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# SYSPOR – Kontrol af syreresistente sporedannere i syrnede ikke-kølede mejeriprodukter

## SYSPOR – Control of acid resistant sporeformers in acidic dairy products at ambient storage

Mejeribrugets
ForskningsFond

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# Final report

## for collaborative projects funded via the Danish Dairy Research Foundation (DDRF)

### 1. Title of the project

**Danish: Kontrol af syreresistente sporedannere i syrnede ikke-kølede mejeriprodukter (SYSPOR)**

**English: Control of acid resistant sporeformers in acidic dairy products at ambient storage (SYSPOR)**

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### 4. Sources of funding

Danish Dairy Levy Fund

Arla Foods amba

### 5. Project period

**Project period with DDRF funding:** 01/2021 – 02/2024

**Revised, if necessary:** 01/2021 – 07/2024

### 6. Project summary

**Danish:**

SYSPOR fokuserer på syrnede (pH ≤ 4,6) mejeriprodukter, fx yoghurt, drikkeyoghurt, smoothies, som ikke opbevares på køl og eksporteres til områder med tempereret eller tropisk klima. I dag er der ikke retningslinjer for varmebehandling af ikke-kølede syrnede produkter. Modsat for ikke-kølede mildt sure produkter (pH>4,6), hvor der er klare mål for varmebehandling mht. patogener og fordærvende mikroorganismer. Derfor anvendes i dag forskellige varmebehandlinger, som ikke nødvendigvis giver god kontrol med fordærvende mikroorganismer, eller der overbehandles, hvilket går ud over kvaliteten.

Formålet er at opnå viden om den mest optimale varmebehandling og formulering af ikke-kølede syrnede mejeriprodukter af høj kvalitet for kontrol af syre-resistente sporedannere. Relevante syre-resistente sporedannere

identificeres, deres D-værdier bestemmes, varmebehandlingsmål foreslås, hurdle-effekter (organiske syrer & varme-behandling) på vækst af sporedannerne evalueres, og endelig valideres effektiviteten heraf i pilot-skala.

Som resultat af projektet blev der skabt en kollektion af 56 sporedannende mikroorganismer, som viste sig relevante for at kunne vokse i syrnede mejeriprodukter, og heraf blev de 16 mest relevante bakteriestammer udvalgt til sporeproduktion. Der er gennem varmebehandingsprotokoller etableret D- og Z-værdier af de mest potente syreresistente stammer af fordærvende sporedannere. Samtidig er grænseværdier for koncentration af mælkesyre og kalium-sorbat for germinering og vækst af sporer fastlagt for opbevaring ved to temperaturer over en 12-ugers lagringsperiode.

Der er skabt viden om, hvordan hurdler gennem produktformulering kombineret med varmestress kan give effektiv kontrol af de relevante sporedannere. Endelig blev protokollerne valideret af varmebehandlinger kombineret med kaliumsorbat i en mejerifremstillet drikkeyoghurt for at kunne kontrollere udvækst af bakterielle endosporer og svampes ascosporer over lagring udenfor kølekæden igennem 12 uger ved hhv. 25°C og 40°C.

**English:**

SYSPOR focuses on acid dairy foods, i.e. yoghurts, yoghurt drinks, smoothies, stored at ambient temperatures for exports in countries with temperate or tropical climates. Currently specific guidelines lack for heat treatment of ambient acid foods (pH ≤ 4.6). This is unlike for ambient low acid foods (pH>4.6), where clear heat treatment targets exist for both pathogens and spoilage microorganisms. The lack of specific guidelines means that different heat treatments are used, which may not offer a high level of protection against spoilage microorganisms or are overly strict and result in inferior products from a quality point of view.

The objective is to gain knowledge on how to optimally heat treat and formulate ambient acid dairy products to control acid resistant sporeformers and ensure safe and high-quality products.

This was done by identifying key species of acid resistant sporeformers, determine the D-values, propose a target heat treatment, evaluate hurdle effects (organic acids and heat treatment) on growth of sporeformers, and finally validate the effectiveness hereof in a dairy product.

A collection of 56 sporeforming microorganism strains relevant for acid dairy products was created, from which the 16 most relevant bacterial strains were selected for spore production. Heat treatment protocols were established and the D-values and Z-values of the most potent acid-resistant strains of spoiler sporeformers were obtained, and outgrowth limits were established in relation to lactic acid and potassium sorbate concentrations at two temperatures during 12 weeks. Knowledge on efficient hurdle combinations based on product formulation and heat stress was obtained for relevant sporeformers. Finally, the hurdles were validated with a lab scale heat treatment of a drinking yoghurt formulated with potassium sorbate for the control of endospores and ascospores during storage for 12 weeks at ambient (25°C) and at elevated temperature (40°C).

## 7. Project aim

**Danish**

Formålet med projektet er at opnå viden om mest optimale varmebehandling og formulering af ikke-kølede syrnede mejeriprodukter af høj kvalitet for kontrol af syreresistente sporedannere. Relevante syreresistente sporedannere identificeres, deres D-værdier bestemmes, varmebehandlingsmål foreslås, hurdle-kombinationers (organiske syrer & varmebehandling) effekt på vækst af sporedannerne evalueres, og endelig valideres effektiviteten heraf.

Hypotesen er, at varmebehandling og formulering af syrnede mejeriprodukter er effektive hurdles, der kan sikre mod fordærvende syreresistente mikroorganismer ved lang tids opbevaring under ikke-kølede forhold.

**English:**

The project aims to obtain knowledge on the most optimal ways of heat treatment and formulation of ambient acidic dairy products of high quality to control acid resistant sporeformers. Relevant acid resistant sporeformers are identified, their D-values determined, heat inactivation kinetics collected, hurdle combinations (organic acids and heat treatment) effect on growth of sporeformers evaluated, and finally the efficiency of hurdle combinations is validated.

The hypothesis is that heat treatment and formulation of acidic dairy products are efficient hurdles, which can control spoilage by acid resistant microorganisms during long storage time at ambient conditions.

## 8. Background for the project

The background and objectives for the project stemmed from a lack of specific guidelines for the dairy industry for the heat treatment of ambient acid foods (products with pH ≤ 4.6) (e.g. thermised yoghurt, acidified dairy drinks and smoothies). This is unlike the situation for ambient low acid foods (pH > 4.6) where there are clear targets for both pathogens (e.g. *Clostridium botulinum*, $F_o$ = 3 minutes) and spoilage microorganisms (e.g. *Clostridium sporogenes*, $F_o$ = 5-6 minutes) [1-3].

The lack of specific guidelines for ambient acid dairy products has resulted in different heat treatments being applied in this product group that may not offer the same level of protection against spoilage microorganisms or that may be overly strict and result in inferior products from a quality point of view. As examples, to inactivate sporeformers that may grow in acidic ambient stable products, a heat treatment of 5-10 min at 95◦C is recommended [4] but specifically for yoghurt drinks, a heat treatment of 110◦C for 60 seconds has been recommended for the control of *B. licheniformis* [5]. This lack of specific guidelines has also resulted in considerable variation in the heating regimes of acid ingredients provided to the dairy industry (e.g. yoghurt preps) where for the same type of product the intensity of the heat treatment may range from 5 minutes at 85◦C to 10 minutes at 90◦C. Many acid resistant sporeformers will not be inactivated with the above heat treatments, having thus the potential to cause spoilage while some of the survivors like *B. licheniformis* may produce components that can potentially contribute to food poisoning although their role in foodborne illness is currently under debate [6]. Processors should also be aware that in the case of under-processing of acid foods, growth of some species of surviving mesophilic bacilli and moulds during ambient storage may raise the pH to a point at which *C. botulinum* could start to grow and produce toxin (phenomenon referred to as **metabiosis**) [1, 7-9]. Lastly, it is currently unknown if such a pH increase is also possible through growth of surviving thermophiles and this point is of particular concern for exports in regions with tropical climates like the Middle East or Southeast Asia. The risk of spoilage and metabiosis is considered to be negligible in chilled stored acid dairy products because acid resistant sporeformers like *Alicyclobacillus acidoterrestris* or *Bacillus licheniformis* cannot grow at refrigeration temperatures (≤8◦C) [10, 11]. It is unclear how big this risk is for ambient stored acid dairy products because unlike other types of acid foods marketed ambient (e.g. tomato sauce) there is no long history of safe production in combination with large consumption volumes.

The objective of the project is to gain knowledge on how to optimally heat treat and formulate acid, ambient stored dairy products to control acid resistant sporeformers and ensure safety and high-quality products.

## 9. Sub-activities in the entire project period

The project consisted of 5 work-packages (WP). Milestones are given in the Gantt chart on next page.
The main focus was on **acid dairy foods with pH≤4.6**, i.e. thermised yoghurts and yoghurt drinks, fermented or acidified dairy drinks that are to be stored at ambient temperatures of relevance for exports in countries with temperate or tropical climates.

**WP1. Prepare a collection of acid resistant sporeformers of relevance for acid dairy products to be stored ambient**
Sub-activities:

a) Conduct a literature search for species of sporeforming microorganisms present in milk and/or dairy ingredients (e.g. fruit preparations for yoghurts) that may survive currently used heat treatments for acid dairy products (pH<4.6) and spoil them during storage at normal (<35◦C) and abusive ambient temperatures (≥35◦C)
b) Identified species of relevance will be gathered from public culture collections (e.g. DSMZ) or when available from Arla's culture collection.

**WP2. Evaluate the effect of common heat treatments applied to acid dairy products on the inactivation of spores of acid resistant sporeformers**

Sub-activities:

a) Perform a literature search on suitable spore production methodology for each species of the culture collection that has been created in WP1
b) Produce spores for strains of the WP1 culture collection
c) Use a capillary tube method to estimate the D- and z- values for spores of each WP1 culture collection strain at pH values relevant to acid dairy products (pH 3.7-4.6)
d) Create a mapping of heating conditions that inactivate aciduric sporeformers (combinations of temperature and time for relevant pH values for acid foods) and suggest a target heat treatment to achieve a 5-6D reductions of inoculated spores.

**WP3. Evaluate the growth potential of acid resistant sporeformers, in laboratory media in the presence of different hurdles (±organic acids, ±heat stress)**

Sub-activities:

a) Evaluate **growth potential** of each strain of the WP1 collection in laboratory media acidified with either HCl or lactic acid at pH 4.6 (the highest pH that acid foods/acidified foods can possess)
b) For the faster growing strains of each species estimate **minimum inhibitory concentrations** of organic acids relevant for dairy (e.g. lactic acid) against sporeformers at pH 4.6
c) Investigate the inhibition of faster growing strains identified in the first step of this WP in the presence of **multiple hurdles** i.e. sub-lethal heat stress in combination with sub-inhibitory concentrations of organic acids identified in the second step of this WP.
d) Map hurdle combinations that can inhibit spore growth for different product pH gradients.

**WP4. Validation and protocol for heat inactivation**

For a selected number of strains, the target heat treatment identified in WP2 is aimed to be validated in dairy matrices of relevance as typically there is a difference between heat inactivation results from batch processes that do not take into account shear force or other physical stress and continuous processes used by the industry (see also item 15. New projects).

Sub-activities:

a) Selection of suitable dairy products (e.g. yoghurt and yoghurt drinks, acidified/fermented dairy drinks)
b) Inoculation with high levels of the most heat-resistant strains identified in WP2
c) Application of the heat treatment guideline defined in WP2 using a Visco Analyzer equipment
d) Sampling and analysis of survivors to determine efficiency of inactivation treatment.

**WP5. Validation of multiple hurdle combinations in preventing growth of acid resistant sporeformers in dairy matrices, under different storage conditions of relevance for long life dairy products**

Sub-activities:

a) Formulation of different products with an identified growth boundary (e.g. organic acid) or a combination of hurdles that prevents growth (mild heat treatment + organic acid)
b) Inoculation of above-mentioned products with a suitable cocktail of strains selected from WP2+WP3

c) Incubation at temperatures of relevance for ambient storage for a period of 3-6 months
d) Sampling and analysis of growth to determine efficiency of product formulations on growth boundaries.

The Gantt chart of SYSPOR:

| | 2021 | | | | 2022 | | | | 2023 | | | | 2024 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WP1 Collection of acid resistant sporeformers | | | | M 1 | | | | | | | | | | | |
| WP2 Heat inactivation & D-values spores | | | | | M2 | | | | M3 | | | | | | |
| WP3 Hurdles effect on growth of sporeformers | | | | | | | | | | M4 | | | | | |
| WP4 Validation and spore heat inactivation protocol | | | | | | | | | | | | M5 | | | |
| WP5 Validation of hurdle combinations on growth | | | | | | | | | | | | | | | M6 |

Milestones

M1: sporeformer collection is completed
M2: spores are produced from the collection
M3: heat inactivation experiments completed, D-values established
M4: hurdle combination experiments completed, hurdles for WP5 selected
M5: Protocol for spore heat inactivation delivered
M6: The combined best hurdles are found

## 10. Deviations

SYSPOR was extended with 5 months in the final project phase, but without a change in the funded budget from the Danish Dairy Levy Fund. This was due to staff resources, as one of the key persons in SYSPOR got involved in another project at the department of food science, which required activities at the same time. It was only the last work package 5, which was extended. The co-funding from Arla Foods amba primarily covering the extended project period (5 months extension in 2024).

## 11. Project results

Identification of microbial species of relevance for the studies

A literature search was performed for species of sporeforming microorganisms present in milk and/or dairy ingredients (e.g. fruit preparations for yoghurts) that may survive currently used heat treatments for acid dairy products (pH<4.6) and are able to spoil the products during storage at normal (<35◦C) and abusive ambient temperatures (≥35◦C).

Identified species of relevance were gathered from public culture collections and when available from Arla's culture collection to create the collection of acid resistant sporeformers. The collection contained 56 strains from culture collections (primarily DSMZ) and 10 industrial isolates, including both bacteria and mould species. The spore collections remained open to be further expanded during the project period. A list of the different species of spores was provided, and in order to manage the amount of work 16 representative strains were finally selected for spore production (Table 1).

**Table 1**. Selected bacterial species for spore production and conditions for sporulation (Pahalagedara, Gkogka, Ravn & Hammershøj, 2023)

| Bacterial species | Strain | Sporulation medium | Incubation temperature and time |
|---|---|---|---|
| ***Alicyclobacillus acidoterrestris*** | DSM2498 | Bacillus acidoterrestris (BAT) agar (DOHLER, Germany) | 45 ºC for 7 days |
| ***Bacillus subtilis*** | DSM10 | Nutrient agar (VWR, Belgium) supplemented with 1 mM $CaCl_2.2H_2O$ (Merck, Germany) and 0.1 mM MnSO4. $H_2O$ (Sigma-Aldrich, Japan) | 35 ºC for 9 days |
| ***Bacillus licheniformis*** | DSM13 | Nutrient agar (VWR, Belgium) supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM $MnSO_4$. $H_2O$ | 35 ºC for 7 days |
| ***Bacillus megatarium*** | DSM32 | Milk agar (Oxoid, UK) supplemented with 1 mM $CaCl_2.2H_2O$ (Merck, Germany) and 0.1 mM $MnSO_4$. $H_2O$ | 35 ºC for 14 days |
| ***Bacillus coagulans*** | DSM2308 | Milk agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM $MnSO_4$. $H_2O$ | 35 ºC for 7 days |
| ***Bacillus smithii*** | DSM4216 | Milk agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 55 ºC for 3 days |
| ***Bacillus pumilus*** | Arla0109.003 | Nutrient agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 35 ºC for 7 days |
| ***Bacillus circulans*** | Arla0126.0011 | Nutrient agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 35 ºC for 7 days |
| ***Paenibacillus odorifer*** | DSM15391 | Nutrient agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 35 ºC for 7 days |
| ***Paenibacillus polymyxa*** | DSM36 | Nutrient agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 35 ºC for 7 days |
| ***Paenibacillus xylanilyticus*** | DSM17253 | Milk agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM $MnSO_4$. $H_2O$ | 35 ºC for 14 days |
| ***Paenibacillus peoriae*** | DSM8320 | Nutrient agar supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 35 ºC for 30 days |
| ***Clostridium pasterianum*** | DSM526 | Potato dextrose agar (Merck, Germany) supplemented supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM MnSO4. $H_2O$ | 35 ºC for 14 days |
| ***Clostridium beijerinckii*** | DSM791 | Tryptone glucose extract agar (Merck, Germany) supplemented with 1 mM $CaCl_2.2H_2O$ and 0.1 mM $MnSO_4$. $H_2O$ | 35 ºC for 30 days |
| ***Clostridium tyrobutyricum*** | DSM664 | Reinforced clostridial agar (Oxoid, UK) supplemented with 0.7 mM $CaCl_2.2H_2O$ and 0.25 mM $MnSO_4$. $H_2O$ | 35 ºC for 30 days |
| ***Clostridium butyricum*** | Arla0110.0032 | Reinforced clostridial agar supplemented with 0.7 mM $CaCl_2.2H_2O$ and 0.25 mM $MnSO_4$. $H_2O$ | 35 ºC for 30 days |

## Production of spores of selected species and evaluation of their growth under acid dairy conditions (pH 4.6) and determine D- and Z-values

The laboratory work was based on a literature search on suitable spore production methodology for each species (Table 1). Experiments were performed to test the suitable sporulation conditions to achieve ≈ $10^7 – 10^8$ CFU/mL spore densities from each bacterial strain of the acid-resistant sporeformer list created in WP1. The growth potential of the spores of 16 strains was evaluated at pH 6.8 (control) and under acidic conditions (pH 4.6), in combination with two ambient temperatures, 25°C and 40°C for a 12-week period based on optical density ($OD_{595nm}$) readings and the results are shown in Figure 1. Here, all the spores germinated at pH 6.8 except *A. acidoterrestris* spores at both temperatures and *B. smithii* at 25°C. Spores of seven species germinated and grew at pH 4.6; *A. acidoterrestris, B. megatarium* (only at 25ºC), *B. subtilis, B. coagulans* (only at 40 ºC), *C. beijerrinckii, C. butyricum,* and *C. tyrobutyricum. C. tyrobutyricum* spores showed a 6 week and 2 week lag-time at 25ºC and 40ºC, subsequently.

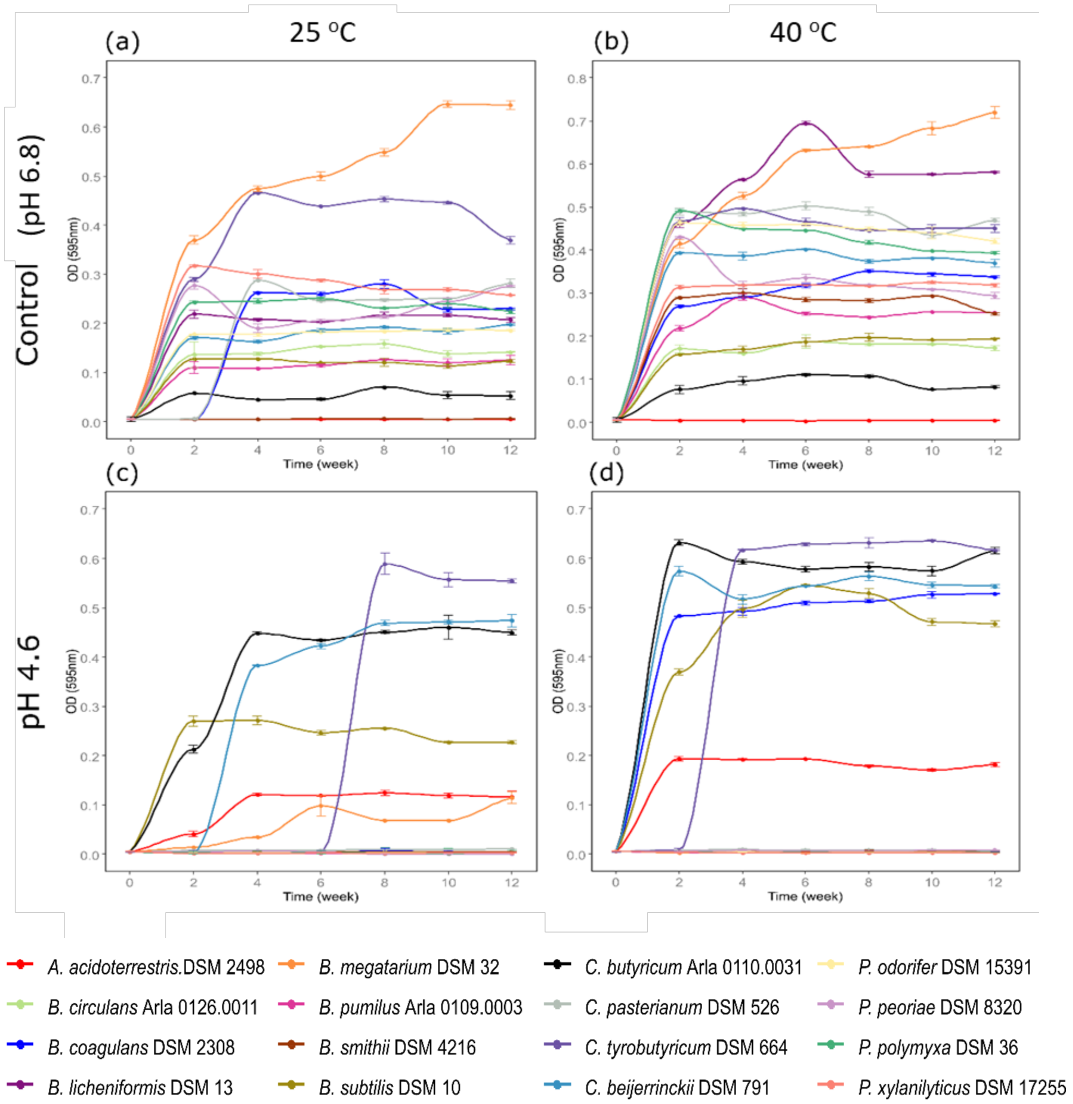


**Figure 1.** Growth of microbial spores at two ambient temperatures and at two pH-levels during 12 weeks of storage (Pahalagedara, Gkogka, Ravn & Hammershøj, 2023).

Furthermore, the presence of 0.4% and 0.8% lactic acid (LA) in the growth medium inhibited the spore growth of all tested bacteria, except for one species that grew at 40°C. Only *A. acidoterrestris* spores were able to germinate and outgrowth at 40°C in the presence of 0.4% and 0.8% LA.

Hence, based on these results four strains of *A. acidoterrestris* were selected for heat inactivation experiments. Literature was reviewed to find out previously reported heat-resistant data for the microorganisms of interest and to select a suitable methodology to estimate thermal inactivation parameters (D- and z-values) of the microbial spores. Heat inactivation data for *Alicyclobacillus acidoterrestris* spores in a yoghurt-based medium (pH 4.6 and 0.8% lactic acid) at 85°C, 90°C, 95°C, 100°C, and 108°C were obtained for spores of four strains by the capillary tube method using an oil bath. The results hereof were used to determine the D- and Z-values for each strain, as shown in Table 2.

**Table 2.** Heat inactivation parameters of four strains of *A.acidoterrestris* spores in a yoghurt-based medium.

| Strain | Temperature (°C) | D value (min) | $R^2$ | z value (°C) |
|---|---|---|---|---|
| ***A. acidoterestris* DSM2498** | 85 | 48.08 ± 0.33 | 0.99 | 8.20 ± 0.07 |
| | 90 | 12.86 ± 0.98 | 0.99 | |
| | 95 | 2.46 ± 0.14 | 0.99 | |
| | 100 | 0.39 ± 0.02 | 0.98 | |
| | 108 | 0.10 ± 0.00* | 0.99 | |
| ***A. acidoterestris* DSM3922** | 85 | 71.41 ± 1.49 | 0.98 | 7.50 ± 0.00 |
| | 90 | 12.44 ± 0.13 | 0.99 | |
| | 95 | 2.31 ± 0.07 | 0.98 | |
| | 100 | 0.28 ± 0.02 | 0.98 | |
| | 108 | 0.08 ± 0.00* | 0.99 | |
| ***A. acidoterestris* DSM3923** | 85 | 65.36 ± 0.60 | 0.98 | 8.16 ± 0.05 |
| | 90 | 9.54 ± 0.44 | 0.98 | |
| | 95 | 2.08 ± 0.25 | 0.98 | |
| | 100 | 0.34 ± 0.00 | 0.97 | |
| | 108 | 0.11 ± 0.00 | 0.97 | |
| ***A. acidoterestris* DSM3924** | 85 | 101.55 ± 1.55 | 0.99 | 7.91 ± 0.08 |
| | 90 | 14.65 ± 0.53 | 0.97 | |
| | 95 | 2.36 ± 0.24 | 0.97 | |
| | 100 | 0.36 ± 0.01* | 0.98 | |
| | 108 | 0.15 ± 0.01 | 0.95 | |

* Data were fit into no tail Geeraerd model, and the D-value was calculated as average of 3D inactivation (Pahalagedara, Gkogka, Ravn & Hammershøj, 2023).

To our knowledge, no existing studies have determined heat treatment parameters (D- and Z-values) for *A. acidoterrestris* in an acidic milk- or dairy based media. We are therefore the first to report heat inactivation parameters D- and z-values for spores of four strains of *A. acidoterrestris* in a yoghurt-based medium (Pahalagedara, Gkogka, Ravn & Hammershøj, 2023). In conclusion, the four strains showed a wide range in D-values which is taken into consideration for further studies, where a cocktail of the four strains were used to ensure the variability was covered. The variability in D- and hence Z-values is important to pay attention to, when heat treating acid dairy products to ensure sufficient inactivation of the spores.

Evaluation of growth of acid-resistant spore-formers in the presence of different hurdles (±organic acids, ±heat stress)

Acid resistant molds are also able to grow in acid dairy products, hence mold ascospores are included in further growth and hurdle studies together with the bacterial endospores of *A. acidoterrestris.* It was decided to focus on *Byssochlamys* ascospores, as they pave a spoilage risk in acid dairy stored at ambient temperature based on literature and experience from Arla Foods. Ascospores were generated of two *Byssochlamys* species; *B.fulva* and *B.nivea.*

First the Minimum Inhibitory Concentrations (MIC) and Minimum Bacteriocidal Concentrations (MBC) of four organic acids (lactic acid, citric acid, potassium sorbate, potassium benzoate) were determined for four strains of *Alicyclobacillus acidoterrestris* and two species of *Byssochlamys*, as given in Table 3.

**Table 3.** MIC and MBC of four organic acids against four bacterial stains, and MIC against two mould species (Pahalagedara, Gkogka, Ravn & Hammershøj, 2024, *In preparation*).

| *A. acidoterrestris* strain | Lactic acid (mg/mL) | | Citric acid (mg/mL) | | Potassium sorbate (mg/mL) | | Potassium benzoate (mg/mL) | |
|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| DSM 2498 | 8 | 12 | 44 | 68 | 0.8 | 1.6 | 0.4 | 1.6 |
| DSM 3922 | 8 | 10 | 26 | 32 | 0.8 | 1.6 | 0.4 | 0.8 |
| DSM 3923 | 8 | 48 | 22 | 32 | 1.6 | 3.2 | 0.8 | 0.8 |
| DSM 3924 | 8 | 16 | 24 | 34 | 0.8 | 1.6 | 0.4 | 0.8 |

| *Byssochlamys* spp. | MIC (mg/mL) | | | |
|---|---|---|---|---|
| | Lactic acid | Citric acid | Potassium sorbate | Potassium benzoate |
| *B. fulva* DSM 1808 | 140 | >140 | 0.8 | 1.6 |
| *B. nivea* DSM 1824 | 120 | >140 | 0.8 | 3.2 |

Lactic acid (LA) exhibited a consistent antimicrobial efficacy across all tested strains of *A. acidoterrestris.* MICs of PS fell within the maximum permitted level (1 mg/mL) of sorbate set by the EU commission for unflavored fermented milk products, except for strain DSM 3923, which MIC was 1.6 mg/mL. For the moulds, LA and citric acid showed poor activity against *Byssochlamys* ascospores, while PS was the most effective against *Byssochlamys* species.

## Evaluation of combined effects of heat treatment and organic acids on growth of bacterial and mold spores

The individual and combined effects of organic acids and heat on the growth of *Alicyclobacillus acidoterrestris* spores were studied on basis of the results of WP1-WP3 in a cocktail of the four strains. The following formulations of organic acids in tryptic soy broth (TSB) medium was studied; no acids added, 0.5 mg/mL PS; 0.8 mg/ml PS, 8 mg/mL LA; 0.5 mg/mL PS + 8 mg/mL LA; and 0.8 mg/mL PS + 8 mg/mL LA. This was combined with heat treatments of; no heat (Control); 75°C for 20 min; 85°C for 10 min; and 95°C for 30 seconds, based on relevant heat regimes for dairy products. The results of growth curves at two ambient temperatures for 12 weeks are shown in Figure 2.

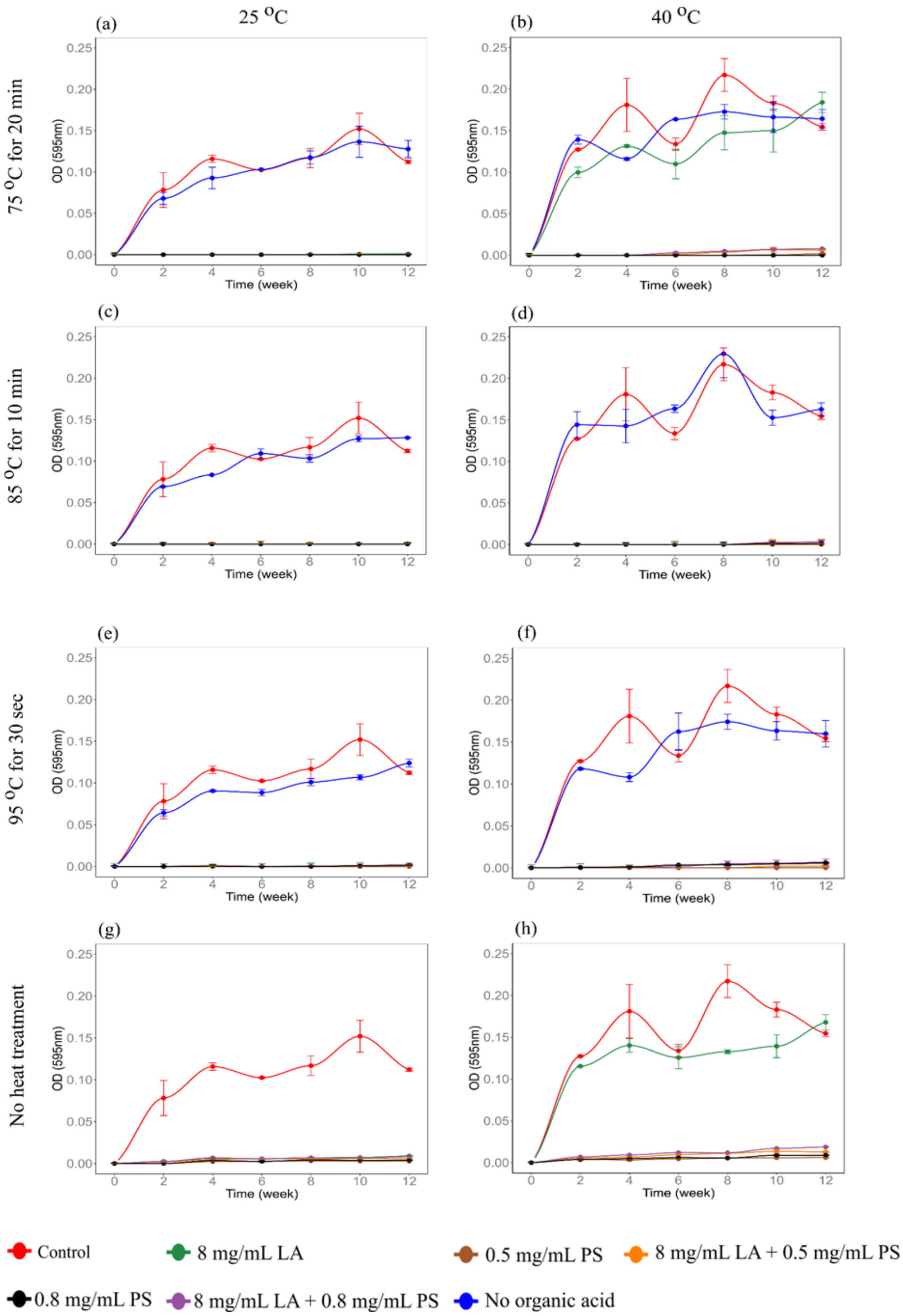


**Figure 2.** Effect of lactic acid (LA), potassium sorbate (PS) and heat treatments on the growth of *A. acidoterrestris* spores in a tryptic soy broth (TSB) medium at two storage temperatures during 12 weeks (Pahalagedara, Gkogka, Ravn & Hammershøj, 2024, *In preparation*).

The results show that heat treatments alone are not able to control the growth. Furthermore, 8 mg/mL LA was effective when combined with heat treatment at 85°C for 10 min, while the presence of 0.5 or 0.8 mg/mL PS was effective alone.

The effect of heat and PS combinations were also studied on the growth of *B. fulva* and *B. nivea* ascospores. Results are not shown here due to limit of space, but in brief it was found that for *B. fulva* (DSM 1808) the combinations of 75°C for 20 min + 0.8 mg/mL PS and 85°C for 10 min + 0.5 mg/mL PS were effective, hence, a synergistic effect of lower heat load and higher concentration of PS, and vice versa. Furthermore, 95$^{o}$C for 30 Sec was also effective alone without PS to control growth. The *B. nivea* (DSM 1824) ascospores were more heat sensitive than *B. fulva* and 85$^{o}$C for 10 min or 95$^{o}$C for 30 sec alone (without PS) inhibited the growth, and the presence of PS alone (0.5 mg/mL or 0.8 mg/mL) was inhibitory too for unheated samples. There were overall only little differences in the growth pattern due to storage temperature (25°C and 40 °C) and no difference in growth under aerobic or anaerobic conditions.

Validation of the protocol for heat inactivation

The results obtained in WP4 were validated by the use of a pilot-scale drinking yoghurt produced at Arla Foods amba with the identified growth boundaries (potassium sorbate) and suggested heat treatments. Two treatments were validated; '75°C for 20 min and 0.8 mg/mL PS', and '85°C for 10 min and 0.5 mg/mL PS' for the growth of *A. acidoterrestris* spores and *B. fulva* ascospores during the storage at normal (25°C) and higher ambient temperatures (40°C) for a period of 12 weeks. For this final validation a cocktail of the four *A. acidoterrestris* strains were used with an initial inoculation level of $10^4$ CFU/g.

For the mold, growth was controlled by the treatment of '75°C for 20 min and 0.8 mg/mL PS' for 4 weeks at 25°C storage and for 12 weeks at 40°C before outgrowth of *B. fulva* ascospores was detected.

The results of bacterial endospore growth showed that only at 40°C *A. acidoterrestris* was able to grow, and only for treatments without PS being present. Here, the CFU increased 3 log-units within week 2-4. Hence, in conclusion addition of potassium sorbate is needed to control growth of acid-resistant *A. acidoterrestris* spores when acid dairy are stored at higher ambient temperatures.

**In conclusion relative to the goals of SYSPOR, we have**

1. created a collection of 56 sporeforming microorganism strains relevant for acid dairy products, from which the 16 most relevant bacterial stains were selected for spore production,
2. established heat treatment protocols and obtained D-values and Z-values of the most potent acid-resistant strains of spoilers of sporeformes, and established outgrowth limits in relation to lactic acid and potassium sorbate concentrations at two temperatures for 12 weeks,
3. obtained knowledge on how to formulate product and heat stress in combination for efficient hurdles against the relevant sporeformers,
4. validated the protocols in pilot scale of heat treatment combined with potassium sorbate results at the dairy for growth control of endospores and ascospores during storage for 12 weeks at ambient temperature (25°C) and at elevated temperature (40°C).

## 12. The relevance of the results, including relevance for the dairy industry

The project results are highly relevant for dairies producing and marketing acid dairy products, e.g. yoghurt, yoghurt drinks and smoothies. The findings on heat treatment and formulation hurdles may require changes in technology or processes at the dairies. Furthermore, the dairies may want to perform challenge test of the results before implementing them. In that case, a calculation of benefit in extended shelf life of the product and market value versus the costs and investments in processing is likely needed.

Danish dairies benefit from the project outcome by gaining knowledge on how to improve production of acid dairy products with extended ambient shelf life, thereby strengthening their market position in regions of temperate and tropical climate. The activities in SYSPOR are targeted at controlling specific spoilage by acid resistant sporeformers that pose a problem for products outside the cold chain. The dairies have for years needed research within this field in order to deliver safe long-life dairy products for the mentioned markets.

We have achieved effects on improved and targeted heat treatment of acid dairy products that can be relevant for implementation at the Danish dairies. The obtained D-values and z-values give quantitative measures at low pH conditions relevant for acid dairy products. Furthermore, we have quantified the levels of lactic acid required in products to control spore growth during 12-week storage at different temperatures.

Based on SYSPOR, possible new research could be relevant for both dairy and other acidic foods, e.g. fruit preparation industries. In SYSPOR, the academia is partnering and collaborating with Arla Foods amba. This facilitated decision making, results discussion and validation focused on possibility to implement the project results in the dairy industry. Furthermore, fruit preparation industries supplying ingredients to e.g. yoghurts, yoghurt drinks and smoothies will also have an interest in implementing the results to deliver high quality ingredients to the dairy industry.

## 13. Communication and knowledge sharing about the project

**Papers in international journals:**

Pahalagedara, A.S.N.W.., Gkogka, E., Ravn, L.W. & Hammershøj, M. 2023. The growth potential and thermal resistance of bacterial spores under conditions relevant for ambient acid dairy products. Food Control, 152, 109841. https://doi.org/10.1016/j.foodcont.2023.109841

Pahalagedara, A.S.N.W., Gkogka, E. & Hammershøj, M. A review on spore-forming bacteria and moulds implicated in the quality and safety of thermally processed acid foods: Focusing on their heat resistance. Food Control. Volume 166, December 2024, 110716. https://doi.org/10.1016/j.foodcont.2024.110716

Pahalagedara, A.S.N.W., Gkogka, E., Ravn, L.W. & Hammershøj, M. Control of *Alicyclobacillus acidoterrestri*s spore and *Byssochlamys* ascospore growth through organic acid and heat treatment. *In preparation, October 2024.*

**Easily read papers:**

Nawarathna, A., Hammershøj, M. & Gkogka, E. 2022. Syreresistente sporedannere i syrnede mejeriprodukter. Mælkeritidende, no. 8, p. 20

Hammershøj, M., Nawarathna, A., Ravn, L.W. & Gkogka, E. 2025. Hvordan syreresistente sporedannere i syrnede mejeriprodukter kan styres. Mælkeritidende, in press.

**Student theses:**

Ravn, L.W. 2022. Effects of incubation temperature on sporulation of three Alicyclobacillis acidoterrestris strain. Report of Research and Development Project in Biotechnology and Chemical Engineering, Aarhus University, 24. October 2022.

Ravn, L.W. Control of Alicyclobacillus acidoterrestris in acid dairy products at ambient storage. MSc Thesis in Chemical Engineering & Biotechnology, Aarhus University, June 2023, 60 pp.

**Presentations at scientific conferences, symposiums etc.:**

Nawarathna, A., Gkogka, E. & Hammershøj, M. Control of Alicyclobacillus acidoterrestris spore and Byssochlamys ascospore growth through organic acid and heat treatment. Abstract for poster, The Danish Microbiological Society: Annual Congress, Frederiksberg, Denmark. 13 November 2023.

Nawarathna, A., Gkogka, E, Ravn, L.W. & Hammershøj, M The growth potential and thermal resistance of bacterial spores under conditions relevant for ambient acid dairy-based products. Poster for International Association for Food Protection (IAFP) European symposium, Aberdeen, United Kingdom. 3.-5. May 2023.

Nawarathna, A., Gkogka, E, Ravn, L.W. & Hammershøj. Control of acid resistant sporeformers in acid dairy products at ambient storage. Oral presentation at Dairy Research Day, Herning, Denmark. 16. March 2023.

**Oral presentations at meetings:**

Hammershøj, M. SYSPOR. DDRF Coordination group 'Technolog & Safety. 21. September 2021.

Nawarathna, A., Hammershøj, M. & Gkogka, E. SYSPOR. Control of acid resistant sporeformers in acid dairy products at ambient storage. DDRF Coordination group 'Technolog & Safety. 11. October 2022.

Nawarathna, A., Hammershøj, M. & Gkogka, E. SYSPOR. Control of acid resistant sporeformers in acid dairy products at ambient storage. Arla Foods Innovation Center meeting 10. Januar 2023.

Nawarathna, A. Sporeformers in food. Poster at Food Festival, Aarhus, 2.-4. September 2022.

Nawarathna, A. Hammershøj, M. & Gkogka, E. SYSPOR. Control of acid resistant sporeformers in acid dairy products at ambient storage. DDRF Coordination group 'Technolog & Safety. 23. October 2023.

Hammershøj, M. Impact assessment of SYSPOR. Danish Dairy Levy Foundation board meeting, Aarhus, 2. May 2024.

## 14. Contribution to MSc and PhD education

One MSc educated, Lasse Wiis Ravn in Chemical Engineering & Biotechnology, Aarhus University, 2023

## 15. New contacts/projects

Based on the outcome of WP4 and WP5 validation results, a project application to obtain knowledge on and determine the difference between heat inactivation results (D-values) from batch processes that do not take into account shear forces or other physical stress and continuous processes used by the industry. Hence, the project application of 'Tailorheat' has been submitted to the DDRF call 2024.

In 'Tailorheat' the collaboration of Arla Foods and Aarhus University will be expanded by partnering with SPX Flow Technology.

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