x TBE buffer at 150 V. Bands were visualized using RedSafe™ (iNtRON Biotechnology) and the UV transilluminator UV solo TS Imaging System (Biometra, An Analytik Jena Company). The obtained band patterns were evaluated visually and grouped according to similarity. Two isolates exhibiting the same band patterns for all three primers were assumed to be the same strain. The combination of three band patterns defines the RAPD type.

2.4. Whole genome sequencing and genome data analysis

To assess the sensitivity of the RAPD PCR method for strain discrimination, whole genome sequencing of 21 *A. flavithermus* strains isolated from milk powder was performed. This included strains of five different dairies and strains of different RAPD type as well as two groups of strains of identical RAPD type and isolated from the same dairy (Supplementary Table A.1). Both groups cover the maximal time of recovery of identical strains and each of these strains was isolated of a different sample.

Genomic DNA was extracted from plate cultures using enzymatic cell lysis (lysozyme, proteinase K) and the QIAamp® DNA Mini Kit (Quiagen). The extraction was conducted following the manufacturer's manual for Gram-positive bacteria with slight modifications. For cell lysis, two inoculation loops of bacteria were suspended in 180 µL lysozyme solution (20 mg·mL^{-1}, Carl Roth) and incubated at 37 °C for 3 h. After heating at 95 °C for 15 min and cooling on ice for 5 min, 20 µL proteinase K and 200 µL Buffer AL were added and incubated at 56 °C for 4 h. Next, 10 µL RNase A (10 mg·mL^{-1}, Thermo Fisher Scientific) was added, mixed thoroughly, incubated at RT for 2 min and followed by enzyme activation at 70 °C for 30 min. After addition of 200 µL ethanol (98–100%), clean-up was proceeded as described in the manual. Genomic DNA was quantified using the Qubit dsDNA HS assay and the Qubit 4.0 fluorometer (Invitrogen by Thermo Fisher Scientific). At least 60 µL of genomic DNA were adjusted to 75–80 ng·mL^{-1} for library preparation.

PCR-free preparation of sequencing libraries was performed with the TruSeq® DNA PCR-free Sample Prep LT kit (Illumina) using an optimized protocol (Huptas et al., 2016). Pooled libraries of 40 pM were sequenced on the Illumina MiSeq platform.

Demultiplexed sequencing data was quality checked, trimmed and filtered using FastQC (v0.10.1, https://omictools.com/fastqc-tool) and the NGS QC ToolKit (v2.2.3, Patel and Jain, 2012). Resulting high quality reads were assembled to draft genomes using SPAdes (v2.5.1, Bankevich et al., 2012).

A multiple sequence alignment based on 92 bacterial core genes and comprising all 21 investigated strains was calculated via the UBCG software (v3.0, Na et al., 2018). Subsequent evolutionary analysis was conducted with MEGA X (v10.0.5, Kumar et al., 2018) applying the General Time Reversible (GTR) nucleotide substitution model with varying rates across sites (+G) and a proportion of invariant sites (+I). After midpoint rooting, the maximum likelihood tree was visualized with the interactive Tree Of Life (iTOL) online tool (v5.5.1, Letunic and Bork, 2007).

Beside the phylogenomic analysis, a core genome multilocus sequence typing (cgMLST) scheme was inferred based on the gene-by-gene approach implemented by the chewBBACA software (v2.1.0, Silva et al., 2018). Default parameter settings were applied in general. The Prodigal (Hyatt et al., 2010) training file of strain WS 5290 was used to create the whole genome MLST scheme and to call alleles. In addition, paralogous loci were removed from the final cgMLST scheme containing 1787 orthologous loci present in all the 21 strains investigated. A minimum spanning tree of allelic differences between strains was created using the goeBURST algorithm and visualized with the PHYLOViZ 2.0 software (Nascimento et al., 2017).

3. Results

3.1. Aerobic thermophilic spore counts and microbial biodiversity in bulk tank milk

To evaluate possible effects of the BTM microbiota on the spore level and microbial composition of resulting powders, we collected samples of German manufacturers for two years. The aerobic thermophilic spore counts in 33 BTM samples were low and ranged between < 1 log cfu·mL^{-1} and 2.1 log cfu·mL^{-1}, at a median of 1.3 log cfu·mL^{-1} (Fig. 3A). Five samples had a very low spore count and it was not possible to isolate an appropriate number of colonies per sample to analyse the biodiversity. Therefore, the microbial composition was determined for 28 samples. Among the 927 isolates analysed, 38 different species belonging to 11 different genera were identified describing the great variety of aerobic thermophilic spore formers in BTM (Fig. 3C, Supplementary Table A.2). Species diversity of BTM in general was much higher than in whey and powder samples; especially *Ureibacillus* spp. and mesophilic *Bacillus* spp. were only detected in BTM (Fig. 3). While obligate thermophilic species of *Anoxybacillus* and *Geobacillus* were rarely detected (0.4–1% mean relative abundance, 7.1–10.7% prevalence), *B. licheniformis* was found in every sample and represented the major fraction of all BTM isolates (82.8%) (Fig. 3C). Other species of the genus *Bacillus* as well as *Ureibacillus* and *Brevibacillus* were identified more frequently (3–8% mean relative abundance), but still not in all samples (32–82% prevalence). The extremely low prevalence of obligate thermophilic bacilli in BTM displays a huge discrepancy between raw material and powder, similar to differences in spore counts.

3.2. Process analyses of skim milk powder production lines

Thermophilic spore counts in SMP were found to exceed critical spore levels of 3 log cfu·g^{-1} whereas thermophilic spores were hardly found in BTM (Fig. 3). Therefore, three different productions of SMP

Table 1
Primers used for RAPD analysis of *A. flavithermus* and *G. stearothermophilus*.

Species	Primer name	Sequence (5′ → 3′)	Annealing temperature	Number of cycles
A. flavithermus	OPO-01	GGCATGACCT	32	30
A. flavithermus	OPI-03	CAGAAGCCCA	30	30
A. flavithermus	OPZ-14	TCGGAGGTTC	32	30
G. stearothermophilus				
G. stearothermophilus	OPBH-04	ACCTGCCAAC	32	30
G. stearothermophilus	OPR-13* [a]	GGACGACCAAG [a]	36	35

[a] Sequence as primer OPR-13 by Seale et al. (2012).

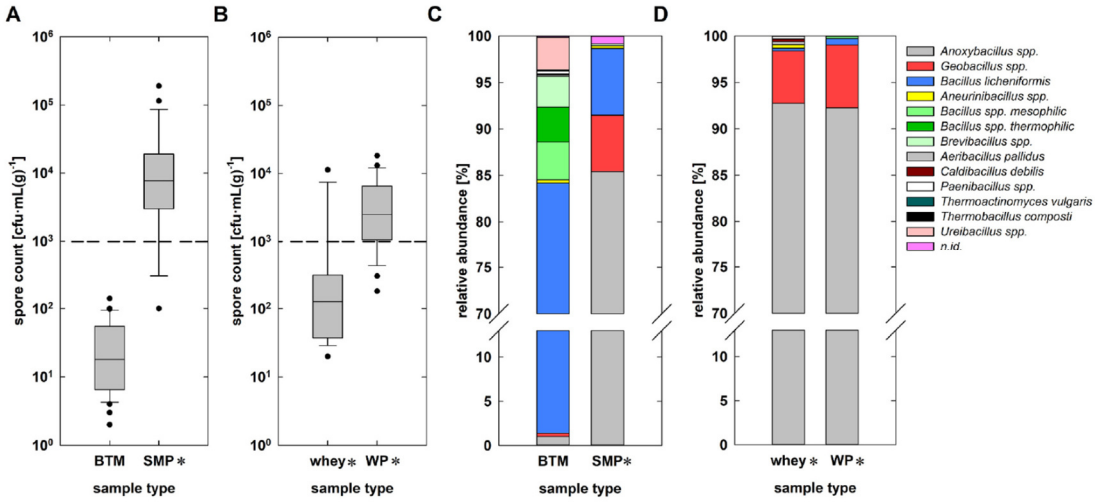

Fig. 3. (A, B) Thermophilic spore counts of German bulk tank milk (BTM, n = 33), skim milk powder (SMP, n = 18), whey (n = 17) and whey powder (WP, n = 20). Boxplots include the median of each sample type. A spore level of 10^3 cfu·mL (g)$^{-1}$ is visualized by a dashed line. (C, D) Average composition, by means of relative abundance, of thermophilic microbiota in German BTM (n = 28), SMP (n = 18), whey (n = 17) and WP (n = 20) determined after heating at 80 °C for 10 min. n.id. = not identified. Species are grouped according to Supplementary Table A.2. Asterisks mark data for whey, WP and SMP that was taken from Dettling et al. (2019).

were monitored over one production run in order to evaluate the impact of the production process and its manufacturing steps on the increase in thermophilic spore level and possibly localize thermophilic growth and sporulation during manufacturing.

While the thermophilic spore counts in BTM of SMP production F were low, they increased in skim milk by almost 3 log levels and reached 4.0 log cfu·mL^{-1} after 18 h of production (Fig. 4A). The onset of increasing spore counts was observed at 6 h and the critical level of 3 log cfu·mL^{-1} was reached after 12 h of processing. The bactofugation step between cream separation and evaporation reduced spore counts of skim milk by about one log level, but after the first evaporation they had increased to approximately the level of skim milk. As the semi-concentrate was collected and stored until the beginning of evaporation step 2, products with low and moderate spore numbers from early production hours were combined with concentrate from the second half having high numbers. During the second evaporation and spray drying, no remarkable increase over the whole production time of almost 28 h was observed (Fig. 4B). Spore numbers in the final SMP were at approximately 3.8 log cfu·g^{-1}.

Based on results of production F, sample collection in SMP production A focused on the first processing steps prior to the storage of semi-concentrate (Fig. 1). The dynamics observed very much resembled those noticed in SMP production F. The thermophilic spore level in BTM was constantly low and there was a pronounced increase in spore count between 8 h and 16 h (=end of processing) in skim milk (Supplementary Fig. A.1). The highest spore level of 5.0 log cfu·mL^{-1} was reached in skim milk and semi-concentrate at the end of processing; the critical level of 3 log cfu·mL^{-1} was reached after 8 h in the semi-concentrate.

Sampling of BTM was skipped for process analysis of SMP production C that was run continuously without storage of semi-concentrate. Between 6 and 8 h, the thermophilic spore count increased massively by two log levels in skim milk (Supplementary Fig. A.2) and spore counts increased further until the end of production after 18 h to end up at 4.3 log cfu·mL^{-1}. The further increase to 4.9 log cfu·mL^{-1} in concentrate and 5.3 log cfu·g^{-1} in the powder at the end of production exactly reflects the physical concentration effect.

All three analyses conducted in different dairies running divergent production strategies clearly showed that the increase of thermophilic bacterial numbers most probably was due to thermophilic growth starting at the initial processing steps during the separation of cream and the pre-heating involved.

3.3. Process analysis of whey powder production

Besides the raw product whey, the sampling of WP included two concentration steps (reverse osmosis and evaporation) and the storage of intermediate products before spray drying the WP (Fig. 1). In contrast to all three SMP productions, WP production of dairy D showed no logarithmic increase of thermophilic spore counts over processing time indicated by a very low standard deviation between 0.1 and 0.3 log cfu·g^{-1} when averaging all data points of one processing step (Table 2). Spore counts increased only by around one log level from approximately 2 log cfu·mL^{-1} in whey to 3 log cfu·g^{-1} in WP, which is even less than the 16-fold increase in dry matter. Thus, apart from the concentration effect, there was no increase in spore count observable.

3.4. Biodiversity of spore microbiota during manufacturing

As a pronounced increase in spore counts was observed for all three SMP productions analysed which cannot be explained by a concentration effect, the thermophilic microbiota was determined to check whether microbial growth is accompanied by substantial changes in the microbiota.

In fact, the thermophilic microbiota of skim milk during SMP production F changed dramatically from the beginning to the end of processing (Table 3). All isolates of early processing stages up to 6 h were identified as *B. licheniformis*, which is also the most abundant species in BTM microbiota (Table 3). In contrast, *A. flavithermus* was the only species isolated from 9 h until the end of processing. This shift was also observed for all consecutive processing steps from the skim milk level (through bactofugation and first evaporation) to the semi-concentrate.

The changes in thermophilic microbiota during SMP production A were similar. At skim milk level, the microbiota initially (0 h) comprised a large fraction of *B. licheniformis* as well as other *Bacillus* species, *Brevibacillus* and *Ureibacillus*, but no *Anoxybacillus*. The fraction of *A.*

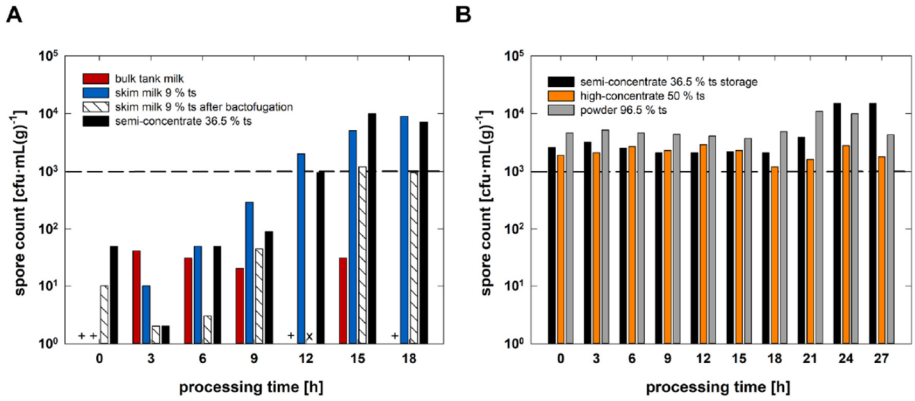

Fig. 4. Development of thermophilic spore counts during skim milk powder (SMP) production F, dependent on processing time and production level. (A) Continuous first part of production until storage of semi-concentrate. Bulk tank milk (red), skim milk 9.0% ts (blue), skim milk after bactofugation (white dashed), semi-concentrate 36.5% ts (black). (B) Second part until finished SMP. Semi-concentrate after storage (black), high-concentrate 50% ts (orange), powder 96.5% ts (grey). +: SC < 10 cfu·mL^{-1}. x: missing sample due to damage during transport. ts = total solids. A spore level of 10^3 cfu·mL (g)$^{-1}$ is visualized by a dashed line. One production batch and one sample per time point were analysed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flavithermus, however, increased to 10% after 8 h and 100% after 16 h (Table 3). This is in agreement with the increase in spore counts and is evidence of pronounced microbial growth with *A. flavithermus* being by far the most competitive species. In production C, obligate thermophiles (*A. flavithermus* and *G. stearothermophilus*) made up the majority (~90%) of the thermophilic microbiota at skim milk, concentrate and powder level at the end of processing. The biodiversity in BTM and during the early hours of the production was not assessed, but the high abundance of thermophilic species in samples with high spore load at the end of processing was similar to the findings in dairy A and F.

3.5. Determination of persisting strains in dairy powder production plants

To gain more insight into the biodiversity of *A. flavithermus* and *G. stearothermophilus* isolates below the species level, strain identities of process isolates of 62 samples were investigated by RAPD PCR. Samples of different production batches of eight German dairies with at least two CIP cleanings in between the samplings were analysed (Fig. 2). PCR controls ensured that patterns were generated reproducibly which enabled good comparability of the around 800 isolates that were typed altogether.

It was revealed that in each dairy unique RAPD types occurred that were not detected in a second factory. A large biodiversity was found among the isolates, as 293 different RAPD types were determined corresponding to a mean relative abundance of each type of 0.3%. However, there was a large variance in diversity and evenness of RAPD patterns in samples between different manufacturers. For dairy G there was a high diversity observed, as altogether 78 RAPD types were detected in nine samples and the richness ranged between seven and 10 different RAPD types per sample. The mean relative abundance of all RAPD types was approximately 1.3%, but for two recurring patterns it was 12 and 13%, respectively. With eight samples, the sample number for dairy C was similar, but there was a lower diversity with only 24 RAPD types detected. While the overall mean relative abundance was 4.2%, two out of 24 RAPD types occurred at a very high relative abundance of 71%. Here, one *A. flavithermus* and one *G. stearothermophilus* strain were isolated very frequently and either one of them occurred in every sample over a period of 14 and nine months, respectively (Fig. 2). The samples were less diverse and only between one and five different RAPD types per sample were observed.

The phenomenon of recurring RAPD patterns in different samples was observed for every dairy and altogether 39 RAPD patterns (13% of all patterns detected) were found in multiple samples of the same dairy. These strains of identical RAPD type were detected in different production batches of every manufacturer (Fig. 2). In some plants only few strains of identical RAPD type were found, in others up to ten different recurring patterns were identified (Fig. 2). Strains of identical RAPD type were even isolated when the product type changed in between the analyses in dairy C and I. Identical RAPD types occur frequently, as for six of eight manufacturers they were found in more than 50% of samples analysed (analysis of 4–8 samples each). Their recurrence extended over periods of up to 24 months (dairy A). Interestingly, in five out of eight dairies at least one strain was identified in samples covering the entire period of analysis (Fig. 2). Dairy D was the only dairy where WP production was sampled and recurring RAPD types were isolated from only 31% of 13 samples within 30 months (Fig. 2). This was by far the least recurrence compared to the other dairies, in particular since this was the production line where the most samples were analysed.

Table 2
Thermophilic spore level and dry matter content of process analysis of WP in dairy D. The spore counts are given as average of all samples per process step. ts = total solids.

Process step no.	Process step	Thermophilic spore count [log cfu·mL (g)$^{-1}$]		Dry matter [% ts]	Number of samples
1	Whey	2.2	± 0.2	6	7
2	Whey retentate	2.7	± 0.3	18	4
3	Whey retentate storage	2.3		18	1
4	High-concentrate	2.7	± 0.1	47	8
5	High-concentrate storage	2.7	± 0.1	47	7
6	Whey powder	3.1	± 0.1	97	8

Table 3
Biodiversity of thermophilic spores during SMP production F and A: Relative abundance of species with respect to processing time and step. BTM = bulk tank milk.

process step	processing time [h]	relative abundance of species [%]								Σ isolates
production F		BacLic		AnoFla		GeoSte		BreAyd		
skim milk	0	100								5
	3	100								5
	6	100								5
	9			100						4
	12			100						20
	15			100						20
	18			100						20
semi-concentrate	0	100								5
	3	100								5
	6	100								3
	9			100						5
	12			100						3
	15			100						20
	18			95				5		20
semi-concentrate storage	0			100						20
	3			84		16				19
	6			100						18
	9			95		5				19
	12			89		11				18
	15			100						20
	18			100						20
	21	5		95						20
	24			100						20
	26			100						19
production A		BacLic	AnoFla	Bac mes	Bac ther	GeoSte	BreAyd	UreThe	n.id.	
BTM	0	74			21			5		19
	8	68		11	21					19
	16	100								14
skim milk	0	40		10	20		10	10	10	10
	8		10							10
	16		100							20
semi-concentrate	0	94		6						16
	8	90				11				19
	16		100							20

BacLic = *Bacillus licheniformis*, AnoFla = *Anoxybacillus flavithermus*, GeoSte = *Geobacillus stearothermophilus*, BreAyd = *Brevibacillus aydinogluensis*, UreThe = *Ureibacillus thermosphaericus*, Bac mes = *Bacillus* sp. mesophilic (*B. circulans*, *B. coagulans*, *B. subtilis*), Bac ther = *Bacillus* sp. thermophilic (*B. hisashii*, *B. kokeshiiformis*, *B. thermoamylovorans*, *B. thermolactis*).

RAPD typing proved to be reproducible in our analysis and we did not find identical RAPD patterns in different dairies. Nevertheless, state-of-the-art phylogenomic treeing and whole-genome molecular typing were performed on representative isolates to further evaluate the discriminatory capacity of RAPD PCR. We selected 21 *A. flavithermus* isolates for whole-genome sequencing and calculated a maximum likelihood (ML) phylogenomy based on 92 concatenated universal bacterial core genes and including 85,641 alignment positions (Fig. 5A). In addition, cgMLST analysis based on 1787 orthologues genes was performed (Fig. 5C). Isolates exhibiting different RAPD patterns were clearly separated in the ML phylogenomy (Fig. 5A) and displayed several hundreds of allelic differences (Fig. 5C). The only exception were isolates 36 and 37 which are genotypically very similar (five allelic differences), but the band pattern of primer OPO-01 clearly differed (Fig. 5B).

Isolates from dairy A and C sharing identical RAPD types formed closely related groups in both the ML phylogenomy and the cgMLST minimum spanning tree. They display no more than 27 differing loci in the cgMLST and a maximum evolutionary distance of 0.000035 substitutions per site. Isolates 27 and 42–45 of dairy C were isolated in Feb-May 2018 (isolate 27, 43–45) and April 2019 (isolate 42). Isolate 44 is grouped in the centre and displays between nine and 24 allelic differences to the other 4 isolates. For strains of dairy A, there is a clear trend between isolation date and number of allelic differences and evolutionary distances. Isolates from samplings in April 2016 (isolates 23 + 48) display six allelic differences, those of March/April 2018 (isolates 46 + 47) only one. Isolates of each pair share identical nucleotide sequences and had an evolutionary distance of two nucleotides between them. Both groups are linked via isolates 23 and 47 having 23 allelic differences. Hence, RAPD typing is in good congruence with cgMLST results and phylogenomic analysis and provides reliable indications on clusters containing highly similar isolates.

4. Discussion

4.1. Thermophilic growth during early processing of powder manufacturing leads to high spore counts and shift in biodiversity

In our previous study of dairy powders and whey, the majority of powder samples exceeded 3 log cfu·g^{-1} and reached up to 5 log cfu·g^{-1} (Dettling et al., 2019). In contrast, thermophilic spore counts of BTM analysed in the present study were much lower ranging between < 1 and 2 log cfu·mL^{-1}, which was found in other studies as well (Coorevits et al., 2008; Kent et al., 2016; McGuiggan et al., 2002). Low BTM spore counts, therefore, appear to be largely independent from the geographical location. Given the low spore level in BTM, an increase by up to three log levels cannot be explained by a physical concentration of spores during the evaporation and drying process, only. Accordingly, high spore counts in powder, in our study, are not directly related to the spore load of raw materials.

The increase in spore counts is accompanied by a massive change of the BTM microbiota during processing. Over time, thermophilic bacteria become dominant in the end product with *A. flavithermus* being by far the most abundant species. In our study, the first processing steps

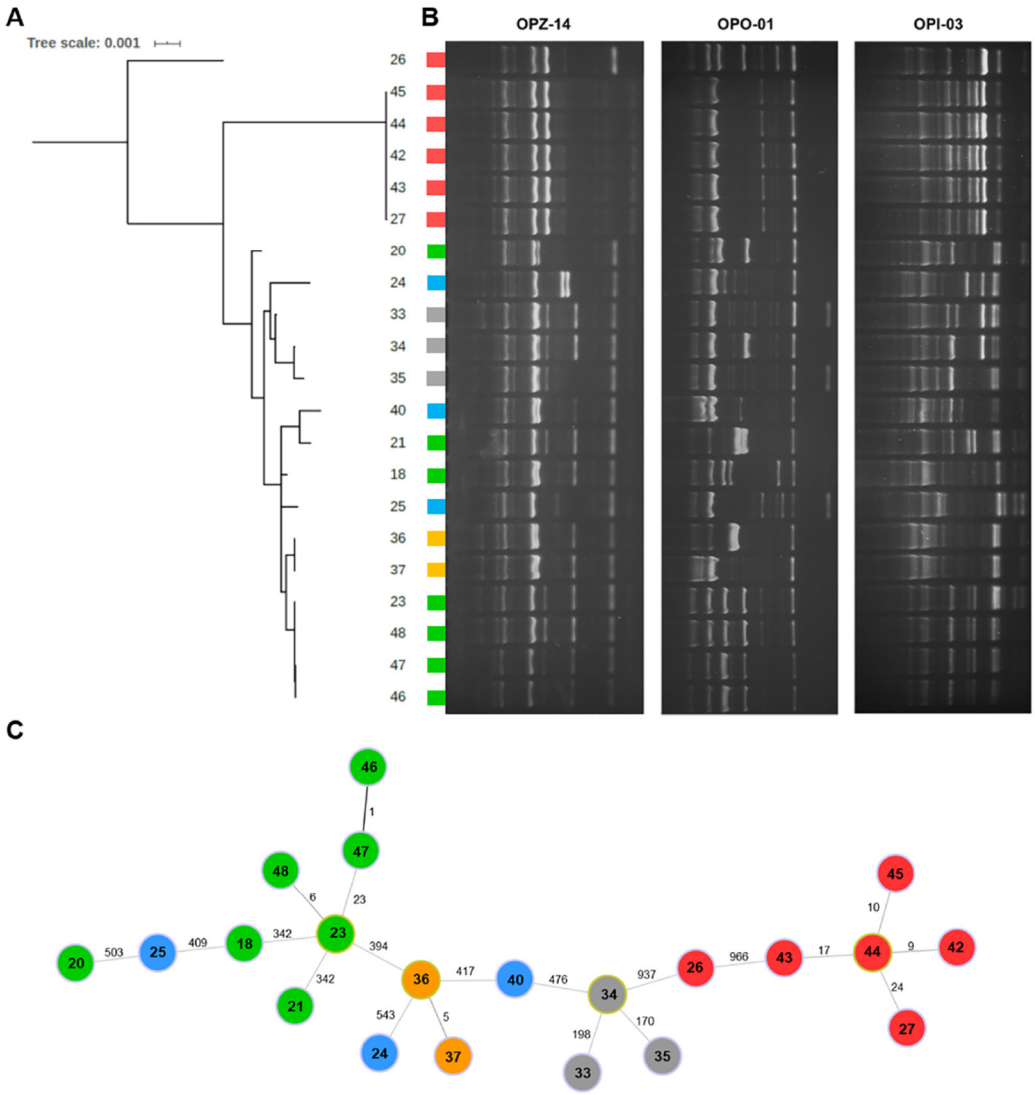

Fig. 5. (A) Maximum likelihood phylogenomy of 21 *A. flavithermus* strains based on the multiple sequence alignment of 92 universal bacterial core genes (total of 85,641 alignment positions). (B) RAPD profiles of primers OPZ-14, OPO-01 and OPI-03, separated on a 2% agarose gel. All PCR reactions shown were separated in one gel run per primer. (C) Minimum spanning tree based on the final cgMLST scheme comprising 1787 orthologous loci. Each node represents one strain and is coloured with respect to the strain's isolation source: dairy A (green), dairy B (blue), dairy C (red), dairy G (grey), dairy H (orange). Allelic differences between strains correspond to numbers at edges. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(initial heat treatment steps, cream separation) in all three different process analyses of SMP were identified as being most critical for the change of microbiota. The major increase in the number of spores was observed at the skim milk level, already, and the BTM microbiota was not detected anymore. The rise of *A. flavithermus* counts resembles exponential growth, although it is unclear, whether this occurs in the fluid milk or in other parts of the processing plant shedding increasing numbers of spores into the product. A few studies reported that thermophilic growth started after 4 and 9 h of processing in preheat sections prior to evaporation during SMP and WMP production, respectively (Murphy et al., 1999; Scott et al., 2007). In addition, Scott et al. (2007) did not detect thermophilic spores in processing steps prior to heating of skim milk that marked the onset of spore formation.

Altogether, detailed process analyses greatly emphasized that pre-evaporation treatments are highly prone to growth of thermophilic spore formers in powder plants. The operation temperatures of, e.g., 57 °C for pre-heating or 55 °C for cream separation lie well within the optimal thermophilic growth range and are very suitable for thermophilic proliferation. The main fraction of spores originates from this early stage of the process. Moreover, processing times up to 18 h

provide enough time for outgrowth of residual spores. Previous analyses of dairy powders partly found *A. flavithermus* as the prevalent species, but powders exhibiting a high abundance of *B. licheniformis* were described as well (e.g. Kent et al., 2016; Scott et al., 2007). Therefore, the species distribution is diverse as summarized by Sadiq et al. (2018) and seems to depend on the production plant, the process and the environment (Pereira and Sant'Ana, 2018; Ruckert et al., 2004).

In contrast to SMP, thermophilic spores in WP analysed in this study originated from whey. Here, obligate thermophilic spores were already highly abundant in raw material feeding the WP production. This microbiota strongly differed from those in BTM and was concentrated through processing (Dettling et al., 2019). The analysis of sweet WP production by Watterson et al. (2014) found similar relations: no influence of processing time and the increase of spore level due to the concentration effect only. As a by-product of cheese making including especially pre-heating processes and incubation at elevated temperatures to proliferate the cheese starter cultures, whey already underwent several processing steps before being used for powder production. Like in SMP production, the change in microbial composition occurs along these steps and as a consequence, they need to be addressed in the future to reduce thermophilic spore contents.

4.2. Persisting thermophilic strains as a widespread phenomenon in powder plants

RAPD typing of powder process isolates identified plant-specific RAPD patterns that recurred in separate production batches for up to 24 months. Although the overall sensitivity of RAPD PCR to discriminate different strains is lower than cgMLST, our data show that identical RAPD types demonstrate a very close evolutionary relationship between strains. For *A. flavithermus*, there is no typing scheme available using cgMLST data to perform epidemiological studies, but there was a scheme proposed for outbreak investigations concerning *Listeria monocytogenes*, a Gram-positive food pathogen with a genome similar in size (Ruppitsch et al., 2015). The authors compared results from pulsed-field gel electrophoresis (PFGE), classical MLST and cgMLST and defined 10 allelic differences as the threshold for differentiating cluster types (CT) based on cgMLST. Outgroup isolates, not related to the outbreak but sharing the same PFGE pattern and MLST sequence type, displayed between 16 and 32 allelic differences. Each of the three clusters of dairies A, C and H with isolates having identical or almost identical RAPD patterns contain *A. flavithermus* isolates with < 10 allelic differences. From all typing data obtained we conclude that each dairy possesses a unique profile of *A. flavithermus* and to a smaller extent *G. stearothermophilus* strains. Some of these strains belong to a cluster of non-identical but very closely related strains that recur in several production batches over long time periods. They are likely to be part of a persisting microbiota that is not fully eliminated between CIP cleaning procedures and re-contaminates subsequent production batches. Small allelic variations in cgMLST of identical RAPD type strains indicate microevolutionary variations acquired over time in the production plant. Hence, this is the first study that identifies persisting thermophilic bacilli in powder plants.

Other studies used RAPD analysis mainly for discriminating between species and partly for finding intra-specific RAPD types (Ronimus et al., 2003; Ruckert et al., 2004; Sadiq et al., 2016; Seale et al., 2012). They were less discriminative and did not detect unique plant-specific genotypes (Seale et al., 2012). Instead, similar genotypes were found throughout the samples and concluded to be ubiquitous across all plants, which was found for powders worldwide (Ronimus et al., 2003; Ruckert et al., 2004; Sadiq et al., 2016). In the present study, the resolution of RAPD typing was greatly increased by the combination of three primers in independent PCR reactions and the high discriminatory potential was confirmed using cgMLST.

Spore forming bacteria persisting in the production environment have been described before. Similarly to *A. flavithermus*, spore forming spoilage bacteria of ESL milk (e.g. *Bacillus cereus*) are hardly found in bulk tank milk while being prevalent in spoiled pasteurized drinking milk suggesting plant-driven recontamination (Doll et al., 2017). The development of an in-house microbiota was in fact previously described for mesophilic *Bacillus cereus* in raw milk silo tanks (Svensson et al., 2004).

Factors that support the survival of spores in production plants include their adherence to stainless steel surfaces that was shown to increase the spore's resistance (Simmonds et al., 2003). Moreover, milk fouling layers provide good growth conditions and hinder the elimination of spores by CIP processes (Hinton et al., 2002; Wedel et al., 2020). Formed biofilms are a protecting environment and potential reservoir as well (Parkar et al., 2004; Zou and Liu, 2018). Cleaning efficiency is impaired by circulating cleaning solutions that do not reach all niches sufficiently which is critical when reservoirs of spores develop in separators, plate heat exchangers (e.g., uneven plates) or gaskets between pipes. These areas must be kept at minimum following the idea of hygienic design. Preventive maintenance and adherence to defined sanitation schedules is highly suggested. Additionally, maturated spores are resistant against regular CIP treatments or heat < 100 °C (Dettling et al., 2019; Wedel et al., 2019; Wedel et al., 2018) and vegetative cells survive and may even proliferate during pasteurisation conditions up to 70 °C (Reich et al., 2017). All these aspects favour the fast and wide distribution of spores in the production plant, their survival and potential settlement. With the start of the next production batch such spores may quickly start to grow and recontaminate the product at a high level. The presence of many persisting strains suggests that the in-house strains are adapted to prevailing conditions, may be able to proliferate very fast and thus have an advantage over those spores being newly introduced into the process.

5. Conclusions

For the powder plants of this study, the influence of bulk tank milk as raw material of powder production on the thermophilic spore load of powders is neglectable, as, in fact, persisting strains of the in-house microbiota of the plant have been demonstrated to prevail in dairy powders. Such recontamination events by persisting strains were not only found for skim milk powder, but also for whey, and they were already known from other products of the dairy industry (e.g. psychrotolerant spores in fluid milk). Therefore, they represent a widespread phenomenon in processes including (pre)heating sections of milk to moderate temperatures. While the colonization of production plants by specific strains is evident, it remains unclear, which genetic and physiological properties may render *A. flavithermus* exceptionally competitive and whether strains adapt by microevolutionary changes acquired during colonization of the production environment.

Strategies to aim for low spore count powders with production times > 10 h need to focus on plant hygiene from the very beginning of the process. Removal of existing reservoirs and prevention of subsequent recolonization will prohibit or at least retard growth and sporulation of thermophilic spores during processing. Optimization of cleaning plans, e.g., application of harsher conditions, higher concentration of cleaning agents, shorter cleaning interval, including chemical disinfection as well as improved hygienic design of equipment and machines are promising measures.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108888.

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Supplementary data

Table A.1: *Anoxybacillus flavithermus* strains for whole genome sequencing, including their strain number, ID of the Weihenstephan Strain Collection (WS), dairy of origin, date of isolation and DDBJ/ENA/GenBank accession number.

Strain no.	WS-ID	Dairy	Date	DDBJ/ ENA/ GenBank accession no.
18	5287	A	16/06/2017	JABJUV000000000
20	5290	A	14/04/2015	JABJUW000000000
21	5292	A	22/04/2016	JABJUY000000000
23	5490	A	22/04/2016	JABJVE000000000
46	5493	A	16/04/2018	JABJVH000000000
47	5492	A	19/03/2018	JABJVG000000000
48	5491	A	20/04/2016	JABJVF000000000
24	5279	B	11/08/2015	JABJUR000000000
25	5291	B	11/08/2015	JABJUX000000000
40	5446	B	16/06/2018	JABJVB000000000
26	5281	C	03/11/2015	JABJUS000000000
27	5364	C	20/05/2018	JABJVA000000000
42	5497	C	30/04/2019	JABJVL000000000
43	5496	C	22/04/2018	JABJVK000000000
44	5495	C	22/03/2018	JABJVJ000000000
45	5494	C	15/02/2018	JABJVI000000000
33	5294	G	20/09/2016	JABJUZ000000000
34	5285	G	23/09/2016	JABJUT000000000
35	5286	G	23/09/2016	JABJUU000000000
36	5448	H	13/11/2017	JABJVC000000000
37	5449	H	13/11/2017	JABJVD000000000

Table A.2**:** Classification of isolates for the analysis of microbial biodiversity in bulk tank milk (BTM), skim milk powder (SMP), whey and whey powder (WP) into categories of species and species groups based on FTIR spectroscopy and 16S rRNA gene sequencing. [1]data for SMP, whey and WP was taken from Dettling et al., 2019. Mean relative abundance per sample = no. of isolates of species per total no. of isolates per sample. Prevalence (fraction of positive samples) = no. of species-positive samples per no. of all samples.

Species	**BTM (n=28)**		**SMP (n=18)[1]**		**Whey (n=17)[1]**		**WP (n=20)[1]**	
	mean relative abundance	prevalence	mean relative abundance	prevalence	mean relative abundance	prevalence	mean relative abundance	prevalence
Aeribacillus pallidus	0.2 %	7.1 %						
Aneurinibacillus spp.	0.3 % *An. thermoaerophilus* *An. aneurinilyticus*	14.3 %	0.3 % *An. thermoaerophilus*	5.6 %	0.4 % *An. thermoaerophilus*	5.9 %		
Anoxybacillus contaminans	0.1 %	3.6 %						
Anoxybacillus flavithermus	0.9 %	10.7 %	85.4 %	100.0 %	92.7 %	94.1 %	92.2 %	100.0 %
Bacillus licheniformis	82.8 %	100.0 %	7.2 %	38.9 %	0.3 %	5.9 %	0.7 %	15.0 %
Bacillus spp. thermophilic	3.7 % *B. hisashii* *B. kokeshiiformis* *B. smithii* *B. thermoamylovorans* *B. thermolactis*	42.9 %						
Bacillus spp. mesophilic	4.1 % *B. altitudinis* *B. circulans* *B. coagulans* *B. plakortidis* *B. pumilus* *B. shackletonii* *B. sonsorensis* *Bacillus sp.nov.* *B. sporothermodurans* *B. subtilis*	39.3 %						
Brevibacillus spp.	3.3 % *Br. agri* *Br. aydinogluensis* *Br. borstelensis* *Br. brevis* *Br. gelatini*	32.1 %	0.2 % *Br. aydinogluensis*	5.6 %			0.2 % *Br. aydinogluensis*	5.0 %
Caldibacillus debilis	0.1 %	3.6 %						
Geobacillus spp.	0.4 % *G. stearothermophilus* *G. thermodenitrificans*	7.1 %	6.1 % *G. stearothermophilus* *G. thermodenitrificans*	27.8 %	5.7 % *G. stearothermophilus / thermoleovorans*	11.8 %	6.8 % *G. stearothermophilus*	35.0 %
n.id.	0.1 %	3.6 %	0.8 %	5.6 %	0.6 %	5.9 %		
Paenibacillus cookii					0.3 %	5.9 %		
Paenibacillus spp.	0.3 % *P. baerengoltzii* *P. campinasensis* *P. lactis*	14.3 %						
Thermoactinomyces vulgaris	0.1 %	3.6 %						
Thermobacillus composti	0.1 %	3.6 %						
Ureibacillus spp.	3.5 % *U. suwonensis* *U. thermosphaericus*	35.7 %						

Dettling, A., Doll, E., Wedel, C., Hinrichs, J., Scherer, S., and Wenning, M. (2019). Accurate quantification of thermophilic spores in dairy powders. *Int Dairy J* 98:64-71.

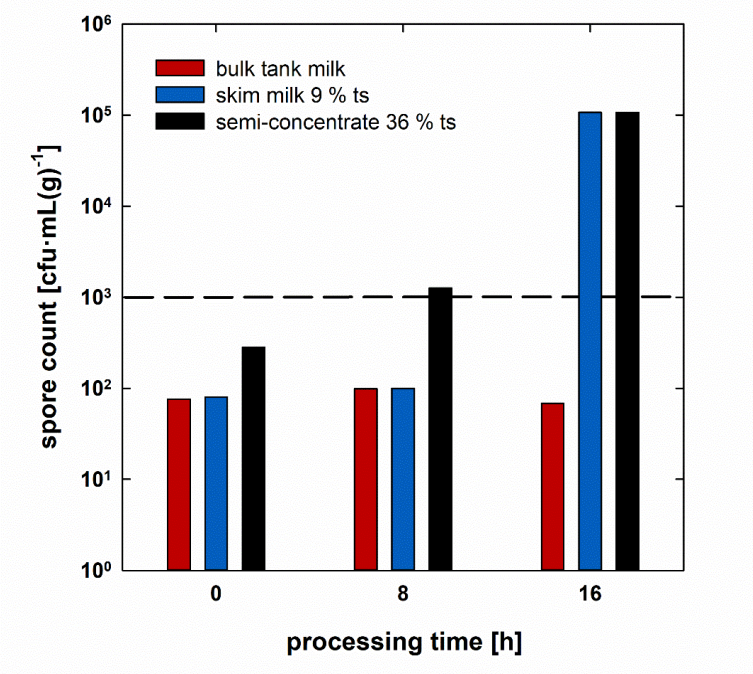

Figure A.1: Development of thermophilic spore count during skim milk powder production in dairy A dependent on processing time and production stage. Bulk tank milk (red), skim milk (blue) and semi-concentrate 36 % ts (black). A spore level of 10^3 cfu·mL(g)$^{-1}$ is visualised by a dashed line. ts = total solids. One production batch and one sample per time point was analysed.

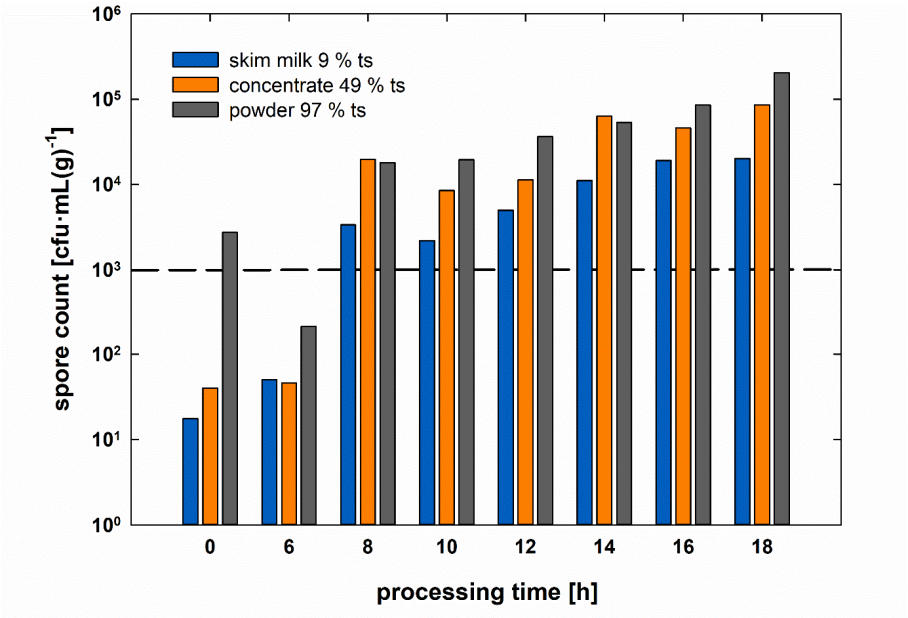

Figure A.2: Development of thermophilic spore count during skim milk powder production in dairy C dependent on processing time and production stage. Skim milk (blue) and concentrate 49 % ts (orange), powder 97 % ts (grey). A spore level of 10^3 cfu·mL(g)$^{-1}$ is visualised by a dashed line. ts = total solids. One production batch and one sample per time point was analysed.

3 Part 3: "Phenotypic and genomic characterisation of *Anoxybacillus flavithermus* strains originating from bulk tank milk versus powder production including persister strains"

Drafted manuscript

ABSTRACT

Thermophilic *Anoxybacillus flavithermus* is a highly abundant part of the milk powder microbiota, but can hardly be detected in non-processed milk. Therefore, a significant change of the thermophilic microbiota during processing occurs in many production plants. Moreover, highly similar isolates recur in plants as persisting strains of the in-house microbiota. This study characterises 41 *A. flavithermus* strains of bulk tank milk (n=18) and milk powder origin (n=23, incl. eight persisting strains) both, phenotypically and genomically. We demonstrate faster growth and better sporulation of powder isolates in milk as well as better survival of heat (95 °C) and NaOH (1 %, 65 °C, 10 min) treatment, conditions similar to often used plant cleaning regimes. It is suggested that phenotypic traits render powder strains highly competitive to remain in the production process, whereas bulk tank milk spores are more easily removed. Phylogenomics reveal a clear evolutionary distance between dairy and non-dairy isolates. Moreover, phenotype-associated pan genome analysis indicates genetic determinants of phenotypic differences. The repetitive NaOH stress to bulk tank isolates suggests the selection of specialised strains for settlement in the production plants rather than their adaptation to production conditions. Knowledge about determinants of specialisation and settlement of persisting *A. flavithermus* strains is needed to prevent further increasing spore counts due to recontamination events and guide back to powders of lower spore count.

INTRODUCTION

Anoxybacillus flavithermus is a thermophilic, spore forming bacterium which is ubiquitous in various habitats like geothermal areas or dairy environments (Khalil et al., 2019, Pikuta et al., 2000, Saw et al., 2008, Tasara et al., 2017). The habitat milk is of particular interest as the level of thermophilic spore counts determines the quality of powdered dairy products when traded globally. While other thermophilic bacilli like

Geobacillus stearothermophilus or mesophilic bacilli like *Bacillus licheniformis* were isolated from milk powders as well*, A. flavithermus* was more prevalent in German powders (Dettling et al., 2019, Pereira and Sant'Ana, 2018, Sadiq et al., 2018). In contrast, the abundance in non-processed raw materials is low in samples worldwide (McGuiggan et al., 2002, Miller et al., 2015, Scott et al., 2007, Yuan et al., 2012, Dettling et al., 2020). Thereby, thermophilic spore loads in bulk tank milk (BTM) samples are mostly $< 10^2$ cfu·mL^{-1}, whereas powders also exceed $> 10^5$ cfu·g^{-1} (Dettling et al., 2019, Hill and Smythe, 2012, Kent et al., 2016).

Discrepancies between raw material and final product of powder productions are often high, and the thermophilic load of powders is not necessarily linked to BTM quality (e.g. Ruckert et al., 2004, Scott et al., 2007, Dettling et al., 2020). For powders with a high abundance of *B. licheniformis*, the BTM spore load and microbiota in final products was partially linked to the powder (e.g. Miller et al., 2015). Moreover, preferably the increase of spore levels during the first steps of milk processing due to growth and sporulation of thermophilic spore formers, particularly of *A. flavithermus*, occurred during pre-evaporation heating and separation of milk (Murphy et al., 1999, Scott et al., 2007, Dettling et al., 2020). Production time longer than 6 - 7 h leads to a higher proportion of thermophilic spores in the product, for example during concentration steps for cheese manufacture (Kable et al., 2019) or during cream-separation for skim milk powder production (Dettling et al., 2020). An in-house microbiota of the production plant as a reasonable contaminant of subsequent production batches leading to high spore counts is assumed, particularly for *A. flavithermus* (Burgess et al., 2010, Miller et al., 2015). Similarly, this was found for mesophilic *Bacillus cereus* as spoilage bacterium of drinking ESL milk (Doll et al., 2017, Svensson et al., 2004). The isolation of several recurring *A. flavithermus* and *G. stearothermophilus* strains for up to 24 months from eight different production lines supports the assumption of resident microbiota (Dettling et al., 2020).

The resistance of thermophilic spores towards harsh environmental influences like desiccation, UV radiation or high pressures and their relatively low sensitivity towards heat and chemical treatments as applied during daily dairy plant routine supports their survival in production plants (Burgess et al., 2010, Dettling et al., 2019, Seale et al., 2012, Setlow, 2014, Wedel et al., 2019, Wedel et al., 2018). Milk fouling deposit on production plant surfaces further enhances the survival and protection of spores for a

longer time (Hinton et al., 2002, Wedel et al., 2020). Additionally, thermophilic spores can adhere on stainless steel surfaces and develop biofilms, an additional reservoir of spores (Burgess et al., 2010, Coleri Cihan et al., 2017, Parkar et al., 2004, Sadiq et al., 2017, Zhao et al., 2013, Zou and Liu, 2018).

It remains unclear, which determinants will lead to a fast proliferation and the formation of a high amount of spores in order to become dominant in the final product if a resident spore population is assumed. First evidence on a dairy adaptation of *G. stearothermophilus*, a closely related species of *A. flavithermus*, by accessory genome analysis was reported recently (Burgess et al., 2017).

The presented study compares *A. flavithermus* strains that originate from BTM and MP samples in order to identify determinants of their persisting behaviour and understand the settlement of thermophilic spores in plants. Phenotypic analyses focussed on proliferation and sporulation abilities and characteristics of spores that support their survival in the production plant (sensitivity to heat and cleaning solution). The genotypic comparison of whole genome sequences using core genome multilocus sequence typing (cgMLST), phylogenomy of highly conserved core genes and pan genome analysis associated with phenotypic traits estimated the phylogenetic relationship of strains and their genetic differences at the gene level. The potential to adapt to a prevailing stress condition was tested in a multiple stress experiment of BTM strains.

MATERIAL AND METHODS

Bacterial strains

The bacterial strains of *Anoxybacillus flavithermus* in this study were initially isolated from bulk tank raw milk or milk powder processes between 2015 and 2019 in Germany (Table II.1). The isolates were identified using Fourier-transform infrared spectroscopy (FTIR) and 16S rRNA gene sequencing as described previously (Dettling et al., 2020). The strain set includes 17 isolates from bulk tank milk (BTM group) and 24 isolates of milk powder (MP) samples and their production lines (MP group). Overall, isolates originate from 13 different dairies (A-M).

Table II.1: 41 isolates of *A. flavithermus* of this study, their isolation source and categorisation of traits (sensitivity to NaOH and heat, formation of > 6 log spores/mL during 48 h cultivation). BTM = bulk tank milk, MP = milk powder, WS-ID = identifier of the Weihenstephan Strain Collection.

No.	WS-ID	Date isolation	Source	Dairy of origin	Sensitivity		> 6 log spores
					NaOH	Heat	
1	5268	20/02/2017	BTM	G	X	X	
2	5267	07/02/2017	BTM	G	X	X	
3	5269	19/09/2016	BTM	G	X	X	
4	5270	19/09/2016	BTM	G	X		
5	5271	04/09/2017	BTM	I		X	
6	5272	04/09/2017	BTM	I		X	
7	5276	23/01/2017	BTM	I	X		X
8	5367	22/01/2018	BTM	I		X	
9	5368	22/01/2018	BTM	I			X
10	5369	22/01/2018	BTM	I			X
11	5265	12/04/2016	BTM	J	X	X	
12	5289	12/04/2016	BTM	J		X	X
13	5266	23/01/2017	BTM	K	X	X	
14	5273	04/09/2017	BTM	L	X		X
15	5274	04/10/2017	BTM	L	X	X	
16	5275	04/10/2017	BTM	L	X		
17	5366	22/01/2018	BTM	L	X	X	
18	5287[c]	16/06/2017	MP	A	X		X
19	5288	16/06/2017	MP	A	X		X
20	5290[c]	14/04/2015	MP	A		X	
21	5292[c]	22/04/2016	MP	A	-	-	-
22	5444[a]	19/03/2018	MP	A		X	
23	5490[a,c]	22/04/2016	MP	A		X	
24	5279[c]	11/08/2015	MP	B	X		X
25	5291[c]	11/08/2015	MP	B			
26	5281[c]	03/11/2015	MP	C		X	X
27	5364[a,c]	20/05/2018	MP	C			X
28	5445[a]	05/04/2018	MP	D		X	X
29	5451	16/04/2018	MP	E	-	-	-
30	5277[b]	06/07/2015	MP	F			X
31	5280	30/09/2015	MP	F		X	X
32	5282	05/03/2016	MP	F	X		X
33	5284[c]	20/09/2016	MP	G	X	X	X
34	5285[c]	23/09/2016	MP	G	-	-	-
35	5286[c]	23/09/2016	MP	G	X		X
36	5448[a,c]	13/11/2017	MP	H			X
37	5449[a,c]	13/11/2017	MP	H			X
38	5283	15/04/2016	MP	J	X		X
39	5278	16/07/2015	MP	M	X	X	X
40	5446[a,c]	13/03/2018	MP	B		X	X
41	5450[a]	07/05/2018	MP	G		X	

[a] persister strains (isolated of at least two charges of one production line)
[b] Dettling et al., 2019 [c] Dettling et al., 2020

Cultivation of thermophilic *Anoxybacillus*

All *Anoxybacillus* strains were cultivated at their optimal growth temperature 55 °C if not indicated differently. From cryo-conserved cultures, stored at -80 °C, each isolate was regrown on tryptic soy agar (TSA, Oxoid) before use.

First cultures in 10 mL UHT milk (1.5 % fat, local distributor) were cultivated overnight (55 °C, 150 rpm). On the next day, second cultures in 50 mL (growth and sporulation analysis, sensitivity of spores) or 10 mL (temperature-dependent growth limits) UHT milk were inoculated 1:1000. The thermophilic cell and spore count of the first cultures were quantified by plating serial dilutions in ¼ Ringer's solution (Merck) on TSA to determine the inoculum. The total cell count (TCC) of the culture was measured by diluting and plating the sample directly, whereas the determination of spore count (SC) required heating the sample at 80 °C for 10 min first (Dettling et al., 2019). Controls were cultivated in parallel of each experiment and checked for sterility of the cultivation medium.

Cultures for analysis of **growth and sporulation** were incubated for 48 h. Then the thermophilic cell and spore count was determined, and the cultures were used for testing the sensitivity of formed spores. Cultures for analysis of the **temperature-dependent growth limits** were cultivated at the test temperature, and the thermophilic cell count was quantified after 48 h. The test temperatures were 28, 30, 34, 37, 40, 45, 60, 62, 65, 68 and 70 °C. At temperatures higher than 60 °C, the cultures were kept in plastic bags in order to prevent extensive desiccation. Cultures were assigned growth positive if the thermophilic cell count increased by more than 1 log level withing 48 h for at least two out of three replicates.

Sensitivity of thermophilic spores to heat and cleaning solution

The 48 h bacterial cultures were analysed for the sensitivity of formed spores towards heat at 80 °C and 95 °C (1) and caustic cleaning solution using 1 % NaOH similar to usual dairy plant cleaning (2).

(1) Three aliquots of 1 mL culture were prepared for the heat treatment in a thermal laboratory shaker (TS basic, CellMedia). Two samples were treated at 80 °C, one sample at 95 °C. The heating time of 10 min started after the set temperature T ± 1 °C was reached. The temperature was controlled continuously using a reference tube. As a reference for data analysis, sample one was cooled directly after 80 °C were reached

(= 80 °C, 0 min). After cooling to RT, the thermophilic spore count of each sample was measured in duplicate by plating on TSA. Each strain was analysed in three independent biological replicates.
(2) For cleaning solution analysis, 1 mL culture was added to 9 mL preheated NaOH solution of 1.11 % (*w*/*w*) strength in order to work at a final concentration of 1 % NaOH. Hereafter, all preparatory steps were conducted immediately in order to minimise side effects due to the addition of the NaOH solution only. The mixed sample was heated in the water bath (WMB14, memmert), which was adjusted to 65 °C and kept at 65 °C ± 1 °C for 10 min. A reference tube was used for continuously controlling the temperature. After cooling to RT, the remaining spore count was measured in duplicate by plating on TSA. Each strain was analysed in three independent biological replicates.

Experimental adaptation of *A. flavithermus* to repeated stress treatments

The initial cultivation of four BTM strains from cryo-conserved cultures on TSA (strain no. 2, 6, 7, 14) started the adaptation experiment (Supplement Figure II.8). Three colonies of each strain were then chosen and cultured independently in separate pre-cultures of 10 mL UHT milk to obtain a total of twelve lines (2A−C, 6A−C, 7A−C, 14A−C). Hereof, the initial culture of the circular proceedings, where regrowth and stress treatment were alternating, was inoculated 1:1000. The cultures for regrowth of bacteria were cultivated in 40 mL UHT milk at 55°C while shaking at 150 rpm. After 20 h and for the stress treatment, 25 mL of the bacterial culture were added to 15 mL preheated 2.66 % (*w*/*w*) NaOH solution, to obtain a final concentration of 1 % NaOH, and mixed immediately. The culture was then heated at 65 °C ± 1 °C for 10 min in a temperature adjusted water bath where the temperature was controlled continuously using a reference tube. After cooling to RT, the culture was centrifuged and washed two times in 1 mL ¼ Ringer's solution. This concentration step was used to transfer all remaining spores to the next round of regrowth and remove cell debris and other milk residues. The remaining pellet was dissolved in 1 mL UHT milk and added to 40 mL UHT milk to serve as inoculum of the next cycle of the experiment. The experiment was conducted for a total of 29 cycles of regrowth and stress treatment. For monitoring of the proceedings, the TCC and SC of regrown cultures were quantified for cycle one to three and from then on every third cycle. After 29 cycles, the 12 evolved strains were

tested for their phenotype. This included the analysis of growth and sporulation, the sensitivity of spores to heat at 95 °C and cleaning solution of 1 % NaOH.

Whole genome sequencing and phylogenomic analysis

The extraction of genomic DNA, subsequent whole genome sequencing and data processing (quality check, trimming, filtering, assembly of draft genomes) was conducted as described previously (Dettling et al., 2020). In brief, the QIAamp® DNA Mini Kit (Qiagen), enzymatic cell lysis (lysozyme, proteinase K) and the RNase treatment were applied during DNA extraction. For sequencing on the Illumina MiSeq platform, libraries were prepared using the TruSeq® DNA PCR-free Sample Prep LT kit (Illumina). Processing of the sequencing data used FastQC (v0.10.1, https://omictools.com/fastqc-tool), the NGS QC ToolKit (v2.2.3, Patel and Jain, 2012) and SPAdes for assembly (v2.5.1, Bankevich et al., 2012).

For phylogenomic analysis, a multiple sequence alignment based on 92 bacterial core genes was calculated through the UBCG software (v.3.0, Na et al., 2018). One analysis included the 41 *A. flavithermus* strains, and another analysis included the strain set extended by 12 publicly available genome sequences of *A. flavithermus* at the National Center for Biotechnology Information (NCBI, Supplement Table II.2). For both strain sets, the General Time Reversible nucleotide substitution model including varying rates across sites and a proportion of invariant sites (GTR +G +I) at 50 bootstrap replications was applied for evolutionary analysis using the software MEGA X (v10.0.5, Kumar et al., 2018). The maximum likelihood tree was then midpoint rooted and visualized using the interactive Tree Of Life online tool (iTOL, v5.5.1, Letunic and Bork, 2007).

The core genome multilocus sequence typing (cgMLST) scheme was created by the chewBBACA software (v2.1.0, Silva et al., 2018) as described previously (Dettling et al., 2020). Allelic differences were then visualised in a minimum spanning tree and using the PHYLOViZ 2.0 software (Nascimento et al., 2017).

Pan genome analysis and association of genotype and phenotype

Draft genomes were annotated *de novo* using Prokka (v1.12, Seemann, 2014). The pan genome of *A. flavithermus* strains was built using the annotated genomes (protein-coding only) and Roary (v3.13.0, Page et al., 2015). At default settings (blastp=95 %), the core and accessory genome was inferred as the strain-dependent presence and

absence of genes. These genes were associated with specific phenotypes (= traits) using pan-genome-wide association studies (panGWAS) of Scoary (v.1.6.16, Bynidsrud et al., 2016). With this, the topology of UBCG analysis was included for phylogenetic analysis. Genes were considered as being significantly associated with the trait at a naïve p-value < 0.05.

Sets of trait-associated genes were analysed for their functionality using the Kyoto encyclopedia of genes and genomes (KEGG) databases (Kanehisa and Sato, 2020). First, the BLASTKoala tool assigned the KEGG Orthology to the given amino acid sequence of each gene. Orthologs were then analysed for their function in metabolic pathways, genetic information processing and signalling and cellular processing using the database of KEGG pathway maps. The associated genes were also categorised into protein families of a specific function using the BRITE hierarchy database.

Statistics

Shapiro-Wilk normality test was performed on data to test for normality. For normal data, the variance was tested using the F-test. To determine differences in sensitivity of spores to heat and NaOH between the BTM and MP group, the two-sided student's classical t-test was used. Similarly, data on the increase in SC for evolved strains 6A-C and 7A-C, the increase in TCC of 14A-C, the sensitivity to NaOH of strain 6A-C, 7A-C and 14A-C and the heat sensitivity of all 12 evolved strains was tested. The comparison of the increase in TCC and SC between BTM and MP strains and analysis of all other data of the 12 evolved strains was conducted using the Wilcoxon Rank Sum test. All tests were carried out using Rstudio version 1.2.5001. A p-value $p < 0.05$ was considered significant (*), $p < 0.01$ as highly significant (**).

RESULTS

Growth and sporulation of *A. flavithermus* in UHT milk

The temperature growth range of all 38 tested *A. flavithermus* strains in UHT milk is extensive and ranged from 30 to 68 °C (Figure II.1). All BTM strains grew from 34 to 62 °C, whereas growth of all MP strains was restricted to 37 – 55 °C. At the upper growth limit, the cut-off was stringent, and the increase by 3 °C to 65 °C prevented the

growth of two-thirds of the 38 strains. A higher proportion of MP strains grew at temperatures lower than 34 °C.

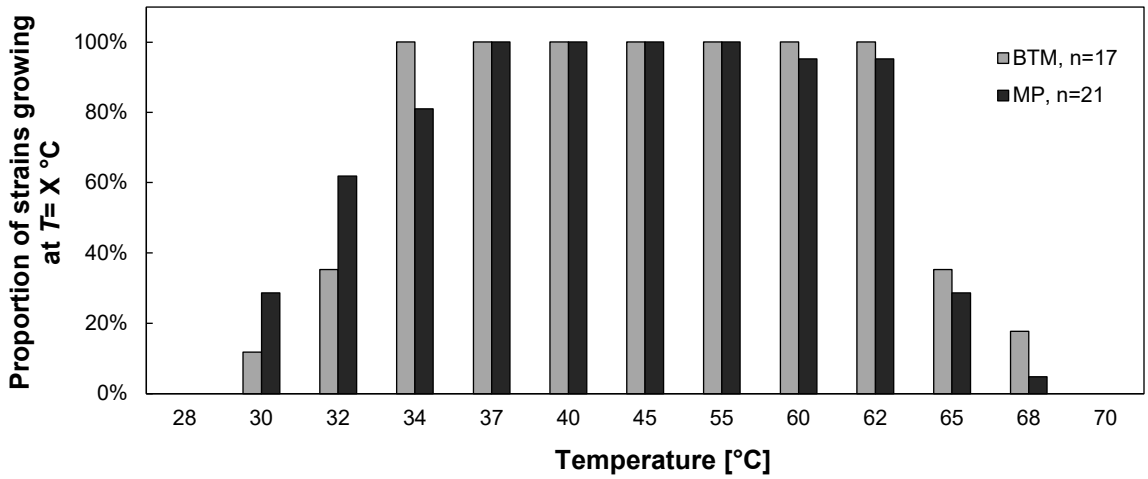

Figure II.1: Growth of *A.flavithermus* strains isolated from bulk tank milk (BTM, n=17) and milk powder (MP, n=21) between 28 and 70 °C.

At 55°C and identical experimental conditions, MP strains showed a more substantial increase in cell and spore count than BTM strains (Figure II.2A). Notably, sporulation was significantly different, and the spore count of MP strains increased between 4.8 and 7.0 log spores·mL^{-1}, whereby one MP outlier at a lower increase of 4.3 log spores·mL^{-1} was observed (no. 25). In contrast, spore formation within the BTM group was highly variable and covered 2.9 to 6.9 log spores·mL^{-1}. The inoculum was between 2 – 5 log cfu·mL^{-1} cells and 1 – 3 log cfu·mL^{-1} spores for both groups. The relation of low inoculum levels leading to lower increases was not given, and it was observed both, an extended increase at low as well as at high inoculum levels. This is valid for both groups.

All strains with an increase of the spore count by more than 6 log spores·mL^{-1} are considered as good spore formers and trait-positive for panGWAS (pan-genome-wide association studies, trait spores).

Sensitivity of spores of *A. flavithermus*

Thermophilic spore counts of 38 test strains were reduced by both heat and NaOH treatment (Figure II.2B, C). The initial spore count SC (80 °C, 0 min) before the treatment was between 3 and 7 log cfu·mL^{-1}, dependent on the strain. At 95 °C, spores

of BTM strains were reduced by up to 2 log levels and significantly more sensitive than MP spores (Figure II.2C). For two strains (1x BTM, no. 16; 1x MP, no. 33), spores were not detectable after the treatment. Here, the value of the detection limit was included in the calculations. This means the sensitivity is at least as high as given.

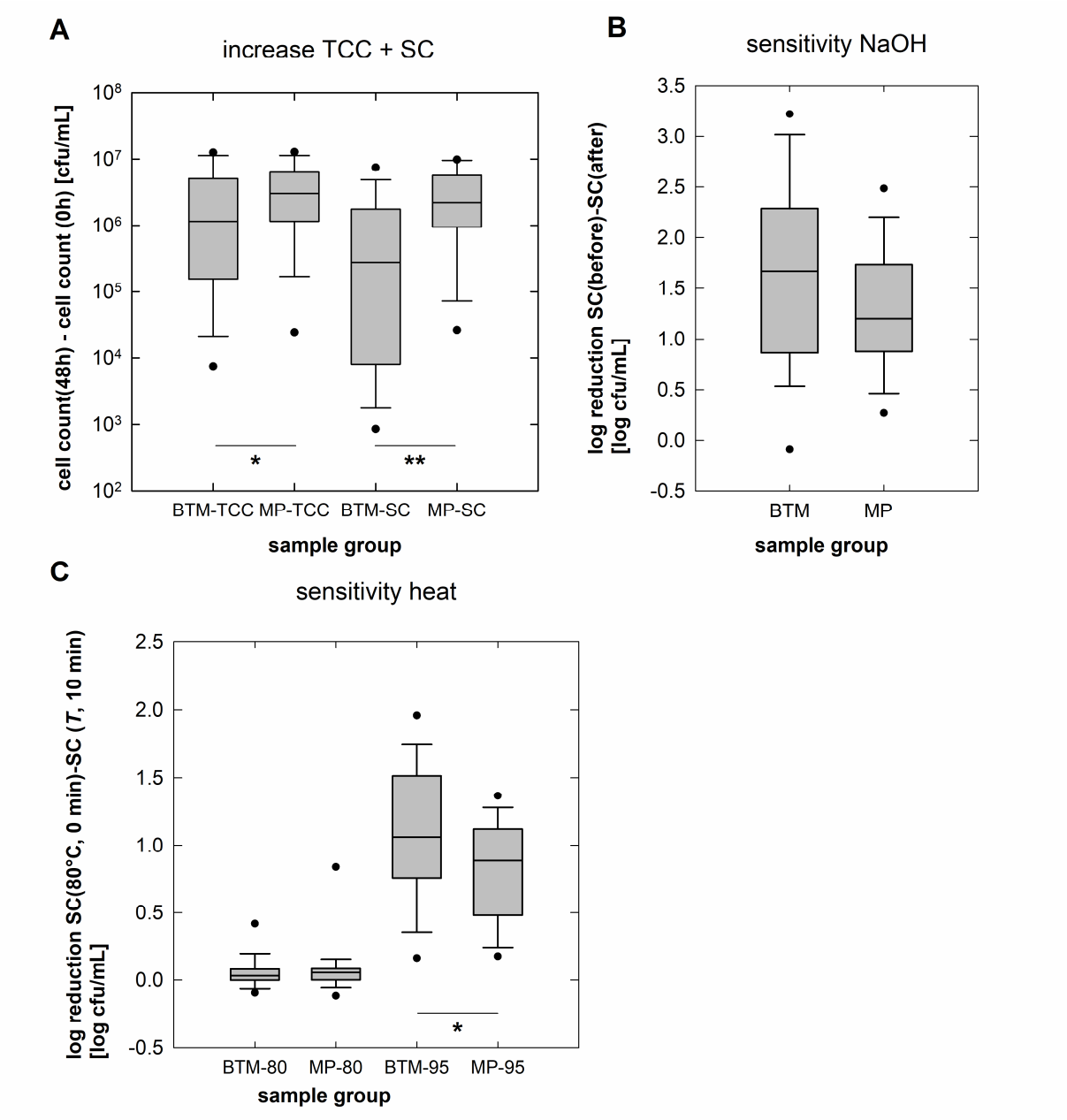

Figure II.2: Phenotypic analysis of *A. flavithermus* strains of bulk tank milk (BTM, n=17) and milk powder (MP, n=21). (A) Increase TCC + SC: Increase of total thermophilic cell (TCC) and spore count (SC) within 48 h cultivation in UHT milk at 55 °C. (B) Sensitivity NaOH: Logarithmic reduction of spore count due to 1 % NaOH treatment (65 °C, 10 min). (C) Sensitivity heat: Logarithmic reduction of spore count due to heat treatment at 80 °C and 95 °C for 10 min. Level of significance between BTM and MP group is marked: * $p < 0.05$, ** $p < 0.01$. Each strain was analysed in three independent biological replicates.

The sporicidal effect of only the addition of NaOH solution to the spore cultures was tested as being neglectable (reduction < 0.5 log levels for 22 tested strains, data not shown). Consequently, it was sufficient to include the spore count of the bacterial culture before (SC before) and after the treatment for the examination of the effect (log reduction SC(before)-SC(after)). Overall, the effect of the NaOH treatment at 65 °C was more pronounced than the heat effect and up to 3.2 log levels of spores were inactivated (Figure II.2B). Spores of five BTM (no. 1, 3, 4, 5, 16) and one MP strain (no. 26) were not detectable after the treatment. As stated before, the inactivation effect is at least as strong as given and could be higher, especially for the BTM group. Spores of MP strains exhibited a remarkable trend to a higher resistance towards NaOH. At a median of 1.2 log levels, they were reduced less than BTM spores (median = 1.7 log levels).

For both treatments, the sensitivity of spores varies in the BTM group. It includes strains of extended resistance as well as the most sensitive ones. MP strains display lower variability than BTM spores, particularly for NaOH treated spores the range is about 1 log level smaller. All strains at a reduction higher than the median of the overall treatment (95 °C = 0.9 log; NaOH = 1.4 log) were considered as sensitive and trait-positive for panGWAS (trait heat sensitivity and NaOH sensitivity).

Based on the combination of each of three phenotypic criteria (heat sensitivity, NaOH sensitivity and spores, Supplement Figure II.9), six strains were grouped as good spore formers of enhanced resistance to heat and NaOH. Their sensitivity was lower than the overall median, and more than 6 log spores were formed. Vice versa, seven strains are of reduced spore formation, and the spores are more sensitive.

Phylogenomy of *A. flavithermus* isolates

The phylogenomy of 41 *A. flavithermus* strains was analysed using two approaches: first, the maximum likelihood (ML) phylogenomy of 92 concatenated universal single-copy genes containing 84,489 and 85,554 nucleotide positions, respectively (Figure II.3A, B). Second, the cgMLST scheme analysis based on 1624 orthologous loci which are present in all strains of this study (Figure II.3C).

The isolation origin of *A. flavithermus* strains is well depicted in the ML phylogenomy (Figure II.3A). Overall, the large cluster of dairy isolates of our study, supplemented by dairy NCBI genomes is evolutionarily distant from isolates of other environments. Non-

dairy isolates expressed an evolutionary distance of 0.04 nucleotide substitutions per site. As an exception, dairy *A. flavithermus* strain B4168 is not part of the dairy cluster. A more in-depth look into the genome characteristics reveal a comparably large genome size of 3.7 Mb and a higher GC content of 43.8 %. Possibly, the identity of strain B4168 is not *A. flavithermus,* but the taxonomy of the *A. flavithermus* cluster was not focussed in this study.

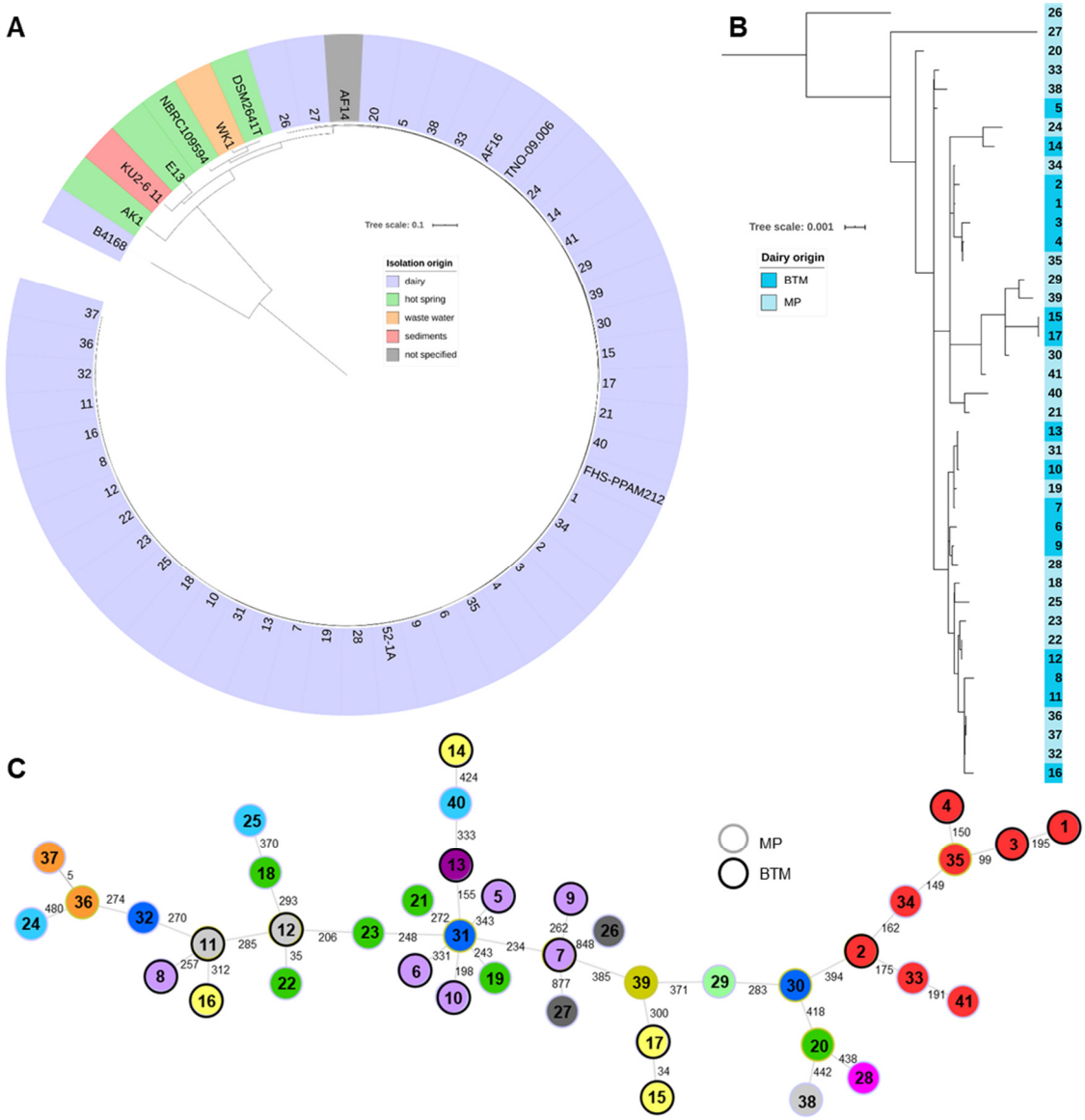

Figure II.3: Phylogenomy of the species *A. flavithermus*. (A, B) Maximum likelihood (ML) tree based on the multiple sequence alignment of 92 conserved core genes including 50 bootstrap replications. (A) ML tree of 53 isolates of different origin (84,489 nucleotide positions). (B) ML tree of 41 strains of this study to differentiate between the dairy origin (85,554 nucleotide positions) (C) Minimum spanning tree based on cgMLST scheme analysis including 1,624 orthologous loci. Nodes represent individual strains, allelic differences between strains are given by numbers at edges. Colours identify isolates of the same dairy. MP = milk powder, BTM = bulk tank milk.

Among the dairy isolates, a general evolutionary gap between the BTM and MP group was not estimated as they do not form two separate clusters (Figure II.3B). Similarly, the cgMLST analysis showed no separation of both groups (Figure II.3C). Though, isolates of dairy G (red in Figure II.3C) show a closer evolutionary relationship among them, compared to isolates of other dairies and form a separate branch of the trees. Nevertheless, there are between 100 and 200 allelic differences in the cgMLST among them. Isolates 26 and 27 exhibited at least 848 and 877 allelic differences in cgMLST and are most distant to the other strains, also to each other.

Pan-genome-wide association studies (panGWAS) and biological function of associated genes

The whole genome sequence assemblies of 41 *A. flavithermus* isolates were on average 2.70 ± 0.07 Mb in length and included 2816 ± 78 annotated protein-coding genes per genome. The pan genome of 41 dairy strains, after Roary and inferred by the strain-dependent presence or absence of genes, was composed of 6293 pan genome clusters. These include 1725 core genes, which are present in 99 – 100 % of all 41 strains (27 % of genes), and 4568 genes representing the accessory genome (73 %). As the total gene number increases by increasing number of genomes and at a relatively constant level of conserved genes, the *A. flavithermus* pan genome of our study is still open (Supplement Figure II.10, definition after Medini et al., 2005).

The strain-specific presence or absence of genes was then analysed for an association with phenotypic traits. Phenotypic data led to the categorisation of 38 *A. flavithermus* strains into the three traits spores, heat sensitivity and NaOH sensitivity. The trait spores includes all good sporulating strains, and the traits heat sensitivity and NaOH sensitivity categorise those strains with spores of enhanced sensitivity. The combination of the three traits groups the heat and NaOH resistant and good sporulating strains and the heat and NaOH sensitive strains of reduced sporulation. Further, two traits represent the isolation source of all 41 strains: MP strains are separated from BTM strains (trait MP), and eight MP strains were previously identified as persisting strains (trait persisters). Even though not included as such, other MP strains may also persist in powder plants, but are not identified yet.

Within the seven traits, between 35 and 112 significantly associated genes were identified (Figure II.4). For all traits, the proportion of hypothetical genes (53 − 66 %)

among the associated genes was higher, and only 34 − 47 % of genes were identified as annotated gene sequences. In comparison, the pan genome of 41 strains was composed of 53 % hypothetical, and 47 % annotated genes while one genome contains between 35 and 41 % of hypothetical genes, on average. Notably and as panGWAS using Scoary implements the analysis of associations to trait-positive and trait-negative strains simultaneously, the results of every trait account for the reverse definition as well, and their additional analysis is not necessary. For example, the analysis of all BTM strains as trait-positive strains led to the association of the identical list of genes compared to the trait MP (data not shown).

Most genes are positively and negatively linked to the trait. In other words, trait-positive strains possess the gene while it is absent in trait-negative strains (Figure II.4). The opposite association was not significant if genes are only positively or negatively associated. If genes are 100 % absent in trait-positive strains and 100 % present in trait-negative strains, these genes are not included in the list.

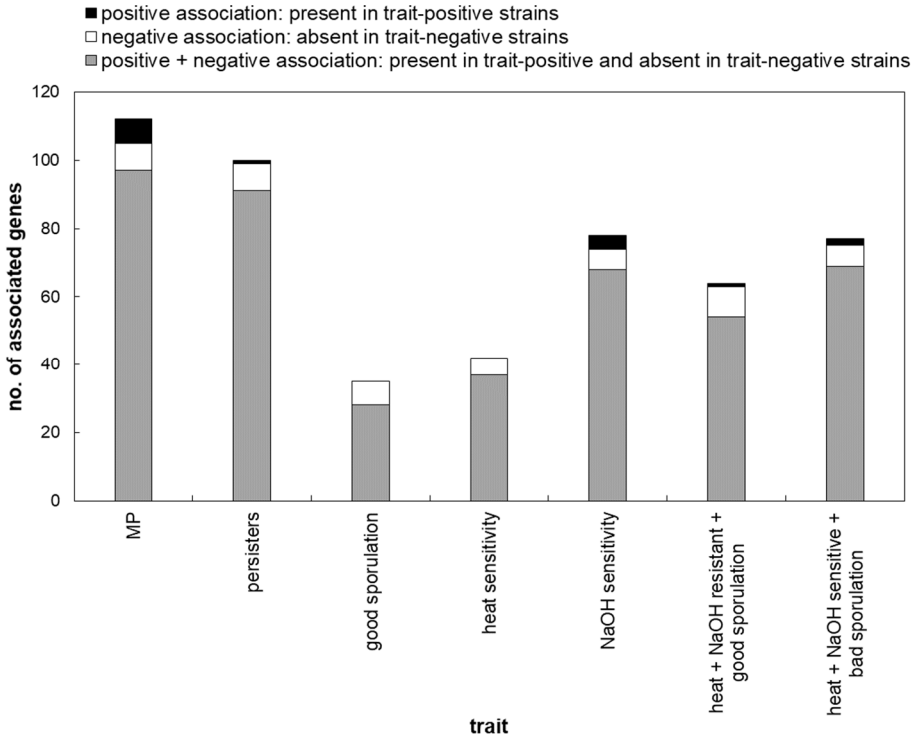

Figure II.4: Distribution of genes of *A. flavithermus* that are significantly ($p < 0.05$) associated with phenotypic traits. The traits heat + NaOH resistant + good sporulation and heat + NaOH sensitive + bad sporulation result from the combination of the phenotype of the three included traits. The total number of genes per trait is divided according to the association of each gene into a positive and negative or only positive or negative association. Positively associated genes are present in trait-positive strains, negatively associated genes are absent in trait-negative strains. MP = milk powder.

Between 22 − 43 % of all genes per trait were annotated to KEGG databases to screen for their function. The remaining genes did not show an appropriate hit. The KEGG annotation using the database PATHWAY and BRITE does not categorise one gene to one function or protein family exclusively. Specific genes often function in more than one pathway and are included in several categories. KEGG PATHWAY annotation associated most and up to 28 hits per trait to metabolism pathways (Figure II.5). Assignments to genetic information and environmental processing as well as cellular processes were of lower prevalence, and a maximum of four hits was observed per trait.

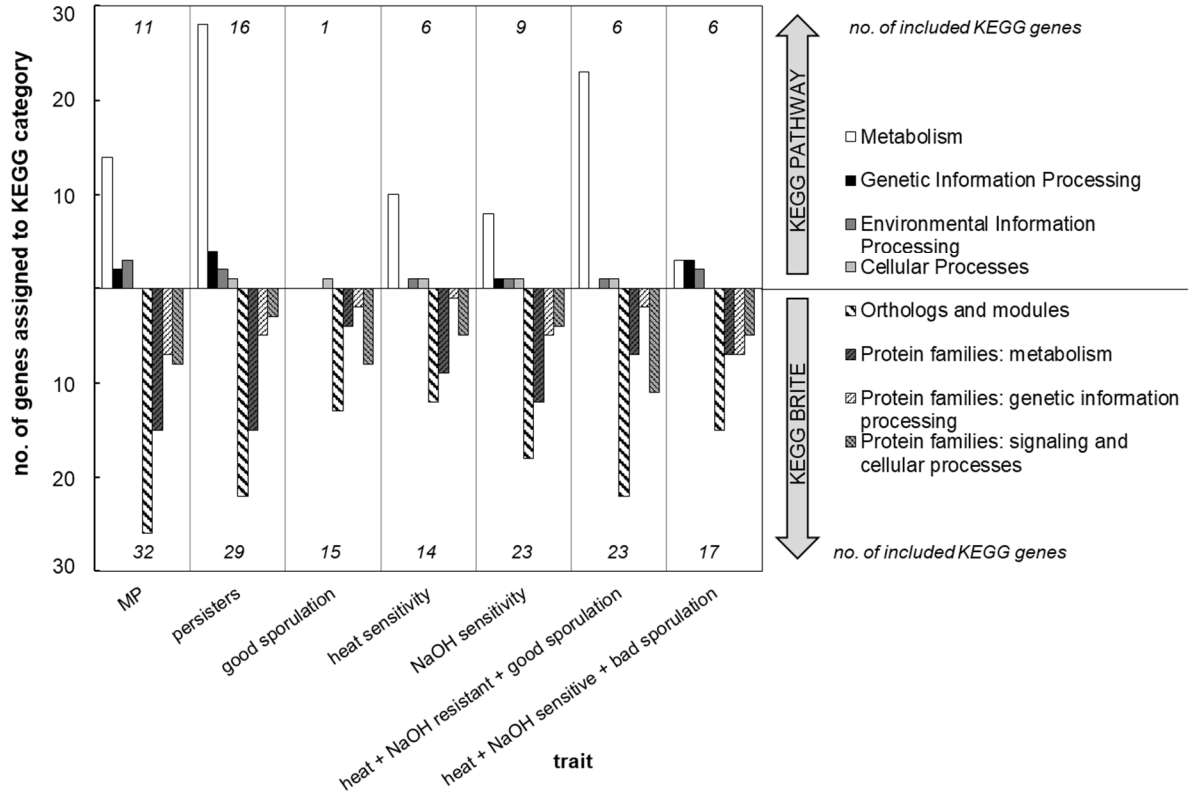

Figure II.5: Functional categorisation of the trait-associated genes according to the two KEGG databases PATHWAY (top) and BRITE (bottom). The total number of KEGG annotated genes per trait is given as the *number of included KEGG genes* for KEGG PATHWAY annotation at the top and for KEGG BRITE annotation at the bottom of the graph. One gene may be categorised into more than one functional group. The traits heat + NaOH resistant + good sporulation and heat + NaOH sensitive + bad sporulation result from the combination of the phenotype of the three included traits. MP = milk powder.

For KEGG BRITE annotations, the more general group of orthologs and modules comprises most gene clusters and between 12 and 26 hits per trait (Figure II.5). Nevertheless, the number of hits is lower than the overall number of KEGG genes per

trait. Since the same KEGG identity was annotated to more than one of the trait-associated genes in some cases, this function is only represented once in the KEGG dataset, which reduced the number of orthologs. The classifications into the three more specific protein families metabolism, signalling and cellular processes and genetic information processing were less abundant (between 1 and 15 hits).

More specifically, most metabolic genes function as enzymes and within the metabolism of carbohydrates. The resistant and good spore forming strains were most prominent in carbohydrate metabolism as well as in the metabolism of nucleotides and other secondary metabolites (Supplement Figure II.11). The MP and persister group are unique for the association with two and three genes for folding, sorting and degradation of DNA (*thiI*, *iscS* and *thiS*). As a tRNA uracil 4-sulfurtransferase (*thiI*), a cysteine desulfurase (*iscS*) and a sulfur carrier protein (*thiS*), they are involved in the sulfur relay system and tRNA biogenesis. Genes of environmental processes involved in signal transduction via the two-component system were found for all traits (Supplement Figure II.12). Besides the pathway functions, KEGG proteins involved in signalling and cellular processes were identified for all traits (Supplement Figure II.12). With this, a high proportion of CRISPR-associated proteins and enzymes of the prokaryotic defence system as well as genes involved in the ABC transporter system were identified. While MP strains exhibited most annotations to transfer RNA and ribosome biogenesis, one to four DNA repair and recombination proteins were omnipresent within the group of genetic information processing and associated with all traits (Supplement Figure II.12). For example, the DNA -3-methyladenine glycosylase *alkA* for base excision repair is associated with the bad sporulating and NaOH and heat-sensitive strains and persister strains, but in opposite direction.

In addition to the functional categorisation using KEGG databases, eight genes of the sporulation process were found to be associated with the traits. This includes a spore germination protein (*gerPB*), other sporulation proteins (*gerPF*, *spoIIR)* and sporulation kinases (*kinA*, *kinB)* which are associated with the trait good sporulation (*gerPF*, *spoIIR*), persisters (*gerPB*), heat sensitivity (*gerPB*, *kinA*), heat and NaOH resistant, good spore formers (*gerPB*), and MP (*kinB*).

Moreover, between 7 – 18 % of associated genes were annotated as transposases which are part of the mobilome for movement and exchange of genetic material.

Effect of multiple stress treatments on the phenotype of *A. flavithermus* strains

Spores of BTM isolates of *A. flavithermus* in this study exhibited a higher sensitivity to the NaOH treatment than MP spores. Therefore, four stress-sensitive BTM strains (no. 2, 6, 7 and 14) were tested for their potential to adapt to this stress condition in a long-term adaptation experiment. The three strains 2, 7 and 14 were initially of high sensitivity to NaOH, expressing a reduction higher than 1.4 log levels. At 0.9 log levels reduction, strain 6 was more resistant. A good spore yield between 4 and 6 log cfu·mL^{-1} in a 20 h bacterial culture was one essential requirement for strain selection.

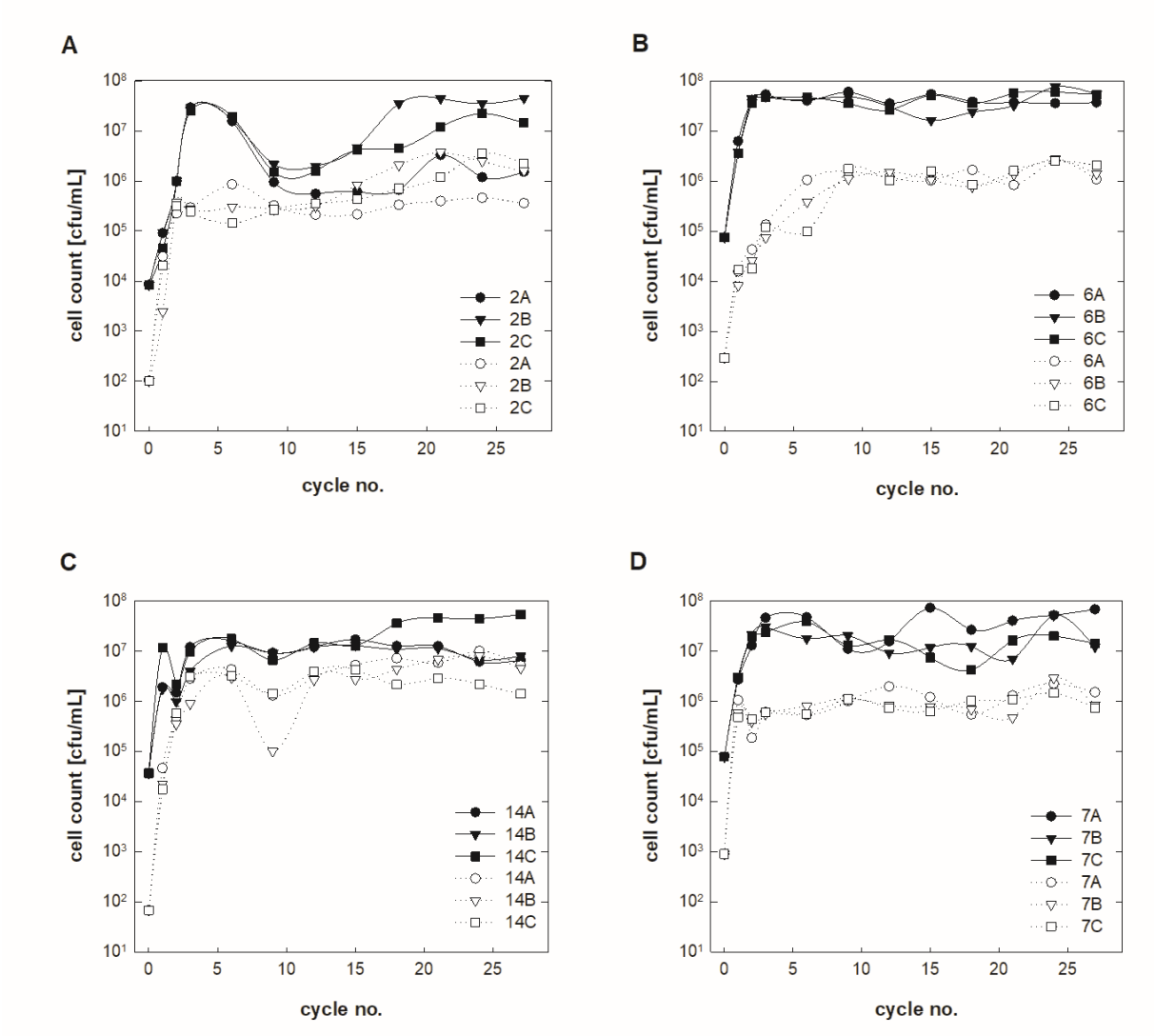

Figure II.6: Thermophilic total cell (black) and spore count (white) in 20h-cultures of 12 evolved *A. flavithermus* strains during the long-term adaptation experiment. Cycle 0 is the inoculum of the first culture and cycle 1-27 represent the counts in the 20h-culture in UHT-milk of each stress cycle. (A) strain 2A-C (B) strain 6A-C (C) strain 14A-C (D) strain 7A-C. Solid and dotted lines are included for a better visualisation and are not based on calculations.

Moreover, the number of spores surviving the stress treatment was between 3 and 6 log cfu·mL^{-1} which is an appropriate bottleneck size to be transferred to the next cultivation.

Alternating stress treatment and cultivation of surviving spores were successfully conducted 29 times for all three parallel lines per strain (2A−C, 6A−C, 7A−C, 14A−C; Figure II.6). In the first cycle, the transfer size between the stress treatment and the next bacterial cultivation was between 3.3 and 5.2 log spores (strain 2A and 7A, respectively).

The course of TCC and SC in 27 regrown 20 h-cultures of the evolving strains 6A-C and 7A-C was very homogeneous among the descendants of the same strain (Figure II.6B, D). The lineages of strain 2 and 14 had a higher degree of sporulation in regrown cultures, at least from cycle ten on (Figure II.6A, C). Interestingly, TCC and SC of 14C were diverging into a lower degree of sporulation from cycle 15 while this was not observed for 14A and B. At the same time anomalies in colony morphology of 14C on agar plates were observed. The colonies appeared in a very bimorph shape, including tiny as well as swarming colonies. Using FTIR-spectroscopy (after Dettling et al., 2020) for identification of different colony types, bacterial contamination of this lineage could be excluded.

After 29 cycles of regrowth and stress treatment, phenotypic characteristics that showed differences between BTM and MP strains were investigated and compared between the initial and evolved strains. The lineages of three out of four strains showed a tendency for better sporulation and growth in 48h-cultivations in milk, compared to their initial strain (Figure II.7A, C). This was particularly pronounced for descendants of strain 2 where the spore count increase in 48h-cultures was up to 2.5 log cfu·mL^{-1} for strain 2B and 2C. Additionally, an increase in TCC after 48 h was detectable for 2A−C, which was not possible for the initial strain 2. The analysis of the sensitivity of spores of the 12 evolved strains did not indicate an evident trend (Figure 7B, D). The sensitivity was relatively similar to each initial strain or a tendency to an increased effect was observed. The relatively high standard deviations of the sensitivity analysis complicate the observation of clear trends, especially if they would only be small. Strain 14C was conspicuous during phenotypic analysis as bacterial cells could only be quantified for one out of four trials in 48h-cultures and growth could not be replicated.

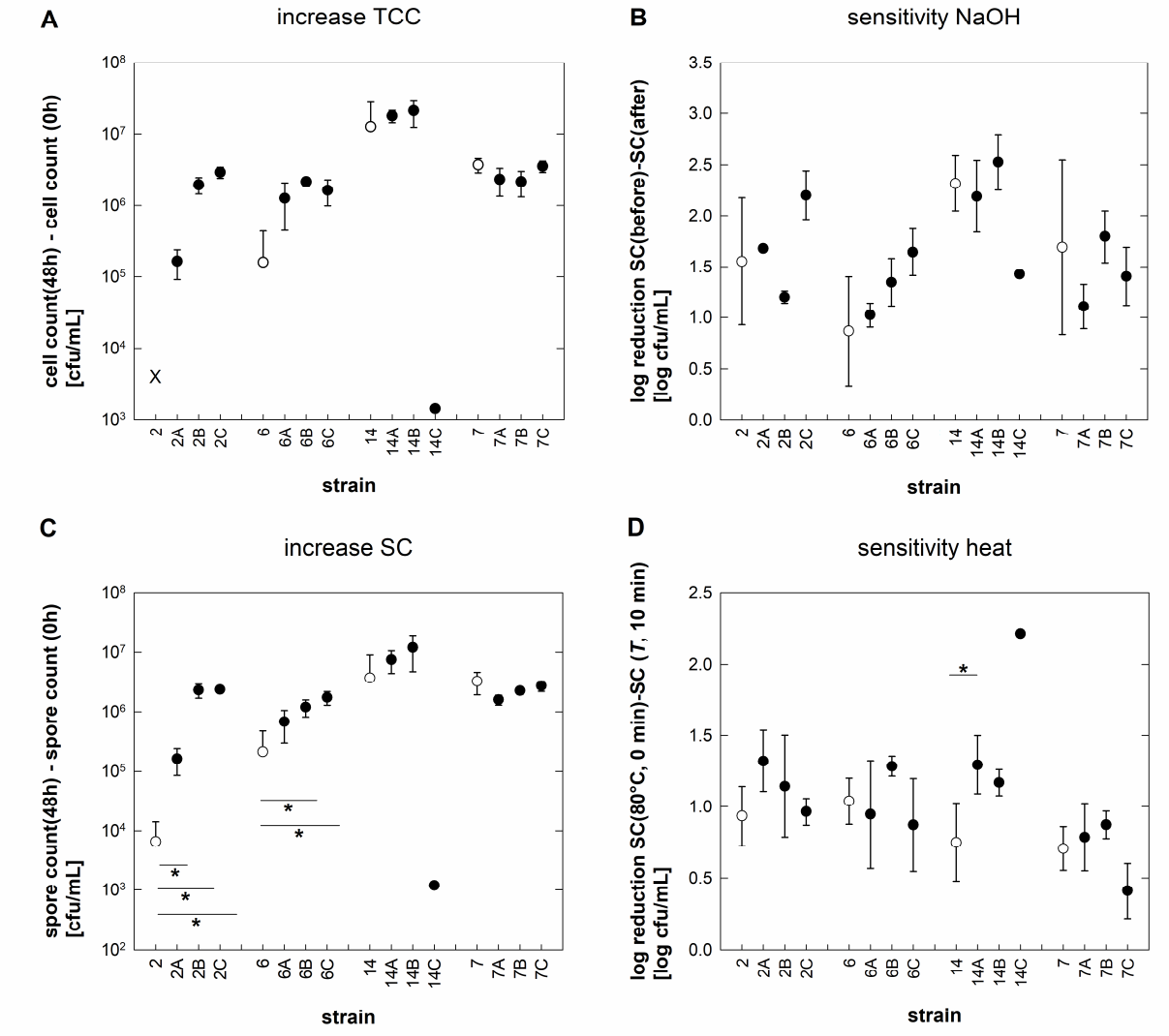

Figure II.7: Phenotypic analysis of 12 evolved *A. flavithermus* lines (● black; 2A−C, 6A−C, 14A−C, 7A−C) compared to their initial strain (○ white; 2, 6, 14, 7). (A) Increase of thermophilic total cell count (TCC) and (C) spore count (SC) within 48 h cultivation in UHT milk at 55 °C. For strain 2, no increase of TCC within 48 h was detectable, which is marked with a X. (B) Logarithmic reduction of spore count due to a 1 % NaOH treatment at 65 °C for 10 min and (D) due to a heat treatment at 95 °C for 10 min. The level of significance between the initial and evolved strain is marked at $p < 0.05$ (*). Each strain, except of strain 14C, was analysed in three independent biological replicates. As growth was inappropriate, only one data point could be obtained for 14C.

DISCUSSION

A. flavithermus strains of MP origin differ in phenotype from BTM isolates

A. flavithermus was initially isolated from a hot spring in New Zealand and described to grow at temperatures between 30 and 72 °C, optimally at 60 °C (Pikuta et al., 2000). Our dairy isolates exhibited a somewhat more narrow temperature growth range, and the upper growth limit was reduced to 68 °C. The limit of 68 °C was already pointed to use as a differentiation tool between *Geobacillus* and *Anoxybacillus* species previously (Burgess et al., 2010). Moreover, the dairy isolates showed a tendency to an optimal temperature lower than 60 °C when cultivated in milk. Additionally, the product stream passes several heating areas at temperatures < 50 °C, e.g. in continuous plate heat exchangers where the temperature increases stepwise, during powder processing. This could explain why a higher degree of MP strains can grow at lower temperatures, especially at 30 and 32 °C. Process isolates are exposed to lower temperatures as they pass such areas or originate from residual biofilms thereof.

Better growth and sporulation abilities of MP isolates, compared to BTM isolates, allow for a faster proliferation and formation of a higher amount of spores. This is advantageous for the presence of theses strains in powdered products. On the one hand, the probability of many completely maturated spores to occur is higher within a larger amount and when sporulation starts earlier. The full maturation of spores is required to develop the optimal resistance, which then increases the chance of survival (Dettling et al., 2019, Sanchez-Salas et al., 2011). On the other hand, a faster growth enables for outcompeting or overgrowing other strains of lower performance, possibly BTM strains that are newly introduced into the production plant. Better growth of MP strains in milk and compared to non-dairy *A. flavithermus* WK1 was already observed (Zhao et al., 2018). Additionally, thermophilic *Anoxybacillus* and *Geobacillus* MP strains show potential for a better proliferation in an environment that was supplemented by external cations (especially Ca^{2+} and Mg^{2+}) similar to real milk conditions (Somerton et al., 2012).

The resistance of thermophilic spores to heat at 80 °C was already established and found among all isolates. Interestingly, previous analyses of spores determined a minor reducing effect due to heat ≤ 100 °C (Dettling et al., 2019, Sadiq et al., 2016, Wells-Bennik et al., 2018). In contrast, spores of the comparative analysis here showed

a certain degree of sensitivity. Especially spores of BTM strains were significantly more sensitive, which was not observed before. Similarly, thermophilic spores of six *A. flavithermus* strains were only reduced by a maximum of 1 log level due to a caustic treatment at 1 % NaOH in a previous study using other strains (Wedel et al., 2019). This was only found for 34 % of 38 strains in our study, and the majority of strains exhibited a higher sensitivity.

At least to a certain degree, working with spores of liquid bacterial cultures instead of pure spore suspensions may cause the observed effect as the spore heat sensitivity was shown to depend on the growth phase (Dettling et al., 2019). Besides the maturation status of the spore population, the sporulation environment also influences the spore resistance (Bressuire-Isoard et al., 2018). Nevertheless, a high proportion of spores should be fully maturated and exhibiting the maximum resistance after cultivation for 48 h. As most isolates of the previously cited studies originated from processed milk or other processed food products, the acquisition of an increased heat resistance could also result from the specialisation of strains from the production environment.

Since the BTM group phenotype was very variable, there are also excellent performers similar to MP strains among them. This holds for fast growth, a high spore yield and enhanced resistance of spores. If these strains enter a production plant, there is a good potential for survival in the plant, thus, for their presence in the product. Among the BTM group of our study strain no.10, which was reduced by 0.2 ± 0.2 log cfu·mL^{-1} only (95 °C) would be a suitable candidate, representing the NaOH resistant outlier as well. For NaOH analysis, the removal of strain no. 10 from the dataset strengthened the difference between both groups to a level of significance $p < 0.05$, expressing an enhanced resistance of MP spores.

Furthermore, the continuously stressed and NaOH-sensitive BTM strains of the adaptation experiment did not evolve into a more NaOH-resistant and MP-like phenotype. At least for the test settings and the time frame of the experiment (approximately 350 bacterial generations and 29 cleaning treatments), an adaptation to the stress condition is not supported. When assuming a regular and daily plant cleaning routine by the manufacturers, the time frame equals approximately one month. This is rather short when compared the time of up to 24 months, where persisting strains were isolated previously (Dettling et al., 2020). The specialised

characteristics of process isolates seem to be based on the natural diversity of *A. flavithermus* strains. Such strains are very suitable to be selected during processing and possibly settle as the persisting microbiota of the plant. However, other processing conditions and longer time may still trigger the adaptation to the processing environment but need to be evaluated and tested. The combinatory stress of cleaning procedures and biofilm formation, for example, could be analysed.

Altogether, isolates of dairy powder processes express good growth and sporulation characteristics in milk conditions. This renders them highly competitive to contaminate milk powders, and a high spore yield of enhanced resistance enables the continuous existence. Since the specialised phenotype was also observed for some BTM isolates and did not evolve through continuous cleaning stress, their settlement in powder plants as persisters is likely.

Genotypic relations of *A. flavithermus* isolates of different origin

The two genera *Anoxybacillus* and *Geobacillus* are closely related with respect to their genome (Bezuidt et al, 2016, Goh et al., 2014). A high proportion of accessory genes and the occurrence of an open pan genome enables the exchange of genetic material which may lead to environmental adaptations. The association of many mobilome genes in our *A. flavithermus* pan genome supports this. Such genes were also found strain-specifically for *Bacillus* species (Kim et al., 2017) and form a basis for a potential niche adaptation to the dairy environment.

Indeed, the ML phylogenomy of the species *A. flavithermus* separated dairy from non-dairy isolates based on estimated evolutionary distances. Similarly, the previously available dairy isolates at the NCBI form a separate cluster due to the analysis of gene content similarity (Khalil et al., 2019), which is also observed for the closely related species *G. stearothermophilus* (Burgess et al., 2017).

Evidence on the dairy adaptation of *G. stearothermophilus* at the genetic level was found by the presence of a putative lac operon (Supplement Figure II.13, Burgess et al., 2017). According to the *S. aureus* lactose operon annotation, the genes *lacA*-*lacD* of the first part function in the tagatose-1,6-diphosphate pathway. The second part of the operon are the genes *lacE*-*lacG* which function in the lactose / galactose specific phosphotransferase transport system for lactose utilisation in the bacterial cell (Götz et al., 2006). Interestingly, the consecutive genes *lacF*, *lacG* and the beta-

galactosidase *cbgA* for lactose / galactose utilisation were present in the *A. flavithermus* core genome of our study. Hereby, *lacF* and *lacG* are two subunits of the lactose transport system permease. The lactose-binding protein *lacE* was present in all but three BTM strains. Besides, the tagatose kinase *lacC*, which occurred primarily in MP isolates and was present in three BTM and nine MP strains, the first part of the putative operon could not be found. Zhao et al. (2018) found lactose utilisation genes in dairy *A. flavithermus* strains only, especially beta-galactosidase (*lacG*) and lactose transporter system genes for better use of lactose. Together with phenotypic characteristics, e.g. enhanced growth in milk media, this is good evidence for how specialisation of dairy isolates could be determined.

Gene associations for genetic information processing, partly enhanced for MP and persisters, enables better protection of genetic material. For example, DNA repair and recombination proteins such as the DNA glycosylase *alkA* are involved in the continuity of the correct DNA sequence. Hereby, *alkA* is mostly absent in NaOH and heat sensitive and bad sporulating strains but present in persister strains. Additionally, a better protection and barrier function of the cells through associations of the prokaryotic defence system, in particular CRISPR associated and transporter system proteins, allows for enhanced resistance and reduced exchange with the surrounding environment (Innamorati et al., 2020). For *B. subtilis*, at least 12 % of genes are expressed during sporulation (Galperin et al., 2012). This attributes the sporulation genes an essential role, mainly as associations with *A. flavithermus* phenotypes were found. Associated signal transduction processes, e.g. via the two-component system, may also function in the sporulation process.

Overall, genotypic associations of *A. flavithermus* strains indicate the importance of sporulation, protection and metabolic process genes to describe their phenotype and isolation origin. The latter is very well depicted by the phylogenomy, which allows for distinguishing between strains of different environments. Genetic determinants of different dairy *A. flavithermus* phenotypes, e.g. better sporulation and spore resistance, are evident but need further support, for example, using alternative techniques like transcriptomics.

CONCLUSIONS

Recurring *A. flavithermus* strains are problematic in milk powder productions and lead to high spore levels. Evident phenotypic differences between BTM and MP isolates express the advantages of process isolates in sporulation capability, the spore resistance to inactivation treatments and fast proliferation over BTM isolates. This leaves MP isolates to be more specialised to prevailing conditions and hinders the efficient removal from the plant. First indications on genetic determinants of the phenotypic observations were found but require more research for their approval. Nevertheless, the estimated evolutionary relations of *A. flavithermus* depict the evolved settlement into the dairy niche, independent from the colonisation of specific production plants.

The exposition of BTM strains to multiple NaOH stress did not lead to the development of enhanced resistance similar to MP strains, whereas improved growth and sporulation was partly achieved. Therefore, the adaptation of non-specialised strains to the production environment and triggered by cleaning stress cannot be assumed so far. The selection of already specialised strains for settlement in production plants is seen more reasonable than the acquisition of specialisation by microevolutionary adaptation to the production environment over time, at least for the applied conditions and evolving time. Among the BTM isolates, suitable candidates were well characterised. The analysis of potential adaptation to other stressors, e.g. alternating caustic and acid cleaning or in combination with biofilm formation, could be the next steps to shed more light into the colonisation of powder plants.

To target the high spore levels, the settlement of thermophilic spore formers in milk powder plants has to be prevented. Additionally, the removal of persisting strains will bring the production plant back to a low spore condition. Both require directed and optimised cleaning and most importantly, disinfection strategies.

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SUPPLEMENT

Table II.2: *Anoxybacillus flavithermus* strains available at the NCBI platform and their NCBI WGS or Refseq/Genbank identifier.

Strain	NCBI WGS ID	Refseq/Genbank ID
WK1		NC_011567.1
TNO-09.006	AMCM01	
AK1	APCD01	
NBRC 109594	BARH01	
AF14	LUFB01	
AF16	LUCQ01	
52-1A		NZ_CP021838.1
DSM2641T		CP020815.1
KU2-6_11	PEDM01	
FHS-PPAM212	SBBW01	
B4168	LQYU01	
E13	AVGH01	

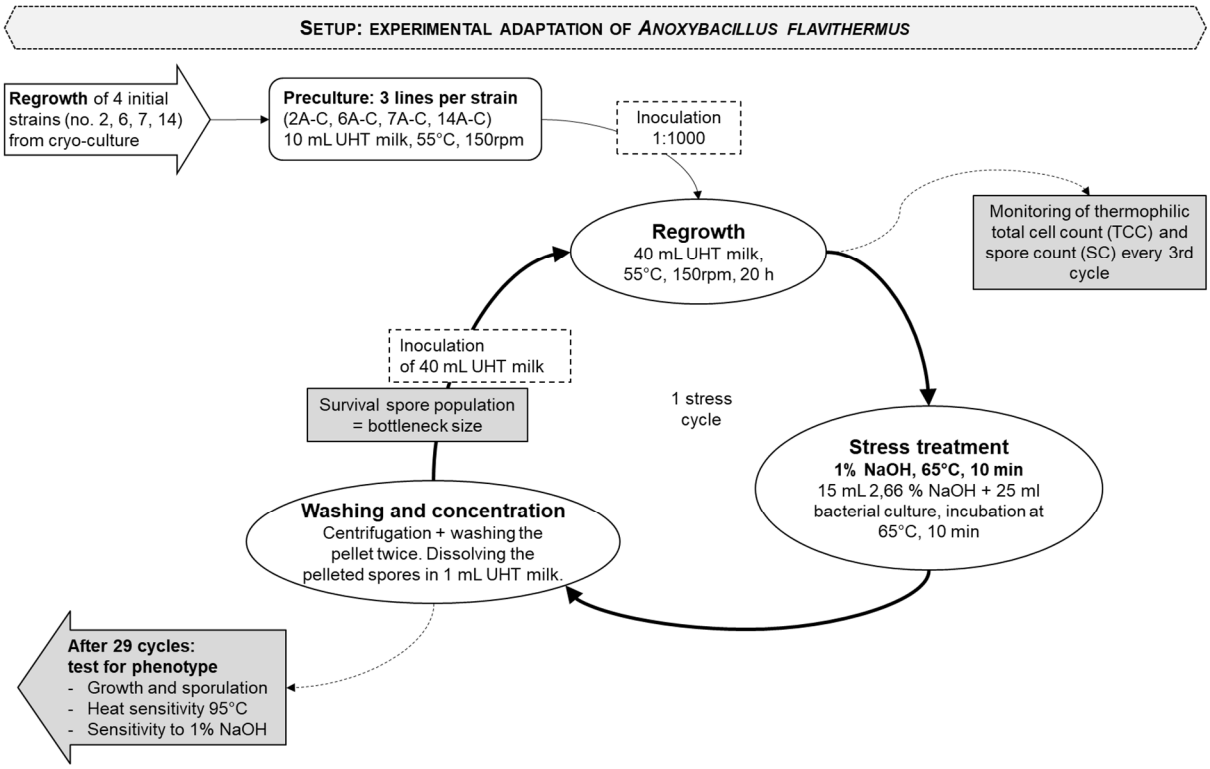

Figure II.8: Experimental setup for the evolutionary adaptation experiment of sensitive *A. flavithermus* strains from bulk tank milk. One experimental cycle consists of the regrowth of the bacterial culture, followed by the stress treatment to the spores of the bacterial culture and the inoculation of the next cycle after the survival spore population is washed and concentrated.

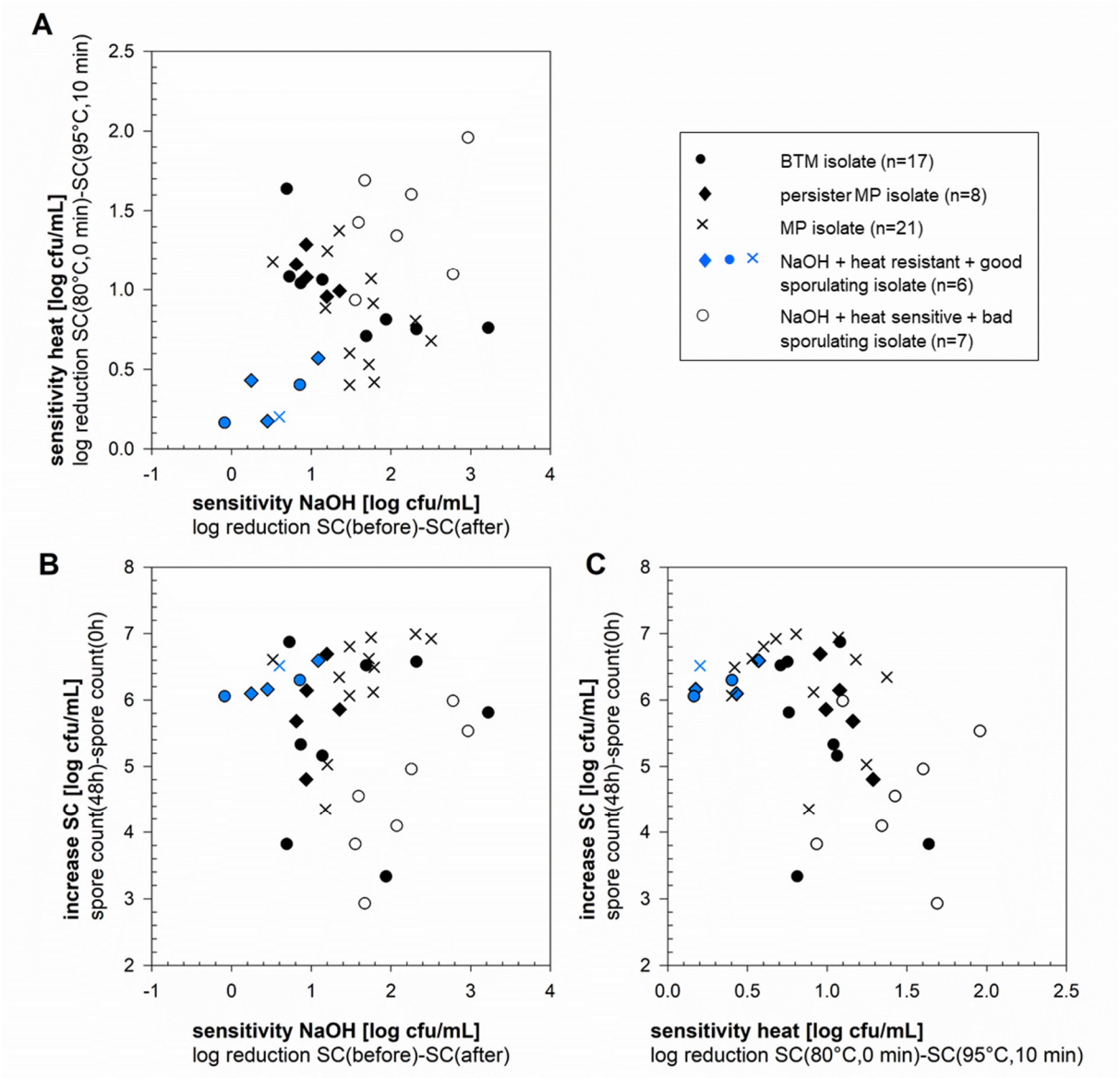

Figure II.9: Combination of the three phenotypic traits sensitivity heat, sensitivity NaOH and SC increase. The two groups “resistant+spores” and “sensitive-spores” include those strains that were higher / lower than the median of the overall outcome in all three characteristic values. (A) sensitivity heat vs. sensitivity NaOH (B) increase SC in 48h vs. sensitivity NaOH (C) increase SC in 48h vs. sensitivity heat. BTM = bulk tank milk, MP = milk powder, SC = spore count.

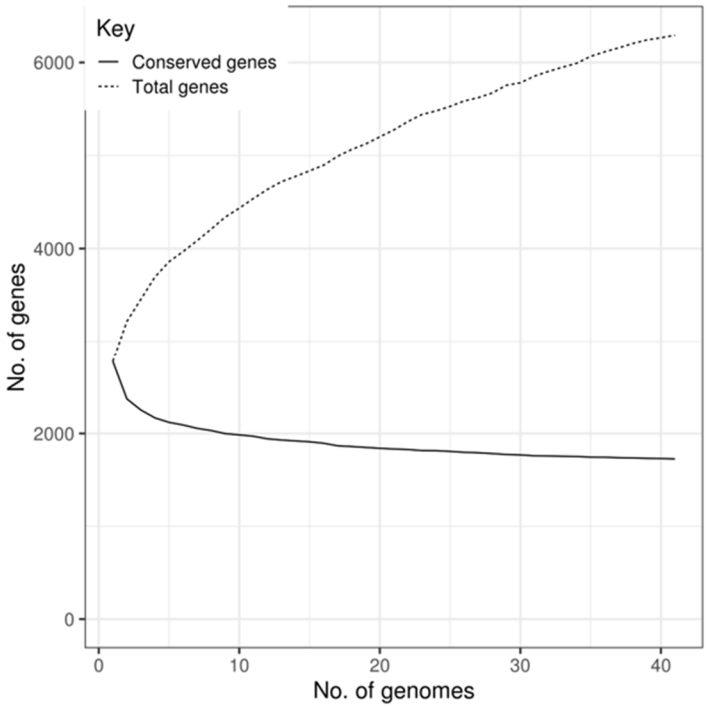

Figure II.10: Dependency of the number of conserved and total genes on the number of genomes for pan genome analysis of 41 dairy *A. flavithermus* strains.

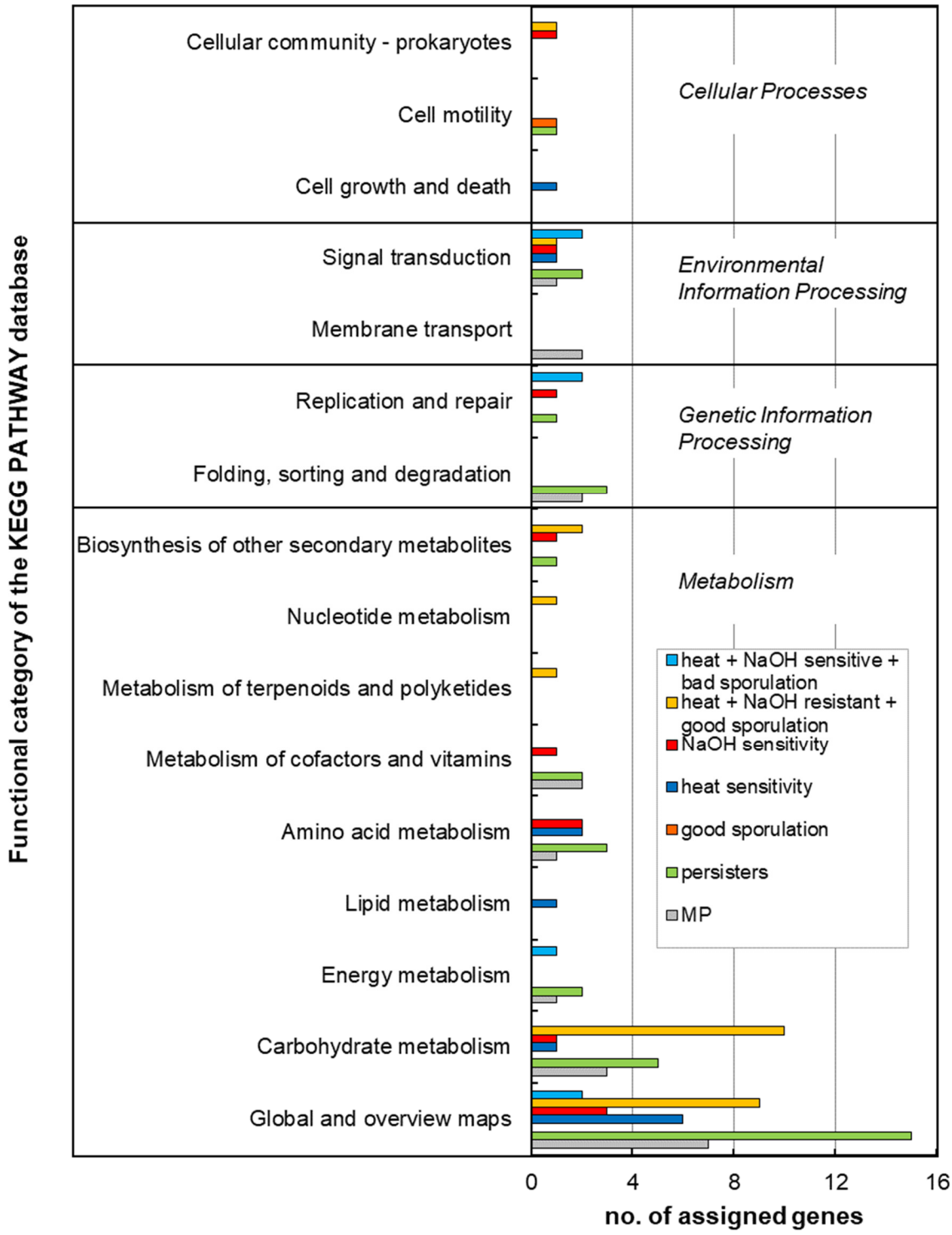

Figure II.11: Functional sub-categories of four KEGG PATHWAY database groups (Cellular processes, environmental information processing, genetic information processing and metabolism) in respect to the number of assigned genes per trait. The traits resistant+spores and sensitive-spores result from the combination of the phenotype of the three traits +spores, sensitivity heat and sensitivity NaOH. One gene may be categorised into more than one category. MP = milk powder.

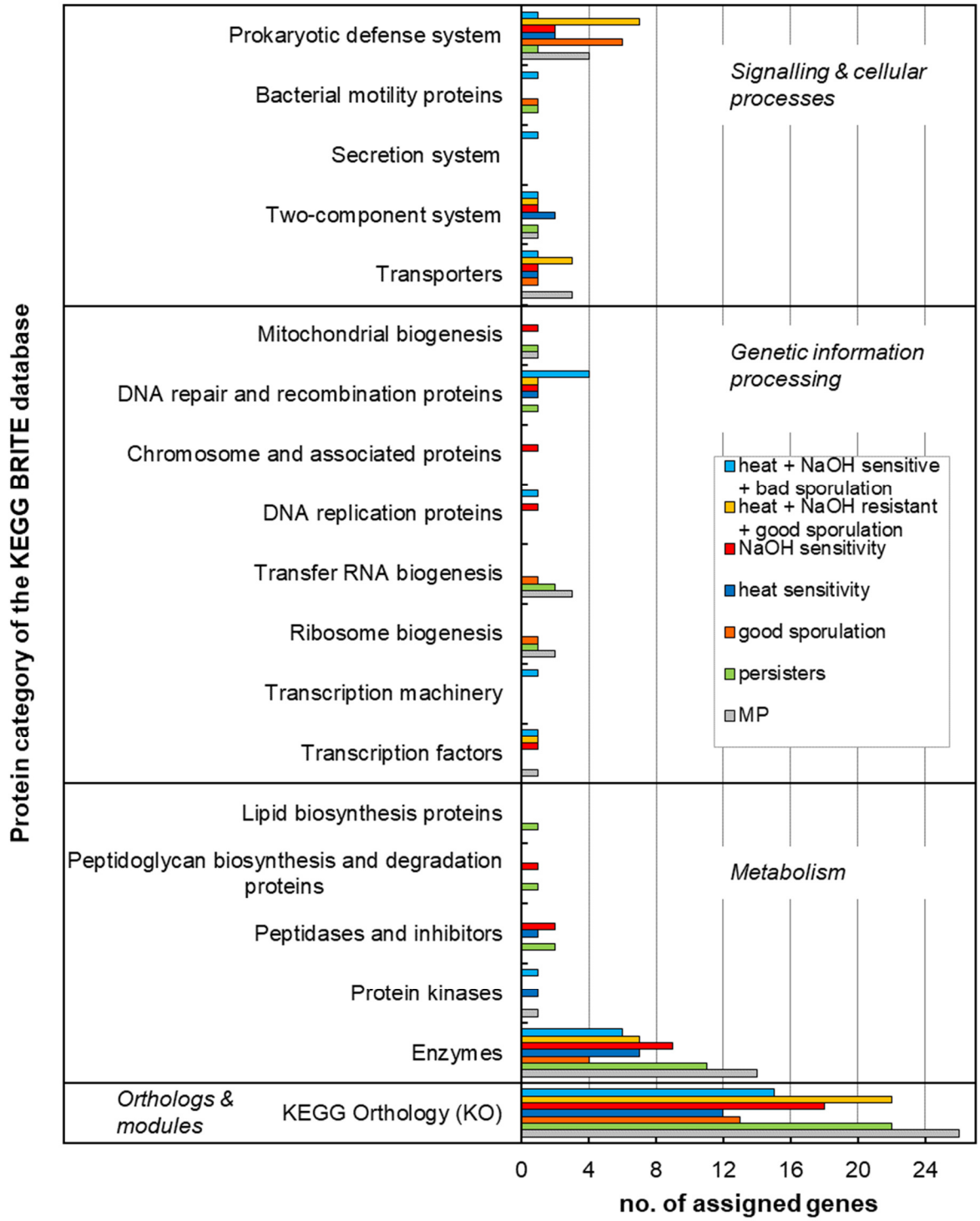

Figure II.12: Protein sub-categories of four KEGG BRITE database groups (Signalling and cellular processes, genetic information processing, metabolism and orthologs and modules) in respect to the number of assigned genes per trait. The traits resistant+spores and sensitive-spores result from the combination of the phenotype of the three traits +spores, sensitivity heat and sensitivity NaOH. One gene may be categorised into more than one category. MP = milk powder.

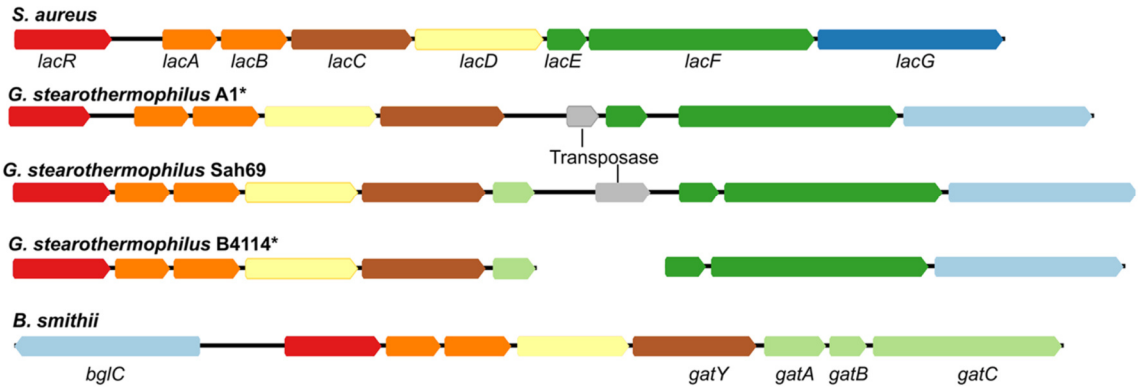

Figure II.13: Putative *lac* operon of dairy *G. stearothermophilus* from Burgess et al. (2017). Comparison of the organisation of *lac* genes. Annotations are based on the assigned KEGG identity of each gene. Colours represent those genes belonging to the same KO group and/or KEGG enzyme entry. The *lac* operon in *S. aureus* and the putative lac operons in strains A1, Sah69, B4114 as well as *B. smithii* (which showed the highest similarity to the putative *lacA*, *lacB* and *lacC* genes from strain A1). Those strains marked with an asterisk were isolated from the dairy environment. The gene organisation of the putative *lac* operon in strains P3 and D1 was syntenic with that of A1. The *gatABC* operon encodes a galactitol transport system and *gatY* a component of the GatYZ tagatose aldolase as described by Van der Heiden et al. (2013). GatY and LacD both belong to the same enzyme group (EC 4.1.2.40).

III GENERAL DISCUSSION

The thermophilic spore load of milk powder is an essential parameter for product quality. The categorisation into low- or high-spore powder determines the sales prize and, consequently, the economic value of the product for the producer. During the last decade, customers intensified their claims towards more and more strict spore loads, which led to the rejection of powders with enhanced spore load, especially challenging German producers. To combat the thermophilic spore problem, this work focused on the production environment, the production process, and entering raw materials to identify the origin of high spore counts in powder. Moreover, the essential contaminant species were characterised. Derived measures may then guide back to a low-spore condition. The first part discusses the effect of different heating conditions on the quantification of thermophilic spores. Then, research hypotheses on the origin of thermophilic spores in powder are developed based on the obtained results of this work.

Heat treatment at 80 °C for 10 min allows for reliable quantification of thermophilic spores

For the determination of endospores, a thermal treatment is applied to only inactivate vegetative cells while spores of enhanced heat resistance survive. Thereby, mesophilic spores are known to be less heat resistant than thermophilic spores (Andre et al., 2013, Kent et al., 2016, Lucking et al., 2013). A temperature of 80 °C is officially established to inactivate mesophilic vegetative cells while not affecting the survival of spores (Frank and Yousef, 2004, VDLUFA, 1985). Also, highly heat resistant spores are quantified following a thermal treatment at 106 °C (ISO/TS 27265:2009). In between those temperatures, there is a vast “grey” area of different temperature conditions for thermophilic spores, and the methods have not been harmonised to date.

The partial survival of pasteurisation conditions at 63-73 °C by vegetative cells of thermophilic bacilli suggested that their inactivation might need higher temperature than 80 °C as it is applied for mesophilic spores (Reich et al., 2017). However, seven *A. flavithermus* and *G. stearothermophilus* test strains were very sensitive to a 80 °C thermal treatment (Dettling et al., 2019). More intense heating did not extend the inactivation effect.

The resistance of thermophilic spores to heat treatments at 100 °C is already well established (Sadiq et al., 2016b, Wells-Bennik et al., 2018). Inactivation was only observed at temperatures > 100 °C (Wedel et al., 2018, Yuan et al., 2012). Consequently, the intensity at 100 °C would be well suited for the inactivation of vegetative cells without affecting the thermophilic spore population. However, the reduction of the thermal treatment to 80 °C would save time for testing and energy resources, if the validity is still given. Spore suspensions of *A. flavithermus* and *G. stearothermophilus* indeed demonstrated high resistance to temperatures between 80 ─ 95 °C and heating times up to 30 min (Dettling et al., 2019). For processed samples of whey, WP and SMP, the intensification of the treatment from 80 to 98 °C and 10 to 30 min led to the continuous reduction of the initially detectable spore population (Dettling et al., 2019). As facultative thermophilic bacilli like *B. licheniformis* are of high abundance in many milk powders (Kent et al., 2016, Yuan et al., 2012), one could assume that the selective inactivation of this more heat-sensitive spore fraction causes the observed reduction. Remarkably, mesophilic spores were only of low prevalence in the analysed samples where the diversity of species was limited (Dettling et al., 2019). Obligate thermophilic *A. flavithermus* was the most abundant and often the only species isolated per sample. Due to the composition of the thermophilic microbiota, the reduction of the spore counts is not exclusively due to the inactivation of heat-sensitive mesophilic spores.

Instead, the heterogeneity of heat sensitivity within the spore population in processed samples may lead to the continuous inactivation effect at temperatures < 100 °C. The spore resistance was shown to depend on the environment (Mtimet et al., 2015, Nguyen Thi Minh et al., 2011), is species- and strain-specific (Durand et al., 2015, Lucking et al., 2013, Sadiq et al., 2016b), and the maximal resistance was only acquired after full maturation (Dettling et al., 2019, Sanchez-Salas et al., 2011). Spores that are formed at sub-optimal conditions or not fully maturated, e.g. forespores, are more heat sensitive and inactivated due to treatment of lower intensity. Similarly, a higher heat sensitivity of spores from planktonic cultures, compared to biofilms (e.g. on production surfaces) was observed (Simmonds et al., 2003). If powders manufactured under comparable conditions are to be analysed for their total thermophilic spore count, a temperature < 90°C will deliver more realistic results than

treatments at higher temperatures. Therefore, the application of pasteurisation at 80 °C for 10 min for the detection of thermophilic spores, as it is the standard for mesophilic spores, is highly suggested in order to prevent the underestimation of spore counts (Dettling et al., 2019). The harmonisation of methods will intensely increase the comparability between manufacturers and different studies.

Hypothesis 1: Thermophilic spore formers enter the production plant via raw materials to be concentrated and present in the product

Milk powder processing removes water and increases the dry mass of the product up to 10-fold. If no other reservoirs shed additional spores and if they are not removed through processing, the spores of raw materials or other influxes would be physically concentrated to produce the high yield in powder. Besides the spore level, the microbial composition of the spore population in comparison to the microbiota in powder is crucial.

The thermophilic spore level in BTM is only very low, < 10^2 cfu·mL^{-1} (Kent et al., 2016, McGuiggan et al., 2002, Scott et al., 2007, Dettling et al., 2020). However, the diversity of bacterial species is vast and comprises mesophilic spores of extended temperature growth range as well as obligate thermophiles (Dettling et al., 2020). The most common BTM representative is mesophilic *B. licheniformis* whose abundance is often > 80 % in one sample (Coorevits et al., 2008, Miller et al., 2015, Dettling et al., 2020), while the prevalence of thermophilic *A. flavithermus* and *G. stearothermophilus* is only 7 ─ 10 % (Dettling et al., 2020). In contrast, the thermophilic spore counts in powder are higher and reach up to 10^5 cfu·g^{-1} (Dettling et al., 2019, Hill and Smythe, 2012, Kent et al., 2016). The difference of up to three log-levels between BTM and powder cannot result from the physical concentration only. However, the independency of spore counts of powder from those in BTM needs verification by the sampling of raw material and final product belonging to one production charge. Since the thermophilic powder microbiota is often composed of mostly obligate thermophilic *Anoxybacillus* and *Geobacillus* (Dettling et al., 2019, reviewed by Pereira and Sant'Ana, 2018, Sadiq et al., 2018), the relation to BTM spores indeed seems not to be given. This accounts at least for powders with a microbiota which is mainly composed of obligate thermophiles. Nevertheless, powder samples of high proportions of mesophilic *B. licheniformis* do

not always relate to the BTM spore population (Miller et al., 2015). This further intensifies the focus on the production plant and process.

Processing steps before evaporation and conducted at thermophilic growth temperatures as well as late production hours are critical for a thermophilic spore count increase. Thermophilic growth was indeed observed in pre-heater sections of WMP processing (Scott et al., 2007), SMP processing (Murphy et al., 1999), and due to initial heat-treatments and separation of cream during SMP production (Dettling et al., 2020). Additionally, Scott et al. (2007) observed a slight rise during evaporation. The increasing spore levels by processing time are accompanied by a substantial change of the microbial composition. *B. licheniformis*, which is prevailing in BTM, is replaced or overgrown by other thermophilic species, mainly *A. flavithermus* (Dettling et al., 2020). Remarkably, the onset of spore formation occurs already at early production time between 4-9 h (Dettling et al., 2020, Murphy et al., 1999, Scott et al., 2007).

In contrast to milk-based products, the whey powder production process does not influence the thermophilic spore level. Here, the spore load of WP is only associated with the physical concentration of the spore load of the entering whey (Dettling et al., 2020, Watterson et al., 2014). This is not surprising as the spore load and thermophilic microbiota of whey already resemble those of powders (Dettling et al., 2019). The processing of whey, where thermophilic growth was observed on concentration steps during cheese making (Kable et al., 2019), is critical for the spore level, similar to the initial processing of SMP powder production.

In conclusion, the thermophilic spore load and microbiota in raw material do not relate to the high spore levels in powder. The physical concentration during powder production is not essential for the increase. Moreover, the observed dynamics reveal that thermophilic growth during processing, particularly at early processing steps and production hours, leads to elevated spore levels.

Hypothesis 2: Recontamination by persisting spore formers in production plants causes high thermophilic spore levels

The enormous discrepancy between BTM and powder and the minor influence of BTM on the thermophilic spore load requires a more in-depth analysis of the transmission route of the prevalent powder contaminants along the product chain. Different batches

of product are composed of the same contaminants if reservoirs of spores in the production plant lead to fast thermophilic growth during production. Newly introduced microbes may be of less importance. Source tracking for persisting bacteria has to differentiate below the species level to distinguish between different, and possibly, to identify identical strains.

Random Amplified Polymorphism DNA (RAPD) PCR analysis is a molecular tool which is easy to perform in the laboratory and widely used to assess strain identities. As introduced in the 1990s, arbitrary primers bind at random positions in the bacterial genome and the amplification of DNA fragments between binding sites leads to the generation of the isolate-specific “fingerprints” (Williams et al., 1990). Advantageously, RAPD analysis does not require the knowledge of the DNA sequence, but the robustness and reproducibility are often doubted. For thermophilic spore formers of both species, *A. flavithermus* and *G. stearothermophilus*, RAPD genotyping is mostly used to differentiate between species (Ronimus et al., 2003, Ruckert et al., 2004, Sadiq et al., 2016a, Seale et al., 2012). To increase the discriminatory capacity to the strain level, a strain-specific RAPD analysis as the combination of three single-primer PCR reactions, instead of only one, was developed (Dettling et al., 2020). For *A. flavithermus* strains, a very close evolutionary relationship of isolates of identical RAPD type was estimated based on the maximum likelihood phylogeny of 92 conserved core genes. Also, strains of identical RAPD pattern display less than ten allelic differences and are therefore closely related according to the more sensitive cgMLST scheme analysis. Hence, the validity of the developed RAPD method to differentiate between strains has been approved using state-of-the-art tools based on whole genome sequences (Dettling et al., 2020).

Strain typing of thermophilic process isolates identifies recurring RAPD types in samples of each of eight German production lines (Dettling et al., 2020). Remarkably, identical and plant-specific strains persisted in production plants for up to two years and were isolated from multiple intermediary samples. In contrast, other genotyping studies were using the less discriminative method and assessed identical genotypes in powders worldwide (Ronimus et al., 2003, Ruckert et al., 2004, Sadiq et al., 2016a). Also, plant-specific types were not detected before (Seale et al., 2012), which

contradicts a specific influence of persisting strains of plant-unique identity on the thermophilic powder microbiota (Dettling et al., 2020).

However, a plant-specific persisting microbiota is very likely to occur (Burgess et al., 2010, Miller et al., 2015). On the one hand, the existence of recurring strains is widely distributed, and the rapid and intensive initiation of growth may not be attributed to low-level contaminants that are newly introduced into the plant. On the other hand, thermophilic spore formers can adhere to stainless steel surfaces (Palmer et al., 2010), form biofilms in milk processing environments (Burgess et al., 2009, Coleri et al., 2017, Sadiq et al., 2017, Zhao et al., 2013), and mineral- and protein-rich milk fouling layers provide suitable proliferation conditions (Visser and Jeurnink, 1997). Moreover, the inactivation of spores is impaired when protected by milk fouling layers (Hinton et al., 2002, Wedel et al., 2020), with increasing resistance of adhered spores (Simmonds et al., 2003), the resistance to heat <100 °C and normal CIP conditions (e.g. Dettling et al., 2019, Sadiq et al., 2016b, Wedel et al., 2019) or the better propensity to attach to stainless steel after surviving the cleaning treatment (Seale et al., 2011). All these factors support the survival and distribution of spores in the production plant, in particular when biofilms and milk fouling residues are not efficiently removed (Parkar et al., 2004, Zou and Liu, 2018). Consequently, reservoirs of persisting spores can develop and are not eliminated by CIP cleaning procedures to recontaminate the next production batches. Developing reservoirs in production areas that are operated at thermophilic growth conditions are very critical, and the proliferation of residual spores is initiated quickly to contaminate the product at a high level. This adds to the rapid and intensive increase of thermophilic spore counts during early powder production steps as discussed above.

The specific colonisation of powder production plants by thermophilic strains is evident. However, it remains to be determined how the bacteria settle and potentially specialise or adapt to prevailing conditions to become persisters.

Hypothesis 3: Powder strains differ from BTM strains and develop as persisters through the adaptation of non-specialised strains to the production process.

Since persistent spores are prevalent in milk powder production plants, they are constitutively exposed to the processing environment and prevailing conditions. In

contrast, BTM spores that are newly introduced into the process originate from a different environment. The adaptation of microbes to a stable environment may result in a specialised phenotype.

Dairy *A. flavithermus*, independent from the exact origin within the dairy environment, grow at a wide range between 30 and 68 °C (unpublished results of II.3). Their optimal growth temperature of 55 °C is slightly reduced compared to other-origin isolates (unpublished results of II.3, Pikuta et al., 2000). Moreover, the proliferation of dairy strains is enhanced in milk conditions (Somerton et al., 2012, Zhao et al., 2018). The differentiation of dairy and non-dairy isolates is also displayed at genome level in the maximum likelihood phylogenomy based on conserved core genes (unpublished results of II.3). All strains of other environmental sources like sediments or hot springs form a separate cluster and display an evolutionary gap to the dairy group (unpublished results of II.3). Likewise, the gene content similarity analysis separates a dairy cluster from other *A. flavithermus* strains (Khalil et al., 2019). For the closely related species *G. stearothermophilus*, whose genome size is slightly larger and up to 3 Mb, a delineation of dairy isolates and built on the core genome is also observed (Burgess et al., 2017). Thus, there are indications of the adaptation of thermophilic spore formers to the dairy environment. Besides, the presence of a putative "*lac*-operon" in dairy *G. stearothermophilus* strains identified first evidence on genetic determinants for dairy adaptation of thermophilic spore formers (Burgess et al., 2017). However, the more specific origin within the dairy environment (BTM versus MP) was not related to a shared evolutionary history (unpublished results of II.3). Small allelic variations in cgMLST scheme analysis of identical RAPD type strains of one process over time, instead, possibly indicate first microevolutionary variations that might be acquired in the production plant (Dettling et al., 2020).

Besides the general differentiation of dairy strains, the phenotype of *A. flavithermus* isolates from BTM differs indeed from powder process isolates (unpublished results of II.3). In particular, a higher spore yield and faster growth to high cell counts indicate an enormous advantage of powder over BTM strains. This is beneficial for efficient initiation of growth during productions. The better survival of heat and cleaning, similar to general CIP treatment, allows MP spores to withstand the applied conditions in the plant (unpublished results of II.3). As the sensitivity of BTM spores is very variable,

more sensitive BTM spores are inactivated and will not endure in the production plant. Interestingly, specific strains of enhanced resistance, similar to powder strains, are observed among BTM isolates as well (unpublished results of II.3).

The settlement of persisting strains in production plants is apparent; process isolates are more specialised to and withstand prevailing conditions. Nevertheless, the specialisation can result from the adaptation of bacteria to the process conditions or is already established in the natural diversity of spores.

During the long-term adaptation experiment, the multiple cleaning stress on BTM strains did not result in an explicit phenotypic specialisation through approximately 350 bacterial generations (unpublished results of II.3). After 29 cycles of alternating stress treatment, and growth of the survival population, the proliferation of the evolved strains was partly improved. Nevertheless, no significant effect on the resistance of the spores in a way that would suggest an adaptation to the stress condition was observed (unpublished results of II.3). Consequently, and at least for the conditions of the experiment, the specific adaptation of persisting strains to prevailing processing conditions is not supported. Notably, specific BTM isolates express a phenotype that is similar to the process isolates. Due to the low fraction of thermophilic spores in BTM (Kent et al., 2016, McGuiggan et al., 2002, Scott et al., 2007, Dettling et al., 2020), such isolates are rarely introduced into the process. Even though the direct effect of the thermophilic BTM spore load on increasing spore counts during processing could not be established (Dettling et al., 2020), the importance of this fraction should not be neglected. The selection of such isolates for settlement as the persisting microbiota is apparent and of considerable relevance to combat increasing spore levels due to in-house persisters. Other triggers of the production environment, e.g. the combination of different stressors, and more stress treatments to mimic a more extended time may still lead to the adaptation of strains, of course, but need to be examined in future experiments.

Preliminary indications for functional genomic adaptation

So far, meaningful and specific genotypic features could not be associated with individual phenotypes, based on pan-genome-wide association studies at the gene level (unpublished results of II.3). Nevertheless, some similar biological functions are

associated with multiple phenotypic traits comprehensively. The combination of the good sporulation phenotype, resistant phenotype and powder origin does then not only focus on specific observations but considers the complexity to converge various properties. For example, the sporulation process includes signal transduction processes and particularly during sporulation initiation the activity of transporter proteins, e.g. for the passage of germinant molecules, and defence proteins. Associated genes of these functions indicate the importance of the sporulation process on multiple phenotypic outcomes (unpublished results of II.3). Additionally, specific phenotype-associated sporulation genes are observed which strengthen the assumption. Regarding the genetic material, functions of genetic information processing are needed to pack the DNA into the spore core and for the general protection of DNA of spore formers. These features are essential during spore formation, and associations to combined phenotypes were found (unpublished results of II.3).

The converged associations of functions with multiple phenotypic characteristics are first indications of genetic determinants for the specialisation of persisting thermophilic bacteria. However, additional research is required to understand the complexity of the biological system of spore forming bacteria in the powder production environment in more depth and elucidate the origin of persistence. Potentially, other phenotypes which were not considered within the selection of criteria yet could provide more insight. The criteria were developed from conditions that may select for specific spores in respect to the processing conditions. Thereby, biofilm formation is another significant problem during processing (Brooks and Flint, 2008, Marchand et al., 2012). The survival of spores and proliferation in respect to biofilms on manufacturing surfaces could be a suitable analysis, in particular when biofilms are not removed during CIP treatments and residues, including spores, remain. A combination of biofilm formation and cleaning treatment is conceivable. Moreover, the survival of spores when incorporated in milk fouling layers is established and could be a criterion for selection or adaptation (Wedel et al., 2020).

Conclusion

Thermophilic spore forming bacteria, mainly obligate thermophilic *A. flavithermus*, are present in high amounts in milk powders. This work showed that the high spore yield

is not primarily caused by the spore load of raw material but originated from the growth of the resident thermophilic microbiota at critical parts of the production as seen during process analyses. Critical processing steps are operated at thermophilic growth temperatures. The operation outside the suitable growth range of *Anoxybacillus* (30-68 °C) would prevent their growth but is not applicable for the effectiveness of the process. The excellent growth and sporulation abilities and enhanced spore resistance of process isolates, where the persistence in production plants for several months to years was established, is beneficial for their continued existence in the plant. Standard plant cleaning treatments are less useful for inactivation of specialised spores. A specialised phenotype and improved survival impair the removal of existing reservoirs which is essential to guide back to a low-spore condition. An optimisation strategy needs to focus on a targeted cleaning and particularly disinfection to remove the persisting microbiota form the production plant to recover good plant hygiene. Subsequently, scheduled cleaning plans will prevent the settlement of specialised strains, possibly introduced by raw materials.

Furthermore, this work delivered the first insights into the origin of persisting thermophilic spores, and dairy isolates were clearly distinguished from isolates from other habitats phylogenetically. Non-specialised BTM spores did not evolve to a specialised phenotype during approximately 350 bacterial generations, whereas many characteristics of powder isolates are advantageous for their survival in production plants. The association of genomic functions of sporulation, metabolism and DNA processing with the specialised phenotype gives indications on determinants for the persistence of thermophilic spores in powder plants. Moreover, some of the rare BTM isolates already expressed an enhanced resistance and excellent proliferation capabilities. The observations show that the low fraction of thermophilic BTM spores is not essential for thermophilic growth in the production right away but could be of importance for settlement into the plant. Based on the results, the persistent microbiota is likely to establish from already specialised strains being introduced into the process rather than from non-specialised strains that develop as persisters through evolutionary adaptation. Nevertheless, the entire complexity of the manufacturing environment was not represented in the adaptation experiment.

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OTHER PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Publications

Reich, C., Wenning, M., Dettling, A., Luma, K. E., Scherer, S., and Hinrichs, J. (2017). Thermal resistance of vegetative thermophilic spore forming bacilli in skim milk isolated from dairy environments. *Food Control* 82:114-120.

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Oral presentations

„Thermophile Sporenbildner in Milchpulvern? – Ein hausgemachtes Problem?“, 17. Fachsymposium Lebensmittelmikrobiologie, Landshut, Germany, April 2017

„Thermophile Sporenbildner in Milch- und Molkenpulver“, Weihenstephaner Milchwirtschaftliche Herbsttagung, Freising, Germany, October 2017

„Vergleichende Analyse von *Anoxybacillus flavithermus* Stämmen aus Milchpulveranlagen und Rohmilch: Persistenz durch Adaption?“, 18. Fachsymposium Lebensmittelmikrobiologie, Kiel, Germany, October 2019

Poster presentations

„Persistent thermophilic spores lead to high spore counts in milk powders”, 5th International ISEKI Food Conference, Stuttgart-Hohenheim, Germany, July 2018

“Persistent spore formers constitute a widespread phenomenon in dairy food production lines”, 26th International ICFMH Conference: FoodMicro 2018, Berlin, Germany, September 2018

“Comparative phenotypic analysis of *Anoxybacillus flavithermus* strains from dairy powder plants and raw milk”, FEMS2019 – 8th Congress of European Microbiologists, Glasgow, UK, July 2019

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EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die bei der TUM School of Life Science der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Thermophilic spore formers in powdered dairy products: Source tracking, population dynamics and genomic characterisation of persisting strains

am Lehrstuhl für Mikrobielle Ökologie, ZIEL – Institute for Food & Health, unter der Anleitung und Betreuung durch Herrn Prof. Dr. Siegfried Scherer ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Ab. 6 und 7 Satz 2 angebotenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistung für mich ganz oder teilweise erledigt.

Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mit bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich einverstanden.

Ort, Datum, Unterschrift

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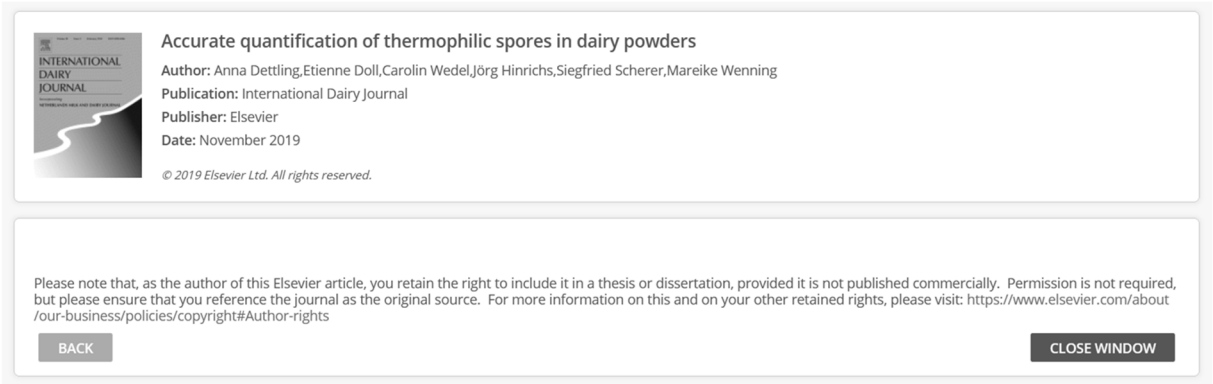

Accurate quantification of thermophilic spores in dairy powders

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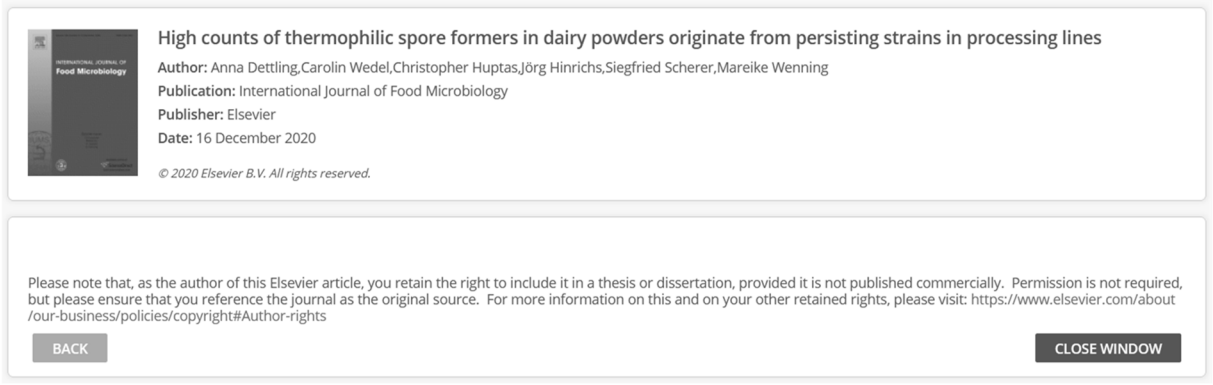

High counts of thermophilic spore formers in dairy powders originate from persisting strains in processing lines

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