NOTE

Bacterial spores isolated from ingredients, intermediate and final products obtained from dairies: thermal resistance in milk

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Received: 16 October 2015 / Revised: 18 December 2015 / Accepted: 18 January 2016 /

Published online: 1 March 2016

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Abstract The ingredients used for the development and production of milk-based products pose the risk of the introduction of new, emerging spore-formers producing highly thermoresistant spores. Therefore, the aim of this study was to examine the heat resistance of spores isolated from dehydrated ingredients, intermediate and final products. Furthermore, the influence of the heating medium (milk or phosphate buffer) on the heat resistance was determined in order to assess which medium is best to use in the context of dairy processing. Sixteen spore-forming strains from seven different species (*Bacillus amyloliquefaciens*, *Bacillus flexus*, *Bacillus subtilis*, *Bacillus thermoamylovorans*, *Bacillus smithii*, *Geobacillus pallidus*, *Geobacillus stearothermophilus*) producing thermoresistant spores were selected after their isolation from diverse food products obtained from local dairies such as cocoa powder, milk powder, spices, and dessert products. Spores produced from the chosen strains were tested regarding their heat resistance at 110, 120, and 125 °C for 30 min. Highly thermoresistant spores surviving a heat treatment for 30 min at 125 °C in milk were produced by *B. amyloliquefaciens* and *G. stearothermophilus*. The inactivation of the spores was higher in milk than in phosphate buffer. This study highlights that not only raw milk but also dehydrated ingredients are important sources of thermoresistant spores in dairy processing. Since

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the inactivation of spores in milk and phosphate buffer turned out to be significantly different, milk rather than laboratory media or buffers should be employed as the model heating system when designing thermal processes for new milk-based products.

Keywords UHT processing · *Bacillus* · *Geobacillus* · Spore · Milk

1 Introduction

Endospores of spore-forming *Bacillus* and related genera are a dormant survival form of the vegetative cells and exhibit a high resistance against environmental factors such as wet and dry heat, radiation, or chemicals (Setlow 2006). In dairy processing, endospores present in raw materials are of major concern because they are able to survive the applied heating processes (Scheldeman et al. 2006; Witthuhn et al. 2011). A large variety of spore-forming bacteria producing highly heat-resistant spores is present in raw milk (Scheldeman et al. 2005). Apart from raw milk, also dehydrated ingredients as used in dairy processing contribute to the spore load of milk products prior to heat treatment (Lücking et al. 2013; Oomes et al. 2007). Postollec et al. (2012) found that 86% of the examined 36 dehydrated ingredients used in food processing were contaminated with a large diversity of spore-forming *Bacillus*. Subsequent outgrowth and enzyme formation of these spores in the final products can result in spoilage and/or toxin formation leading to economic losses for the dairy companies. Considering that especially dehydrated ingredients are traded worldwide and originate from regions with different climatic conditions, new spore-formers producing highly thermoresistant spores may emerge and enter the dairies, thereby challenging the dairy manufacturers.

In milk processing, bacterial spores survive heating processes with moderate heat such as pasteurization and drying. Thus, they are present in various dairy products. In addition, some highly thermoresistant spores are even able to survive UHT heating (see Scheldeman et al. (2006) for a review) although the aim of this process is to inactivate spores (Kessler 2002). These spores are able to spoil final milk products due to their enzymatic activities including proteases and lipases produced during growth (De Jonghe et al. 2010; Lücking et al. 2013).

The composition of dairy products influences the heat resistance of spores. Spores are protected from heat at lowered water activities produced by high solute concentrations as, e.g., in milk concentrates (Kessler 2002; Stoeckel et al. 2013). Also other intrinsic properties of heating media such as pH changes during heating influence the heat resistance (Mathys et al. 2008; Reineke et al. 2011). Therefore, in order to predict the inactivation of spores during technical heating processes using laboratory data, the choice of the heating medium employed for laboratory analysis is crucial. In the context of milk processing, Condron et al. (2015) suggested recently that it is preferable to employ milk as the heating medium as a step towards a harmonized protocol for inactivation experiments. However, in the literature, other heating media are often used to examine the heat inactivation of spores.

This study aimed at giving an overview on the heat resistance in milk of spores previously isolated from the dairy environment, and at assessing if new ingredients introduce highly thermoresistant and new, emerging spore-formers to the final milk products. Furthermore, the heat resistance determined in milk was compared to the heat

resistance of the spores examined in a phosphate buffer solution to provide information about the heating medium to be used in laboratory testing when designing industrial milk heating processes for milk and milk-based formulations.

2 Materials and methods

2.1 Isolation and identification of bacterial strains

Within the entire project, 467 aerobic spore-forming strains were isolated from dairy products, other food samples, and industrial processing environments. Out of these 467 isolates, the spores of 35 representative isolates were tested regarding their thermal resistance in phosphate buffer (0.01 M; pH 7.2) (Lücking et al. 2013). Complementary to the former study, spores of 16 spore-formers were tested in milk in the current study using the same spore batches as in the previous study. These strains were isolated from spoiled and non-spoiled dairy products, ingredients such as spices or cocoa powder, and intermediate products before heating (Table 1).

The isolation of the spore-forming bacteria from the food samples was described in Lücking et al. (2013). Briefly, the samples were diluted with Ringer solution, heated at 100 °C for 20 min, enriched with BHI broth, spread-plated on BHI agar and incubated at 30 and 55 °C in order to gain mesophilic and thermophilic isolates, respectively. After isolation, the bacteria were identified using FT-IR spectroscopy and partial sequencing of the *gyrA* gene (Lücking et al. 2013).

2.2 Preparation of spore suspensions

The preparation of the spore suspensions was performed as described in Stoeckel et al. (2013), except that the sporulation medium from Iciek et al. (2006) was used for thermophilic strains. Briefly, spores were produced from growing cells on sporulation medium agar containing manganese. The spores were harvested after incubation at 30 or 55 °C for up to 10 days using phosphate buffer and were washed eight times in total. The remaining vegetative cells were inactivated via a heat treatment (80 °C, 10 min) and the addition of ethanol to a final concentration of 35%. The final spore suspension was stored in phosphate buffer at <4 °C. The same spore batches were used in this study and the study of Lücking et al. (2013).

2.3 Inactivation experiments

UHT milk (3.5% fat) purchased from a local supermarket was used as a heating medium. The spore suspensions were mixed with the heating medium in a ratio of 1:15 and transferred to screw-capped stainless steel tubes with a volume of 1.5 mL. The tubes were heated for 30 min at 110, 120, and 125 °C in a batch heating system previously described (Dogan et al. 2009). The temperature development in the tubes was monitored continuously and directly in the medium. Approximately 15 s was needed for heating up and for cooling down the medium (Stoeckel et al. 2014a). The holding time started when the desired temperature ± 0.5 °C was reached in the heating medium. The time needed for heating and cooling was neglected as very long holding

Table 1 Survival of spores of sixteen strains after heating for 30 min in milk at 110, 120, and 125 °C

Strain	Origin	Cell count (cfu.mL ⁻¹) before and after heating at different temperatures					
		110 °C		120 °C		125 °C	
		Before heating	After heating	Before heating	After heating	Before heating	After heating
Mesophilic spores							
<i>B. amyloliquefaciens</i> F45PL	Cocoa powder	8.44 ± 0.01	1.48 ± 0.19	8.09 ± 0.03	<1		
<i>B. amyloliquefaciens</i> F85	Wild garlic spice	8.06 ± 0.06	1.02 ± 0.34	8.03 ± 0.03	1.93 ± 0.02	9.04 ± 0.03	1.52 ± 0.15
<i>B. flexus</i> F56	Kaffir lime leaves	8.03 ± 0.09	1.00 ± 0.35	8.11 ± 0.06	<1		
<i>B. subtilis</i> F46	Milk powder	5.79 ± 0.08	<1				
<i>B. subtilis</i> F59	Poppy (baking ingredient)	8.28 ± 0.11	3.62 ± 0.25	8.19 ± 0.01	<1		
<i>B. subtilis</i> F68	Chocolate pudding ^a	7.22 ± 0.00	1.84 ± 0.26	7.14 ± 0.02	<1		
<i>B. subtilis</i> F98	Ginger mixture	6.56 ± 0.02	1.00 ± 0.60	6.34 ± 0.00	1.71 ± 0.34	6.33 ± 0.02	<1
Thermophilic spores							
<i>B. thermoamylovorans</i> F12	Adjusted milk (for rice pudding) ^a	8.19 ± 0.02	<1				
<i>B. thermoamylovorans</i> F42	Cocoa powder	8.07 ± 0.10	<1				
<i>B. thermoamylovorans</i> F61	Poppy (baking ingredient)	6.81 ± 0.03	1.38 ± 0.43	6.83 ± 0.03	1.48 ± 0.21	6.50 ± 0.06	<1
<i>B. smithii</i> F64	Dessert with curd ^b	6.29 ± 0.29	<1				
<i>B. smithii</i> F78	Caraway spice	6.42 ± 0.03	4.83 ± 0.63	6.50 ± 0.01	1.86 ± 0.21	6.09 ± 0.05	<1
<i>B. smithii</i> F93	Cream pudding ^a	7.85 ± 0.03	1.12 ± 0.21	7.81 ± 0.02	1.12 ± 0.29	7.89 ± 0.10	<1
<i>G. pallidus</i> F40	Cocoa powder	7.12 ± 0.12	<1				
<i>G. stearothermophilus</i> F52	Rice pudding ^c	6.60 ± 0.02	6.62 ± 0.31	6.63 ± 0.00	<1		
<i>G. stearothermophilus</i> F74	Cheese spread ^a	6.43 ± 0.18	5.27 ± 0.02	5.77 ± 0.05	1.41 ± 0.34	5.62 ± 0.01	1.00 ± 0.33

^a Intermediate product before heat treatment
^b Final product without visible spoilage
^c Spoiled product

times were examined. The determination of the spore count before and after the heat treatments was performed after serial dilutions in Ringer solution (Merck, Darmstadt, Germany) on tryptic soy agar (TSA; Roth, Karlsruhe, Germany). The agar plates were incubated at 30 and 55 °C for 4 days for mesophilic and thermophilic strains, respectively. Each heating experiment was conducted three times. Additional experiments with phosphate buffer (0.01 M; pH 7.2) as the heating medium are reported elsewhere (Lücking et al. 2013).

2.4 Statistical analysis

The logarithmic reductions of the different strains in the two media were compared groupwise for each temperature examined. A Mann–Whitney rank sum test was performed using SigmaPlot (Systat Software, San Jose, CA, USA). The limit of detection was 10 cfu mL⁻¹. If spores were completely inactivated, they were not examined anymore at the following higher temperature(s). However, for statistical purposes, these missing values were considered as complete inactivation and the logarithmic reduction observed at the foregoing, lower temperature was used.

3 Results and discussion

Due to their ubiquitous nature, spores are present in the raw materials used in dairy processing. Average spore counts in raw milk are 10¹ to 10³ cfu.mL⁻¹ with occasional counts of up to 10⁴ to 10⁵ cfu.mL⁻¹. The sources of the spores in the raw milk are, amongst others, the milking machines and the feed (Scheldeman et al. 2005; Te Giffel et al. 2002). Also non-dairy-based ingredients represent important sources of highly thermoresistant spore-formers in food processing (Lima et al. 2012; Lücking et al. 2013; Oomes et al. 2007). Heat resistance tests of spores are usually performed in buffer systems or laboratory media. Since it is known that the heating medium influences the heat resistance of spores, milk was employed as model heating system in the current study to determine the heat resistance of spores identified as thermoresistant spores (TRS) and previously examined using a phosphate buffer system (Lücking et al. 2013). Out of the 35 strains examined in phosphate buffer, 16 representative isolates were chosen to be further tested in milk. Ten out of these 16 strains originated from dehydrated ingredients such as cocoa powder and spices, four from intermediate products before heat treatment, one from an unspoiled final product and one from a spoiled final product (Table 1). At 110 °C, 69% of the spores survived the applied heat treatment. At 120 °C, still 38% of the spores survived. At 125 °C, two out of the six remaining strains were still active after the applied heat treatment, representing the group of strains producing highly thermoresistant spores (HTRS) (Table 1 and Table 2). The HTRS were produced by strains of the species *Bacillus amyloliquefaciens* and *Geobacillus stearothermophilus*, which belong to the mesophilic and thermophilic isolates, respectively. These HTRS were isolated from wild garlic spice and unheated cheese spread.

The food products found here to contain TRS and HTRS were also shown to contain thermoresistant spores in the literature. Apart from raw milk (Scheldeman et al. 2006), also in cocoa powder (Lima et al. 2012) and spices (Oomes et al. 2007) thermoresistant

Table 2 Comparison of the heat resistance of the tested strains in milk and phosphate buffer

	Milk			Phosphate buffer ^a		
	110 °C	120 °C	125 °C	110 °C	120 °C	125 °C
Number of strains surviving/tested strains	11 / 16	6 / 16	2 / 16	15 / 16	11 / 16	7 / 16
Strains surviving (%)	69	38	13	94	69	44
Mean log reduction	5.1 ^b ± 2.2	6.0 ± 1.0	6.2 ± 1.0	2.9 ^b ± 1.7	5.7 ± 1.0	6.0 ± 0.8

^a Data from Lücking et al. (2013)^b Values are significantly different

spores were found. Spores of thermophilic bacilli isolated from milk powder have been shown to survive UHT heating (Hill and Smythe 2012). Thus, this study confirmed that besides raw milk also the various other ingredients used in dairy processing are important sources of HTRS. All of the species examined in this study were beforehand already isolated from milk (Phillips and Griffiths 1986; Scheldeman et al. 2005). Therefore, exotic ingredients for new milk-based dairy products might not necessarily introduce new species to the products. However, ingredients can introduce new strains of these species that are highly thermoresistant. In order to verify if these strains can cause problems in UHT milk, however, additional tests are necessary to determine their inactivation kinetics as well as their spoilage potential.

The presence of spores in the final product can but does not necessarily lead to spoilage due to growth and enzyme production (Lücking et al. 2013). These authors demonstrated that in milk processing, 50 out of the 98 examined food products without visible trace of spoilage, including final and intermediate products as well as ingredients, were contaminated with potentially heat-resistant spore-formers. However, even if the intermediate products and ingredients are not spoiled, this does not exclude the possibility that heat-resistant spores are able to spoil the final milk products during storage. Typical enzymatic activities responsible for spoilage of the final products are, amongst others, proteases and lipases, which are produced by different spore-forming genera (De Jonghe et al. 2010; Lücking et al. 2013).

Although not additionally tested, some of the spores seemed to show non-log-linear rather than log-linear inactivation behavior, which explains the similar spore counts after the treatments at 110, 120, and 125 °C (F85, F61, F93, and F98). In some cases, it was observed that the spore counts increased slightly after higher heat treatments. Other spores seemed to follow log-linear inactivation (e.g. F59, F74, F78). Stoeckel et al. (2014a) showed that the inactivation of the spores from *B. amyloliquefaciens* F85 was non-log-linear whereas the inactivation of the spores from *Bacillus smithii* F64 was log-linear, which corroborates the results presented in this study. In the study of Stoeckel et al. (2014a), tailing of the spores of *B. amyloliquefaciens* F85 at 120 and 125 °C was already observed after holding times <250 s. At 110 °C, the inactivation was markedly slower. At this temperature, the tailing phase was not reached yet or was about to start after a heat treatment of 30 min. The spores apparently possess a highly heat-resistant fraction that is not inactivated even after holding times of 30 min at 120 and 125 °C as shown in this study. Varying spore counts during the tailing phase of

B. amyloliquefaciens F85 were observed by Stoeckel et al. (2014a), which might explain the increased spore counts after treatments at higher temperatures as observed in this study for a few isolates. This study highlights that spores showing non-log-linear inactivation are able to persist at low spore counts over a wide range of temperatures and holding times (see e.g. *B. amyloliquefaciens* F85), which might in consequence lead to non-sterile final products.

In milk, 69, 38, and 13% of the examined spores survived the heat treatment at 110, 120, and 125 °C, respectively, while in phosphate buffer, 94, 69, and 44% of the spores survived this heat treatment (Table 2). The logarithmic reduction in milk was significantly higher than in phosphate buffer at 110 °C. At 120 and 125 °C, the logarithmic reduction was also higher than in phosphate buffer, although not significantly. In this study, 13% of the spores tested regarding their heat resistance in milk could be assigned to the group of HTRS. In phosphate buffer, Lücking et al. (2013) found that eight out of 35 examined strains (23%) belonged to the group of HTRS. When only the spores used in this study are taken into account, even higher numbers of HTRS in phosphate buffer were found (seven out of 16, thus 44%) (Table 2). All of the tested mesophilic isolates were more heat resistant in buffer than in milk. In contrast, the thermophilic spores showed a more diversified picture with spores showing higher heat resistance in buffer (F40, F42, F52, F64), in milk (F74), the same resistance in the two media (F12, F78) or a temperature-dependent behavior (F61, F93). *B. amyloliquefaciens* F85, identified as HTRS in milk, was also highly heat-resistant when heated in phosphate buffer. Interestingly, *G. stearothermophilus* F74, which was identified as HTRS in milk, was the only strain in this study that consistently showed a lower heat resistance in phosphate buffer than in milk at the three temperatures examined.

Dong et al. (2015) examined the thermal inactivation of *B. amyloliquefaciens* spores in phosphate buffered saline, low-fat and whole milk, and showed that the spores exhibited the same heat resistance apart from at 110 °C. Spores of *Bacillus cereus* IP5832 showed the same heat resistance in phosphate buffer and infant formula at 95 °C (Stoeckel et al. 2013) and it was possible to use the data obtained for the thermal inactivation of *Bacillus subtilis* spores for the modeling of the inactivation in milk (Jagannath et al. 2005). The different findings compared to the data from this study might be explained by the different approaches to determine the heat resistance of the spores (determination of entire kinetics versus “one point” determination after 30 min).

During heating, a pH decrease occurred in milk, while the pH of phosphate buffer remained stable (Reineke et al. 2011; Stoeckel et al. 2014b). The pH of UHT milk and raw milk showed a decrease of about 0.7 and 0.9 pH units, respectively, when heated from 25 to 130 °C (Reineke et al. 2011). When spores of *G. stearothermophilus* and *B. subtilis* were heated in different buffer systems, the inactivation rate was higher in the system that exhibited a pH decrease during heating (ACES buffer; decrease of approximately 1.3 pH units from 20 to 130 °C) than in the system without pH decrease (phosphate buffer) (Mathys et al. 2008; Reineke et al. 2011). Thus, the different pH developments during heating might explain the observed higher heat resistance of the majority of the tested spores in phosphate buffer than in milk seen in this study as it is well-known that spores are less heat resistant at lower pH (Esteban et al. 2013; Kessler 2002). As the inactivation of spores in milk and phosphate buffer is different, it is preferable to employ milk as the heating medium when designing thermal processes for new milk-based formulations. If data that were acquired in phosphate buffer instead of

in milk are used for the design of thermal processes, there is a risk of overprocessing and loss of valuable components of the milk-based products due to an unnecessary high heat treatment.

4 Conclusion

This study confirmed that dehydrated ingredients are an important source of thermoresistant spores in the dairy industry. The usage of exotic, new ingredients can introduce emerging HTRS, which are a threat to the process safety. These spores exhibited a higher heat resistance in phosphate buffer than in milk. For the design of sterilization processes in dairy companies, it is therefore preferable to use milk instead of laboratory buffer systems as the heating medium when examining spore inactivation.

Acknowledgements This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). Project AiF 16012N. The authors thank Jeanette Hauger and Ines Triebel for technical assistance and Meike Samtlebe for revising the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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