Matrix Matters: Context-Driven Metabolic Shifts in *Bacillus cereus* and *Bacillus subtilis*

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Abstract

Spore-forming *Bacillus* species, including pathogenic *Bacillus cereus* and spoilage-associated *Bacillus subtilis*, are major contributors to foodborne illness and product degradation. Understanding their metabolic behaviour in diverse food matrices is essential for improving risk assessment, spoilage prediction, and fermentation control. This study integrates isothermal microcalorimetry and targeted metabolomics to characterize the metabolic activity of *B. cereus* and *B. subtilis* in five nutrient sources: Brain Heart Infusion (BHI) medium, oat drink, milk, pea hydrolysate, and a combined oat-pea matrix. Metabolic heat production was monitored for 24 hours at 30°C. In BHI, *B. cereus* exhibited a shorter lag phase (mean ± sd: 4.3 hours ± 0.8) than *B. subtilis* (7.9 hours ± 1.0) but produced less total heat. Across all food matrices, *B. subtilis* consistently generated more heat. The oat-pea matrix supported the highest growth rates, surpassing oat or pea alone, and showed sugar depletion and accumulation of organic acids, indicating enhanced carbohydrate metabolism. Free amino acid release was matrix- and species-specific: *B. subtilis* had increased levels in oat, while *B. cereus* did so in pea. While *B. cereus* was metabolically active in all matrices, cereulide levels were matrix-dependent: 47.3 ± 1.7 ng/mL in oat, 3.0 ± 0.1 ng/mL in oat–pea, and undetectable in pea. These findings reveal clade-specific and matrix-driven metabolic strategies. This is the first study to combine calorimetry and metabolomics to evaluate *Bacillus* activity in plant-based and dairy matrices. This approach enhances our understanding of microbial physiology in complex food systems and provides a foundation for developing targeted strategies to improve food safety, stability, and product design.

Keywords: *Bacillus cereus*, *Bacillus subtilis*, isothermal calorimetry, microbial metabolism, cereulide, food matrix, plant-based food, metabolomics

1. Introduction

Microbial contamination is a key concern in the food industry (Snyder et al., 2024). In 2023, the United Nations estimated that 19% of all food available at the consumer level was wasted, which has a significant impact on the economy, environment, and public health (UNEP, 2024). The Gram-positive, spore-forming, facultative aerobe *Bacillus* spp. are ubiquitous in nature and include species such as *B. cereus* and *B. subtilis*. *Bacillus* species are usually introduced into the food production system through raw ingredients (Ehling-Schulz et al., 2015). *B. cereus* and *B. subtilis* both produce highly resistant spores, complicating their control with conventional techniques like heat, acid, and cleaning practices (Earl et al., 2008; Rahnama et al., 2023).

Bacillus cereus is an important foodborne pathogen, capable of causing diarrhoea due to enterotoxins or inducing vomiting due to the pre-formed heat- and acid-stable emetic toxin, cereulide (Ehling-Schulz et al., 2004). The *B. cereus* group, also known as *B. cereus* sensu lato, contains many genetically closely related species, including *B. cereus*, *B. mycoides*, and *B. thuringiensis*. *B. cereus* taxonomy has undergone many changes over the years, especially after the introduction of genome sequencing, and numerous nomenclatural frameworks have been used to describe *B. cereus* taxonomy (Carroll et al., 2022).

Foodborne disease caused by *B. cereus* is believed to be grossly underreported. Nevertheless, in 2022, *B. cereus* toxins (both emetic and enterotoxins) caused 306 registered foodborne outbreaks, 3192 cases of illness, 66 hospitalizations, and 2 deaths in the EU and UK alone (EFSA, 2023). *B. cereus* is typically isolated from plant materials (Messelhäusser et al., 2014), and given the increasing development of plant-based ingredients for food production (Kyrylenko et al., 2023). There is a strong need to monitor the prevalence and toxicity of *B. cereus* in plant matrices.

B. subtilis is a key driver of many traditional fermentations based on legumes, seeds, and tubers (Li et al., 2023). A high occurrence of *B. cereus* has been observed in *B. subtilis*-fermented food products, and its presence poses a significant concern for food safety (Akanni et al., 2018; Owusu-Kwarteng et al., 2022). *B. subtilis* is also associated as a spoilage agent in foods, such as slime formation in rice products and ropiness in bread (Dong et al., 2023; Valerio et al., 2008). While interactions between *B. subtilis* and *B. cereus* may influence their behaviour in co-cultures, a detailed understanding of their individual metabolic characteristics and growth dynamics, particularly in food-relevant matrices, remains limited. Elucidating these species-specific responses is essential for predicting their contributions to fermentation, spoilage, and food safety risks, especially in plant-based foods.

An attractive tool for fast detection of microbial activity is isothermal microcalorimetry (IMC). IMC is a non-invasive method based on real-time, accurate measurements of heat flow generated by the

metabolic activity of biological systems, especially in opaque or complex matrices where optical methods are limited. IMC is used as a quantitative proxy for active biomass because heat flow scales with metabolically active cell numbers, with strong correlations reported between calorimetric output and optical or culture-based biomass measurements (Braissant, Bachmann, et al., 2015; Braissant et al., 2010). IMC equipment has become less costly and has demonstrated improved sensitivity and accuracy (Braissant et al., 2010). IMC has recently been successfully applied to improve bacterial detection time in a clinical context (Cichos et al., 2023) and to investigate the metabolic activity of bacteria living in biofilms (Lichtenberg et al., 2022). Despite its potential relevance in non-invasive risk assessment of food, IMC has not yet been explored as a tool for monitoring the growth of pathogenic and non-pathogenic *Bacillus* spp.

While IMC provides valuable insights into the overall metabolic activity of microorganisms, it offers limited specificity regarding the biochemical processes underlying bacterial metabolism. Monitoring changes in key metabolites such as sugars, organic acids, and amino acids enables a deeper understanding of microbial nutrient assimilation while also revealing biochemical changes in the food matrices that are relevant to safety and quality. Sugars serve as primary energy sources for microbial growth, while organic acids are key intermediates in carbon metabolism (Schilling et al., 2007). Free amino acids are an important nitrogen source and can be released either through proteolytic activity or produced through *de novo* synthesis (Sonenshein, 2007). Furthermore, quantifying the heat-stable emetic toxin cereulide is essential for evaluating the safety risks associated with food products contaminated by *B. cereus.*

To bridge this gap, the present study integrates IMC with targeted metabolomics to provide a comprehensive and mechanistic understanding of *Bacillus* physiology in food-relevant matrices. Previous IMC applications in food science have largely focused on microbial growth dynamics, such as lactic acid bacteria performance in sourdough (Mihhalevski et al., 2011), the effect of glucose depletion on growth (Kabanova et al., 2013), shelf life of carrot juice (Alklint et al., 2005), and yeast inhibition by alcohol (Antoce et al., 1997). In contrast, we specifically investigate the metabolic heat production of *B. cereus* and *B. subtilis* in a rich laboratory medium and in dairy- and plant-based liquid matrices (hereafter referred to as matrices), while simultaneously quantifying sugars, organic acids, free amino acids, and cereulide at selected time points (Figure 1). This dual approach of combining IMC with metabolomics enables a novel perspective on *Bacillus* activity in food systems, providing insights that go beyond growth kinetics to inform both food safety and quality management.

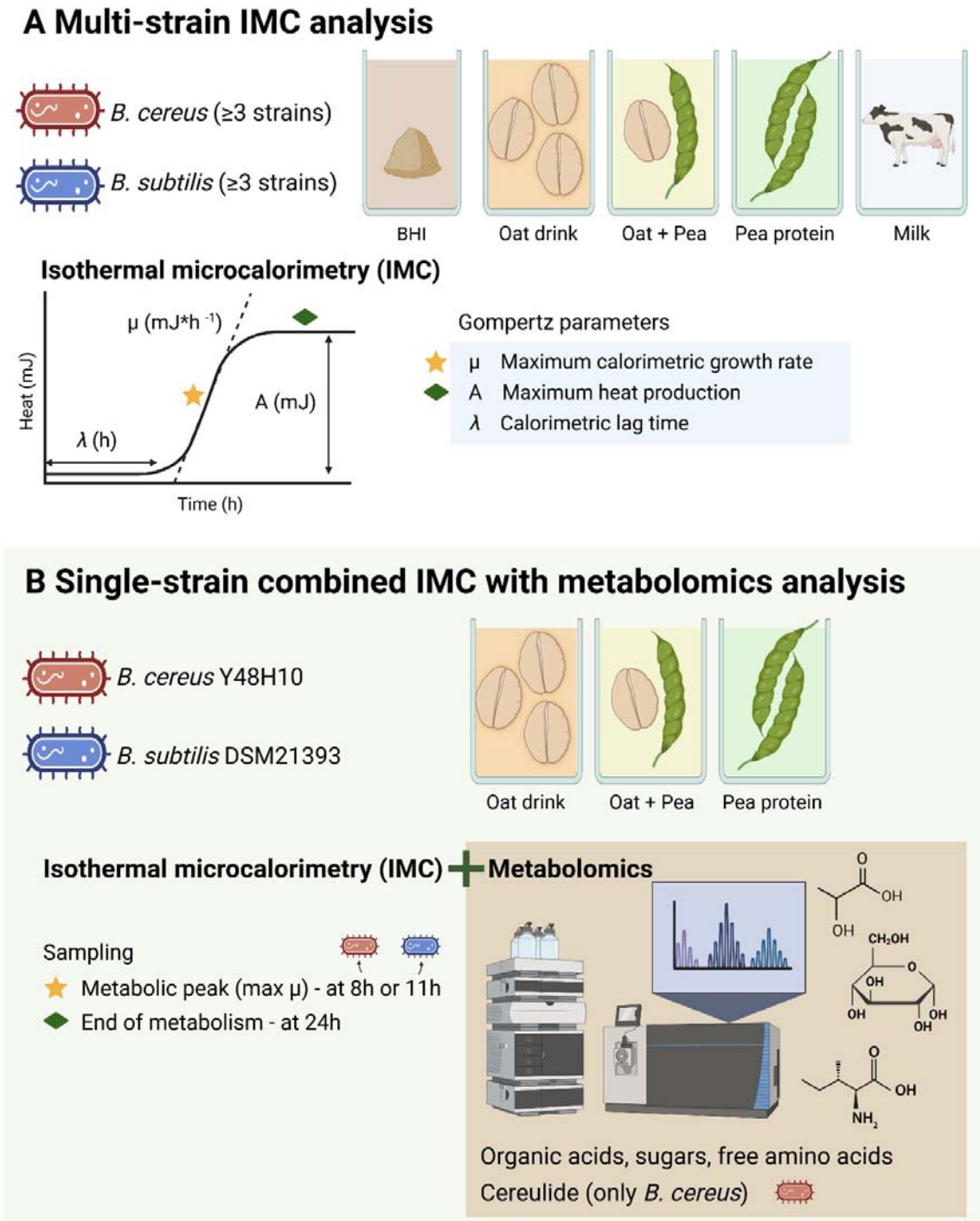

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Figure 1: Experimental workflow combining isothermal microcalorimetry (IMC) and targeted metabolomics. IMC was applied to monitor metabolic activity of *B. cereus* and *B. subtilis* in different food-relevant matrices, and to define sampling points for metabolite analysis. (A) Multi-strain IMC characterization: Sixteen *B. cereus* and eight *B. subtilis* strains were cultivated in BHI broth to

characterize heat flow dynamics over 20 h. A subset of three strains per species was further assessed in four liquid food matrices (milk, oat, pea, oat–pea blend) for 24 h. (B) Integrated IMC–metabolomics analysis**:** One representative strain from each species (*B. cereus* Y48H10 and *B. subtilis* DSM21393) was cultivated in oat, pea and oat-pea matrices. IMC guided sampling at two time points: peak metabolic activity at maximum calorimetric growth rate (star) and end-of-metabolism at maximum heat production (diamond). At these points, pooled samples were analysed for sugars, organic acids, and free amino acids; cereulide was quantified in *B. cereus* only. Figure created in BioRender https://BioRender.com/obt15z8.

2. Materials and methods

2.1 Bacterial strains

Sixteen strains from the *B. cereus* group and eight *B. subtilis* strains were selected based on diversity in toxicity, genetic characteristics, and origin (Table 1). Five of the included *B. subtilis* strains were previously isolated from traditional fermentations in Ghana and Burkina Faso (Kpikpi et al., 2014) (Parkouda et al., 2010; Wiedenbein et al., 2023). Two of the *B. cereus* strains were isolated from African fermented condiments (Thorsen et al., 2010). Strains were maintained in a -80°C glycerol stock and streaked onto Brain Heart Infusion (BHI) agar plates before being cultured in BHI broth.

TABLE 1: Characteristics of the *Bacillus* strains used in this study, based on sequencing and ANIm data for the *Bacillus cereus* group obtained by Skriver et al. (2025) and for the *Bacillus subtilis* strains obtained by Wiedenbein et al. (2023). Asterisk (*) denotes values below species threshold (<96% ANIm). The letter T refers to the type strain.

Strain	**Toxin production**	**Origin isolation**	**Previously assigned taxonomy (ANIm in %)**	**Taxonomy reference**	**Best taxonomic match (ANIm in %)**	**Assembly size (basepairs)**	**GC content**
F4433/73	Enterotoxin	Meatloaf, USA	*Bacillus cereus* (91.68*)	(Midura et al., 1970)	*Bacillus tropicus* (96.26)	5,433,211	35.5
O-216	Unknown	Milk, Denmark	*Bacillus cereus* (91.89*)	(Larsen & Jørgensen, 1999)	*Bacillus pacificus* (99.94)	5,655,403	35.4
LWL3	Enterotoxin	Foodborne outbreak	*Bacillus cereus* (91.68*)	(Dufrenne et al., 1995)	*Bacillus wiedmannii* (96.43), *Bacillus pretiosus* (97.1%)	5,686,534	35.3
59	Unknown	Cream	*Bacillus cereus* (91.72*)	(Stenfors & Granum, 2001)	*Bacillus wiedmannii* (96.56), *Bacillus pretiosus* (96.7%)	5,516,290	35.5
MC67	Emetic	Sandy soil, Denmark	*Bacillus weihenstephanensis*[a] (95*)	(Hendriksen et al., 2006)	*Bacillus mycoides* (95*)	5,802,073	35.5
A536	Enterotoxin	Milk, silotank	*Bacillus cereus* (89.96*)	(Svensson et al., 2004)	*Bacillus mycoides* (97.23)	6,122,672	35.3
NVH 0075-95	Enterotoxin	Foodborne outbreak in Norway	*Bacillus cereus* (92.08*)	(T. Lund & Granum, 1996)	*Bacillus paranthracis* (97.61)	6,044,751	35.3
F4810/72	Emetic	Foodborne outbreak, UK	*Bacillus cereus* (92.09*)	(Turnbull et al., 1979)	*Bacillus paranthracis* (97.62)	5,584,855	35.5
NS117	Emetic	Spruce tree, Norway	*Bacillus cereus* (92.04*)	(Hallaksella et al., 1991)	*Bacillus paranthracis* (97.61)	5,832,990	35.3
LKT1/1	Emetic	Filler material of a building with moisture damage, Finland	*Bacillus cereus* (92.06*)	(Apetroaie et al., 2005)	*Bacillus paranthracis* (97.62)	5,690,232	35.5
Y48H10	Emetic	Iru, Africa	*Bacillus cereus* (92.09*)	(Thorsen et al., 2010)	*Bacillus paranthracis* (97.62)	5,549,004	35.5
Ba18H2	Emetic	Sonru, Africa	*Bacillus cereus*	(Thorsen et al., 2010)	*Bacillus paranthracis*	5,598,436	35.5

			(92.09*)		(97.62)		
SDA GR177	Emetic + Enterotoxin	Milk	*Bacillus cereus* (92.09*)	Svensk Mjolk, Swedish Dairy Association	*Bacillus paranthracis* (97.62)	5,634,738	35.5
SDA MA60	Enterotoxin	Milk	*Bacillus cereus* (92.06*)	Svensk Mjolk, Swedish Dairy Association	*Bacillus paranthracis* (97.62)	5,644,507	35.5
F4430/73	Emetic	Foodborne outbreak, UK	*Bacillus cereus* (92.06*)	(Spira & Goepfert, 1975)	*Bacillus paranthracis* (97.62)	5,661,860	35.5
ISI2489		Rice	*Bacillus cereus* (92.08*)	Arla culture collection	*Bacillus paranthracis* (97.64)	5,541,441	35.5
DSM13019		Boiled hay, Denmark	*Bacillus subtilis* (98.53)	(Wiedenbein et al., 2023)	*Bacillus subtilis* (98.53)	4,044,377	43.6
DSM10 T		Unknown (“Marburg”)	*Bacillus subtilis* (99.99)	(Lilge et al., 2021; Wiedenbein et al., 2023)	*Bacillus subtilis* (99.99)	4,299,854	43.4
DSM21393		potato tubers, Tatarstan, Russian Federation	*Bacillus subtilis* (98.8)	(Wiedenbein et al., 2023)	*Bacillus subtilis* (98.8)	4,100,908	43.9
PRO35		Kantong, Nyankpala, Ghana	*Bacillus subtilis* (98.4)	(Kpikpi et al., 2014; Wiedenbein et al., 2023)	*Bacillus subtilis* (98.4)	4,032,381	43.4
PRO31		Kantong, Nyankpala, Ghana	*Bacillus subtilis* (98.35)	(Kpikpi et al., 2014; Wiedenbein et al., 2023)	*Bacillus subtilis* (98.35)	4,272,877	43.2
PRO64		Kantong, Nyankpala, Ghana	*Bacillus subtilis* (97.9)	(Wiedenbein et al., 2023)	*Bacillus subtilis* (97.9)	4,113,761	43.6
PRO99		Kantong, Navrongo, Ghana	*Bacillus subtilis* (98.2)	(Wiedenbein et al., 2023)	*Bacillus subtilis* (98.2)	3,963,991	43.4
PRO115		Maari, Mansila, Burkina Faso	*Bacillus subtilis* (100)	(Wiedenbein et al., 2023)	*Bacillus subtilis* (100)	4,140,503	43.8

Footnote *(a): B. mycoides* has been used for ANIm comparison, as *B. mycoides* is a later synonym of *B. weihenstephanensis* (Liu et al., 2018).

2.2 Whole genome sequencing

B. subtilis whole genomes included in this work were previously characterized in Wiedenbein et al. (2023), while the *B. cereus* whole genomes were obtained from Skriver et al. (2025).

2.3 Phylogenetic tree construction

Phylogenetic trees were constructed with the up-to-date bacterial core gene (UBCG) method based on the concatenation of 92 core genes found in all taxonomic ranks of Bacteria (Na et al., 2018). FastTree was selected to generate the maximum-likelihood phylogenetic trees (Price et al., 2010). The phylogenetic trees were visualized with Molecular Evolutionary Genetics Analysis version 11 software (MEGA 11) (Tamura et al., 2021). *Halobacillus halophilus* DSM 2266 (type strain) (GenBank: GCA_000284515.1) was selected as the outgroup to root the trees.

2.4 Isothermal calorimetric assessment of *B. cereus* and *B. subtilis* in BHI medium

All sixteen *B. cereus* and eight *B. subtilis* strains (Table 1) were cultivated in BHI broth (Merck). To measure the heat flow of the *Bacillus* strains in BHI medium, the protocol of Beilharz et al. (2023) was followed with a few modifications. Strains were cultured in BHI broth overnight (30°C, 200 rpm) and then diluted with BHI broth to an optical density at 600 nm (OD_{600}) of 0.2. The inoculum was mixed with fresh BHI medium at a 1% inoculation rate to a final volume of 200 µL, corresponding to an initial concentration of 10^6 CFU/mL. The inoculated mixtures were transferred to plastic inserts (calWells™, Symcel AB, Sweden) in the titanium vials of a 48-well calPlate™ (Symcel AB, Sweden). Non-inoculated BHI medium was dispensed into 16 reference vials, one for every two of the 32 sample vials, to account for BHI-dependent effects on system-generated heat. The vials were pre-incubated for 60 min to reach temperature equilibrium. The plates were then incubated in the Calscreener (Symcel AB, Sweden) at 30°C for 20h.

2.5 Isothermal calorimetric assessment of *B. cereus* and *B. subtilis* in food matrices

Ultra-high temperature treated (UHT) whole milk (Geia Food A/S, Czech Republic) and UHT oat drink (Naturli, Denmark) were purchased commercially in Danish supermarkets. For the mixed oat-pea matrix, 5% w/w pea protein hydrolysate (NUTRALYS® PEA F853M-EXP, Roquette, France) was transferred to the store-bought oat drink and autoclaved (121°C, 20 min). For the pea matrix, 5% w/w was added to MilliQ water and autoclaved (121°C, 20 min). The pea and oat-pea matrices were autoclaved prior to inoculation to solubilize the pea hydrolysate and eliminate contaminants present in the pea isolate. In contrast, the UHT oat drink was not further autoclaved, as autoclaving caused growth inhibition by *B. cereus* in preliminary experiments. These differences in thermal processing were considered when interpreting metabolic outcomes.

A selection of three *B. cereus* strains (Y48H10, SDA GR177, MC67) and three *B. subtilis* strains (PRO99, DSM10, DSM21393) were cultivated in the food matrices. Cells were cultured in BHI broth overnight (30°C, 200 rpm), centrifuged at 4500 *g* for 12 minutes, and washed twice in 0.9% NaCl solution. Washed cells were adjusted to OD_{600} of 0.2 before being transferred with an inoculation rate of 1% and a total volume of 200 µL into the different food matrices. Similarly to section 2.5, the inoculated mixtures were transferred to plastic inserts in the titanium vials of the 48-well calPlate and pre-incubated for 60 min to reach temperature equilibrium. Non-inoculated liquid food matrices were dispensed into 16 reference vials, one for every two of the 32 sample vials, to account for matrix-dependent effects on system-generated heat. The calPlate was then incubated in the Calscreener at 30°C for 24 hours.

2.6 Cultivation of selected strains in oat, pea, and oat-pea matrices for metabolic profiling

One strain from each species (*B. cereus* Y48H10 and *B. subtilis* DSM21393) was selected for in-depth metabolic profiling. Washed cells were prepared as in section 2.5 and then inoculated in oat, pea, and oat-pea matrices. The initial pH of each food matrix was measured using a Radiometer PHM250 pH meter (Radiometer Analytical, Denmark). Two biological replicates, each consisting of four technical replicates, were used. Biological replicates were defined as individual colonies streaked from the same BHI plate, while technical replicates originated from the same biological replicate after two rounds of pre-cultivation on BHI broth. Samples were taken at maximum calorimetric growth rate, approximately 8 hours for *B. cereus* and 11 hours for *B. subtilis*, and at the end-metabolic point, 24 hours for both *B. cereus* and *B. subtilis*. Directly after sampling, technical replicates were pooled to obtain a final volume of approximately 800 µL. This pooled sample was analysed for sugars and organic acids (sections 2.7), free amino acids (section 2.8), and the *B. cereus* samples were screened for cereulide (section 2.9).

2.7 Quantification of simple sugars and organic acids

Pooled technical replicates of food samples (section 2.6) were centrifuged at 12000 *g* for 15 minutes at 4°C, and the supernatant was separated and filtered through PTFE membranes with a pore size of 0.45 µm directly into the HPLC vials and stored at -21°C until analysis. An Agilent 1260 Infinity II LC system with refractive index detection (Agilent Technologies, Santa Clara, CA, U.S.A.) was used to quantify simple sugars and organic acids. The mobile phase was water, with a flow rate of 0.5 mL/min. A Cation H Bio-Rad Micro-Guard column (304.6 mm) followed by an Aminex HPX-87H carbohydrate analysis column (300 × 7.8 mm) (Bio-Rad Laboratories, Inc.; Hercules, CA, U.S.A.) was used to separate organic acids and simple sugars isocratically at 35°C. The injection volume of the sample was 10 µL and the run time was 15 minutes.

The chromatographic data were acquired, monitored, and processed using OpenLAB CDS LC Chemstation M8307AA. All compounds were identified through co-injection of authentic standards. Quantification was based on external calibration curves constructed for seven standard solutions for sugars (glucose, fructose, arabinose, galactose, and sucrose) and organic acids (lactic-, acetic-, and citric acid) in concentrations ranging from 0.05 – 10 g/L.

2.8 Free amino acid analysis

Samples (section 2.6) were freeze-dried (Lyovapor L-200, Buchi) for two days. To each freeze-dried sample, 1 mL of 0.1 M HCl was added, and the mixture was ultrasonicated (Branson 2210, Emerson) for five minutes. To the 200 µL sonicated sample, 200 µL of an ice-cold solution of 12.5% trichloroacetic acid was added. After incubation for 2 hours at 4°C, the sample was centrifuged at 10,000 g for 20 minutes. The supernatant was neutralized with 45 µL of freshly prepared 1 M NaOH and then mixed with 195 µL of 50 µM aminocaproic acid (internal standard). Free amino acids were quantified using HPLC as described in Rehlund et al. (2025). All proteinogenic free amino acids were quantified, except cysteine and proline, using external calibration curves. HPLC data were pre-processed using Chromeleon 7.2.7 (Thermo Scientific, MA, USA).

2.9 Quantification of cereulide produced by strain Y48H10 using HPLC-MS/MS

Quantification of cereulide from strain Y48H10 was performed in two biological duplicates. Four technical replicates were pooled together for each sampling time point. Cereulide analysis was performed using ISO 18465 standard (ISO18465:2017, 2017) method with few modifications. Briefly, samples were diluted in 1:10 ratio with HPLC-grade acetonitrile (VWR Chemicals, Poland). Labelled cereulide (^{13}C6-cereulide) was added as an internal standard in each sample at the concentration of 10 ng/ml. The cereulide was extracted by a rotating shaker (300 rpm) for 1 hour followed by resting for 30 minutes at ambient temperature. The solution was decanted twice (first 4500 *g* for 15 minutes and second 12,000 *g* for 15 minutes). A 200 µL of the supernatant was transferred into HPLC vial for LC-MS/MS analysis.

Samples were randomized and analysed on an Elute UPLC system coupled to an Impact II QTOF-MS instrument (Bruker Daltonik, Germany). Chromatographic separation was performed on a reverse-phase Supelco Discovery C18 column (10 cm × 2.1 mm, 5 µm particle size) maintained at 36 °C. The mobile phase consisted of an isocratic elution with 5% phase A (10 mM ammonium formate in water containing 0.1% formic acid) and 95% phase B (acetonitrile with 0.1% formic acid). The flow rate was set to 0.4 mL/min with an injection volume of 5 µL. Mass spectra were acquired in the range of 1000–1300 *m/z* at 12 Hz using electrospray ionization (ESI) in positive-ion mode. Prior to analysis, the instrument was externally calibrated with 10 mM sodium formate in 50% isopropanol and tuned using the ESI-L Low Concentration Tuning Mix (Agilent Technologies, CA, USA). ESI parameters

were as follows: end plate offset, –500 V; capillary voltage, 4500 V; nebulizer pressure, 2.2 bar; dry gas flow, 10 L/min; and drying temperature, 220 °C. MS settings included funnel 1 and 2 RF, 200 Vpp; hexapole, 50 Vpp; ion energy, 5 eV; collision energy, 5 eV; and pre-pulse storage, 5 µs. Peak areas of natural cereulide and isotope-labelled cereulide were extracted using corresponding precursor ions at m/z 1170–1171 and 1176–1177, respectively.

2.10 Data analysis

Isothermal microcalorimetric data were analysed from 1 to 20 hours (BHI medium) or 24 hours (food) after inoculation, as the first hour was needed for thermal equilibration of the instrument. Baseline correction was applied using the heat flow data collected before plate insertion. For 2 out of 96 experiments, an alternative baseline heat flow value was selected to achieve a better fit after plate insertion at 50 minutes. Data from one replicate experiment corresponding to strain *B. subtilis* PRO31 was excluded, due to negative heat flow values at the beginning of the experiment, which was attributed by the instrument manufacturer to moisture evaporation from the vial.

The heat curves (heat accumulated as a function of time) were fitted with the modified Gompertz model to estimate microbial growth characteristics (Zwietering et al., 1990), based on two assumptions (1) that cells remain in the medium, and (2) that heat flow data present themselves with one central peak or a closely similar shape (e.g. peak with a shoulder) (Braissant et al., 2010). Data analysis of the isothermal calorimetry was conducted using RStudio (R Core Team, 2023). Curve fitting and parameter estimation were performed on each experiment using the grofit R package, based on Gompertz modelling, to estimate the maximum heat production (mJ), the length of the calorimetric lag time (h), and the maximum calorimetric growth rate ($mJ \cdot h^{-1}$) (Kahm et al., 2010). The lag time was corrected for the one-hour pre-incubation period.

Statistical tests were performed using the Gompertz parameters related to species, with a significance level of 0.05. Group comparisons between *B. cereus* and *B. subtilis* and between the interspecies clades in BHI medium were performed using unpaired T-tests, and phylogenetic comparisons between *B. cereus* clades were performed using Kruskal-Wallis with Dunn's post-hoc test. Comparison between heat curves obtained in BHI media and food matrices of selected *B. cereus* (Y48H10, SDA GR177, MC67) and *B. subtilis* strains (PRO99, DSM10, DSM21393) were conducted using paired t-test with Bonferroni correction.

Gompertz parameters were square-root transformed to ensure equal variance among groups. A two-way analysis of variance (ANOVA) was performed to examine the effects of *Bacillus* clade (*B. cereus* and *B. subtilis*), food (milk, oat, pea, and oat-pea), and their interaction on the square-root transformed Gompertz parameters. A separate two-way ANOVA was performed to test the influence of all

matrices (foods and BHI medium), *Bacillus* clade, and their interaction on square-root transformed Gompertz parameters. All ANOVA models were fitted using the lmerTest package, which extends lme4 by providing p-values for mixed effects. Post-hoc analyses were conducted using estimated marginal means (EMM) via the emmeans package.

For the metabolomics data, a three-way ANOVA was performed to evaluate the influence of *Bacillus* clade, metabolic time-point (peak vs. end), and food matrix, as well as their interactions, on the content of individual sugars, individual organic acids, and total free amino acids. Biological replicates were included as a random effect using lme4, and significance testing was carried out with lmerTest. Post-hoc analyses were conducted using EMM. Additionally, a two-way ANOVA was conducted to investigate the effects of fermentation (non-fermented vs. fermented) and matrix on total sugars. Tukey post-hoc comparisons were performed between non-fermented and fermented samples within each matrix (oat, pea, and oat–pea). Non-detected compounds were assigned a value equal to half the lowest detected concentration to enable statistical comparisons. Principal component analysis (PCA) was applied to investigate potential interactions between the metabolites of *Bacillus* spp. in food matrices using RStudio (R Core Team, 2023), and a biplot was created using the ggplot2 package.

The quantification of cereulide produced by *B. cereus* strain Y48H10 in food matrices was based on mass spectrometric peak area ratios derived from baseline-corrected chromatograms, using a signal-to-noise ratio (SNR) threshold of 3. The limit of detection (LOD) and limit of quantification (LOQ) were determined from the standard deviation of the noise in 0.1 ng/mL samples, which represented the lowest concentration at which cereulide was not detectable. Inspection and processing of obtained cereulide data were performed using MATLAB 2023b.

3. Results

3.1 Taxonomy and phylogeny

A maximum-likelihood phylogenetic tree was constructed using the UBCG pipeline (Na et al., 2018) from the whole-genome sequences of the *B. cereus* and *B. subtilis* strains. Species names were drawn from the best taxonomic (ANIm) matches rather than the previously recognized taxonomy of the strains (Table 1), since *B. cereus* taxonomy has undergone many recent changes (Caroll et al., 2022). For strains BC59 and LWL3, previously classified as *B. cereus*, ANIm values exceeded the species threshold (>96%) for both *Bacillus wiedmannii* and *Bacillus pretiosus*. However, we adopted *B. wiedmannii* as the taxonomic designation, in line with (Mozhaitseva et al., 2025), who reported genomic similarity between these species and grouped them within a shared cluster. Moreover, *B. pretiosus* remains an effectively published but not validly recognized name under current bacterial nomenclature rules (Oren et al., 2023; Robas Mora et al., 2023), further supporting the use of *B. wiedmannii*.

Five different clades were identified (Figure 2), differentiating the *B. cereus* group strains into Clade A (*B. paranthracis*, *B. pacificus*, and *B. tropicus*), Clade B (*B. wiedmannii/B. pretiosus*), and Clade C (*B. mycoides*), and distinguishing the *B. subtilis* group into Clade D (PRO31, PRO35, PRO64, PRO99), and Clade E (DSM13019, DSM21393, PRO115, and DSM10). In short, we observed a diverse range of *B. cereus* (encompassing five different genomic subspecies) and *B. subtilis* isolates (two distinct clades) to facilitate comparisons between phylogeny and microcalorimetric analysis of metabolic activity.

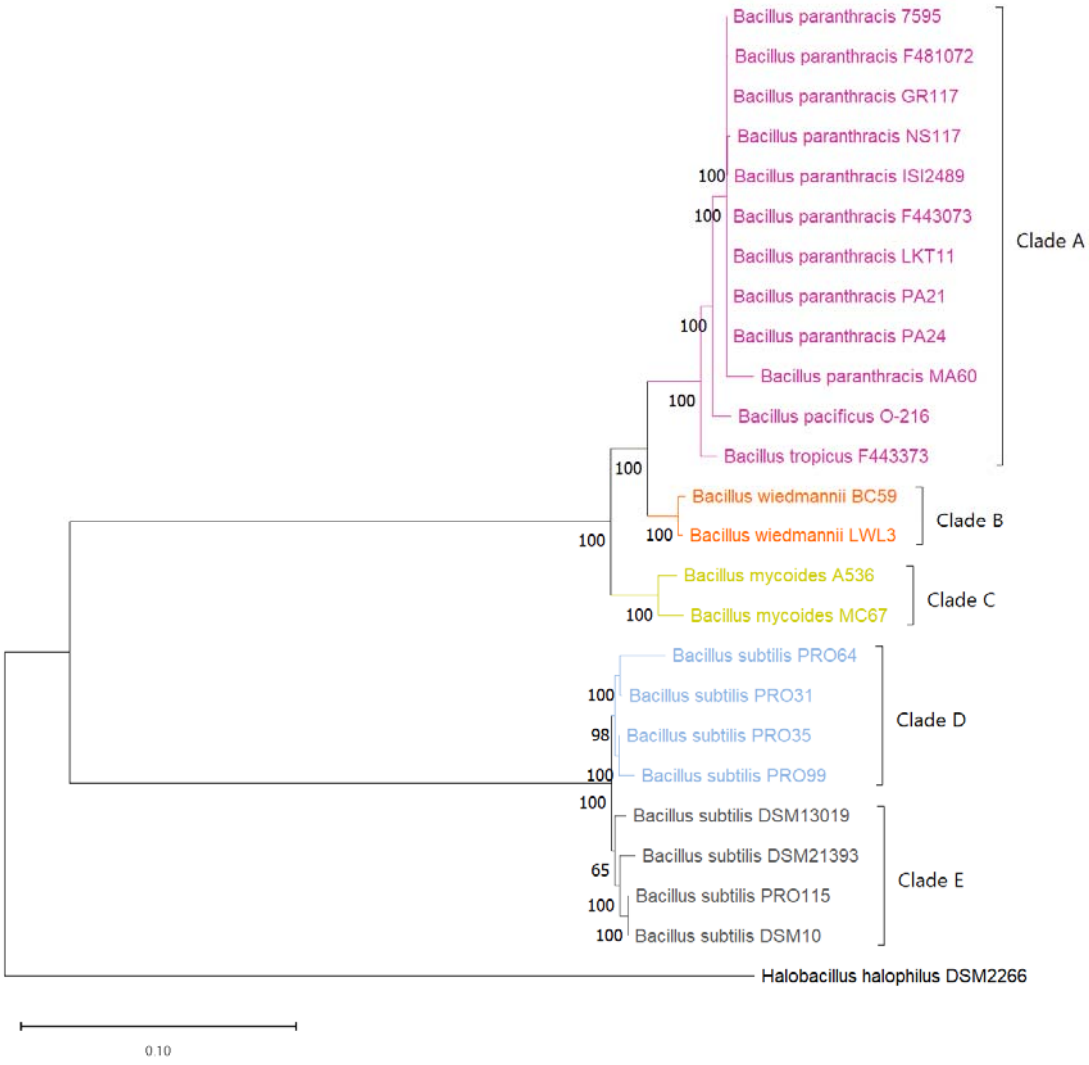

Figure 2: Rooted maximum likelihood phylogenetic tree of *Bacillus cereus* group strains (Clades A, B, and C) and *Bacillus subtilis* strains (Clades D and E) using the up-to-date bacterial core gene (UBCG) pipeline, drawn by MEGA11. The names of the species were drawn from the best taxonomic (ANIm) matches, as displayed in Table 1. *Halobacillus halophilus* DSM2266 was used to root the

tree. Percentage bootstrap values are indicated at branching points, and the scale bar represents 0.01 substitutions per site.

3.2 Metabolic activity of *Bacillus* cultivated in BHI and food matrices

To investigate the metabolic activity of *Bacillus* strains under controlled conditions, we monitored the metabolic heat production profiles of 16 *B. cereus* strains and 8 *B. subtilis* strains. Cultivation was first carried out in BHI medium at 30°C for 20 hours, until the metabolic activity reached a steady-state plateau (Figure 3B). During 20 hours of cultivation, *B. cereus* and *B. subtilis* exhibited a typical sigmoidal pattern in the heat production curves (Figure 3A). The heat flow profiles of both *B. subtilis* and *B. cereus* exhibit one main peak followed by a second shoulder peak (Figure 3B).

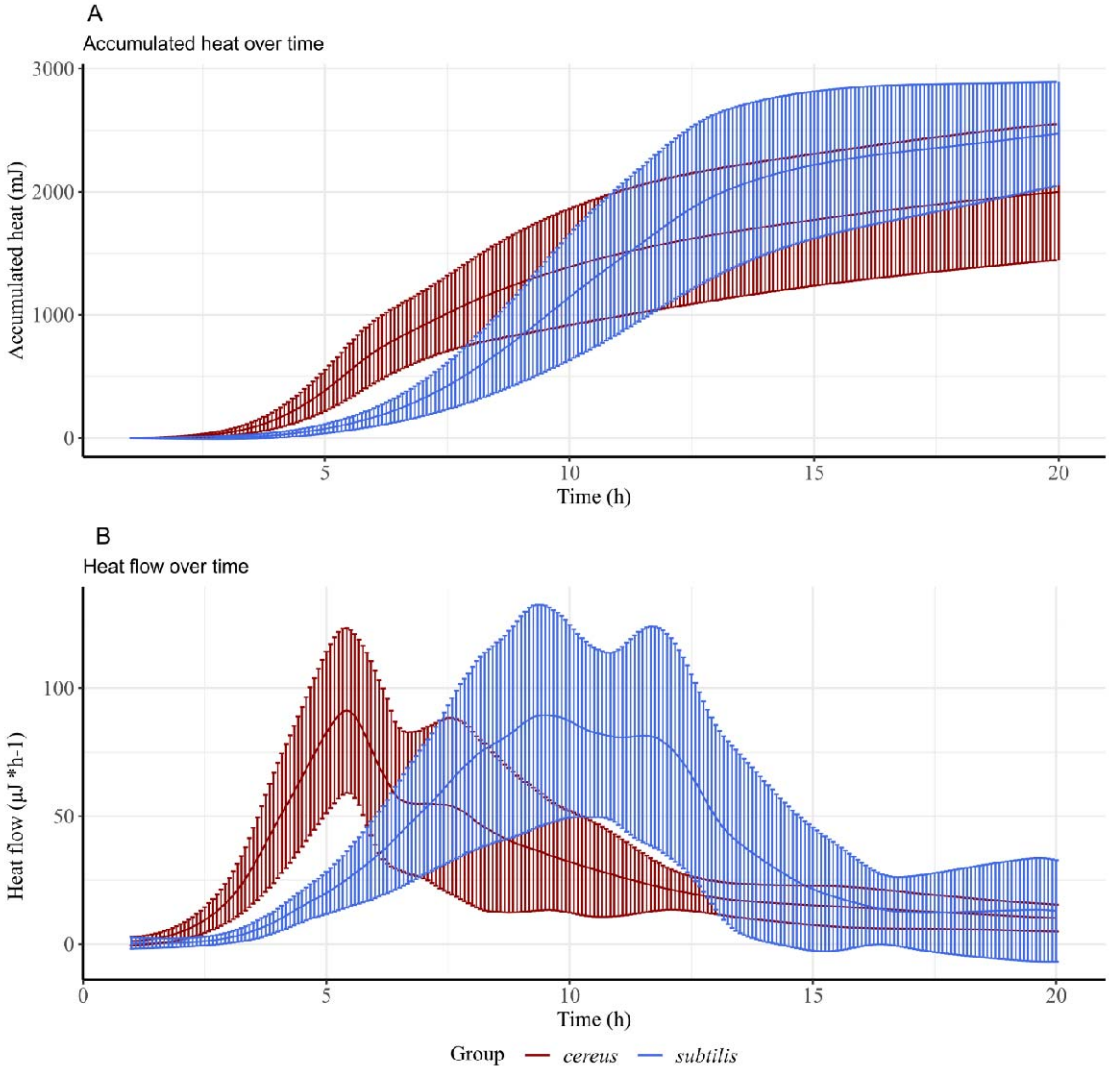

Figure 3: Metabolic heat characteristics of 16 *B. cereus* (red) and 8 *B. subtilis* (blue) strains cultivated in BHI medium, based on four biological replicates divided between two different experiments (A)

Mean values (curve) and standard deviation (error bars) of accumulated heat production over time (B) Mean values (curve) and standard deviation (error bars) of heat flow over time of 16 *B. cereus* (red) and 8 *B. subtilis* (blue) strains.

To gain a better understanding of the metabolic heat profiles, fitted parameters from the Gompertz model, calorimetric lag time (h), the maximum calorimetric growth rate ($mJ*h^{-1}$), and total heat (mJ), were plotted for each *Bacillus* group (*cereus* vs. *subtilis*) (Figure 4A) and the phylogenetic clades within *B. cereus* and *B. subtilis* groups (A, B vs. C; D vs. E) (Figure 4B). Between the *B. subtilis* and *B. cereus* groups, significant differences were observed across all parameters. *B. subtilis* released a significantly higher amount of total heat (2732 mJ ± 462) than *B. cereus* (1941 mJ ± 566) ($p < 10^{-10}$). *B. subtilis* displayed a higher maximum metabolic activity (378 $mJ*h^{-1}$ ± 140) than *B. cereus* (240 $mJ*h^{-1}$ ± 106) ($p < 10^{-5}$). Finally, a longer lag time was observed for *B. subtilis* (7.9 hours ± 1.0) than for *B. cereus* (4.3 hours ± 0.8) ($p < 10^{-16}$).

In addition, differences in metabolic activity were observed within the *B. subtilis* and *B. cereus* groups. Between the *B. cereus* clades, *B. mycoides* strains from clade C produced a significantly lower amount of total heat (971 mJ ± 292) than *B. paranthracis*, *B. pacificus*, and *B. tropicus* strains from clade A (2119 mJ ± 464, $p <10^{-5}$) and *B. wiedmannii* strains from clade B (1839 mJ ± 212, p = 0.01). In addition, the maximum calorimetric growth rate differed significantly between Clade A and B ($p <10^{-5}$) and between Clade A and C (p <10-5). Between the *B. subtilis* clades, clade D strains exhibited a longer lag time (8.6 hours ± 0.8) than Clade E (7.2 hours ± 0.7) ($p <10^{-5}$).

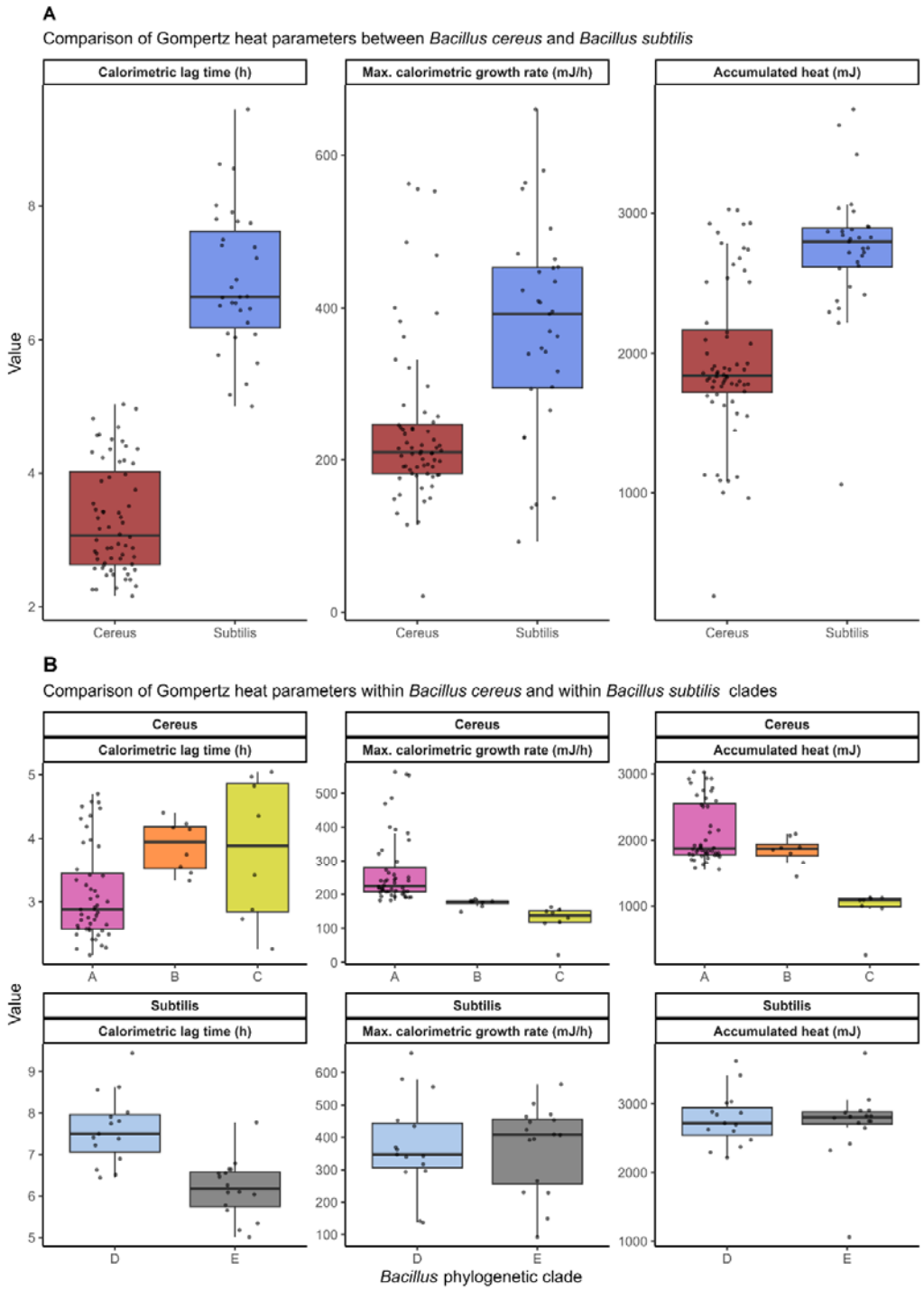

Figure 4: Differences in heat flow characteristics, including calorimetric lag time (h), maximum calorimetric growth rate ($mJ*h^{-1}$), and total heat (mJ) after 20 hours. Calorimetric lag time was corrected for 1 h pre-incubation period. Each dot represents an individual biological replicate (four replicates per *Bacillus* strain) within each phylogenetic group. (A): Differences in heat flow characteristics between the subtilis and cereus groups. (B): Differences in heat flow characteristics between the phylogenetic clades as defined in Figure 2, between Clade A (*B. paranthracis*, *B.*

pacificus, and *B. tropicus*), B (*B. wiedmannii*), C (*B. mycoides*), D (*B. subtilis* PRO31, PRO35, PRO64, PRO99), and E (*B. subtilis* DSM13019, DSM21393, PRO115, and DSM10)

In addition to BHI, three strains of *B. cereus* (Y48H10, SDA GR177, MC67) and three *B. subtilis* (PRO99, DSM10, DSM21393) were cultured in milk (pH 6.5), oat (pH 7.1), pea (pH 6.9), and oat-pea (pH 5.8) at 30°C. Their metabolic heat activity was monitored for 24-hour periods. Gompertz parameters were subsequently derived from the calorimetric data (Table 2).

Table 2: Gompertz calorimetric growth parameters (mean (± sd)) of *B. cereus* (Y48H10, SDA GR177 and MC67) and *B. subtilis* (PRO99, DSM10 and DSM21393) cultivation in milk, oat, pea, and oat-pea matrices.

Species	Food	Calorimetric lag time [hours]	Max calorimetric growth rate [$mJ*h^{-1}$]	Max accumulated heat [mJ]
B. cereus	Milk	7.4 (± 0.5)	150 (± 8)	2040 (± 120)
	Oat	14.5 (±12.8)	184 (± 97)	1696 (± 865)
	Pea	7.7 (± 0.3)	216 (± 65)	1663 (± 714)
	Oat-pea	9.8 (±1.7)	407 (± 103)	1880 (± 665)
B. subtilis	Milk	12.4 (± 2.7)	109 (± 30)	2057 (± 334)
	Oat	11.6 (± 3.1)	211 (± 43)	2452 (± 212)
	Pea	10.4 (±1.3)	275 (± 40)	2065 (± 301)
	Oat-pea	10.9 (± 0.4)	419 (± 28)	2403 (± 232)

B. subtilis exhibited a more consistent calorimetric lag time across food matrices, with a longer lag time in milk (12.4 hours ± 2.7) compared to *B. cereus* (7.35 hours ± 0.5). Maximum calorimetric growth rates varied significantly by substrate and were higher in the combined oat-pea matrix compared to oat ($p = 0.002$, EMM), pea ($p = 0.01$, EMM), and milk ($p < 10^{-4}$, EMM). Across all food matrices, *B. subtilis* produced more metabolic heat than *B. cereus* ($p = 0.02$, EMM) after 24 hours, consistent with observations on BHI medium after 20 hours. When comparing *Bacillus* metabolism between BHI and the food matrices, the calorimetric lag time was significantly lower in BHI than in oat ($p = 0.02$, EMM), while no significant differences were observed between the other matrices.

3.3 Metabolic profiling of *Bacillus* growth in oat, pea, and oat-pea matrices

Targeted metabolomic profiling was performed to assess how matrix composition influences *Bacillus* metabolic activity, with a particular focus on the enhanced metabolic peak observed in the combined oat-pea matrix. One *B. subtilis* (DSM21393) and one *B. cereus* strain (Y48H10) were selected for

cultivation on the food matrices and sampled at two time-points: at peak metabolic activity, e.g. at maximum heat flow (8 hours for *B. cereus*, 11 hours for *B. subtilis*) and at minimum metabolic activity (24 hours for both).

Sugars, organic acids, and free amino acids were quantified in all samples. To interpret the multifactorial data generated from targeted metabolomic profiling (simple sugars, organic acids, and free amino acids), principal component analysis (PCA) was employed to visualize trends in metabolite synthesis and consumption by *B. subtilis* and *B. cereus* cultured in plant-based matrices over 24 hours. PCA revealed that matrix composition was the main driver of sample separation (Figure 5). PC1 (41% variance) was associated with free amino acids, galactose, and fructose. In comparison, PC2 (25% variance) separated the pea matrix from the oat and oat-pea, reflecting lower sugar levels (sucrose, glucose) and higher citrate content.

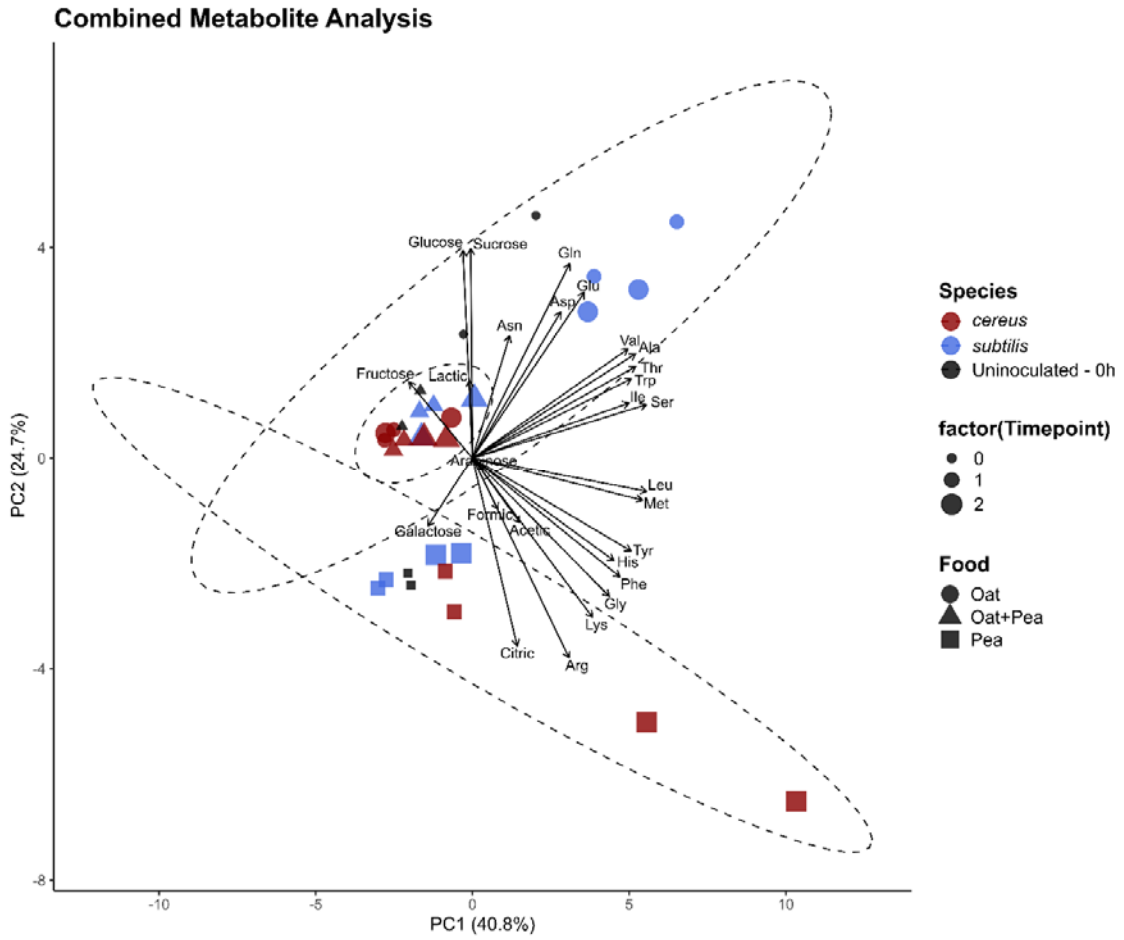

Figure 5: PCA biplot of *Bacillus cereus* Y48H10 (red) and *Bacillus subtilis* DSM21393 (blue) metabolizing free amino acids, simple sugars, and organic acids in oat (circles), pea (squares), and oat-pea (triangles) with two biological replicates. Metabolomic profiles of non-inoculated food

matrices are visualized in black. Ellipses represent 95% of the population for each food matrix (pea, oat, oat-pea).

To gain insight into the factors driving PCA separation, we analysed temporal changes in sugars, organic acids, and free amino acids. Matrix composition strongly influenced sugar and organic acid profiles. Total sugar content, as quantified by HPLC-RID, was significantly higher in the uninoculated oat (49.4 ± 1.8 g/L) and oat-pea (52.6 ± 3.6 g/L, $p = 0.001$, Tukey, Figure 6A) matrices compared to pea (0.74 ± 0.00 g/L, $p = 0.001$, Tukey, Figure 6A). *Bacillus* fermentation significantly reduced the total amount of sugars in the oat-pea matrix ($p = 0.01$, two-way ANOVA, EMM).

Citrate was uniquely present in the pea matrix and absent from both oat and oat-pea (Figure 6B), contributing to its distinct separation in PCA (Figure 5). Cultivation of *B. subtilis* and *B. cereus* led to an increase in total organic acids from peak metabolism (maximum heat flow) to end metabolic phase ($p = 10^{-5}$, three-way ANOVA). The oat-pea matrix showed the most pronounced increase in organic acids, with both species sharply increasing lactic acid levels ($p = 10^{-4}$, EMM, Figure 6B). In contrast, the changes in organic acid levels in oat and pea were more modest ($p > 0.05$, EMM). During *B. subtilis* growth on pea, citrate was consumed, and acetic acid was produced. *B. cereus* produced more acetic acid than *B. subtilis* after 24 hours ($p = 10^{-4}$, EMM).

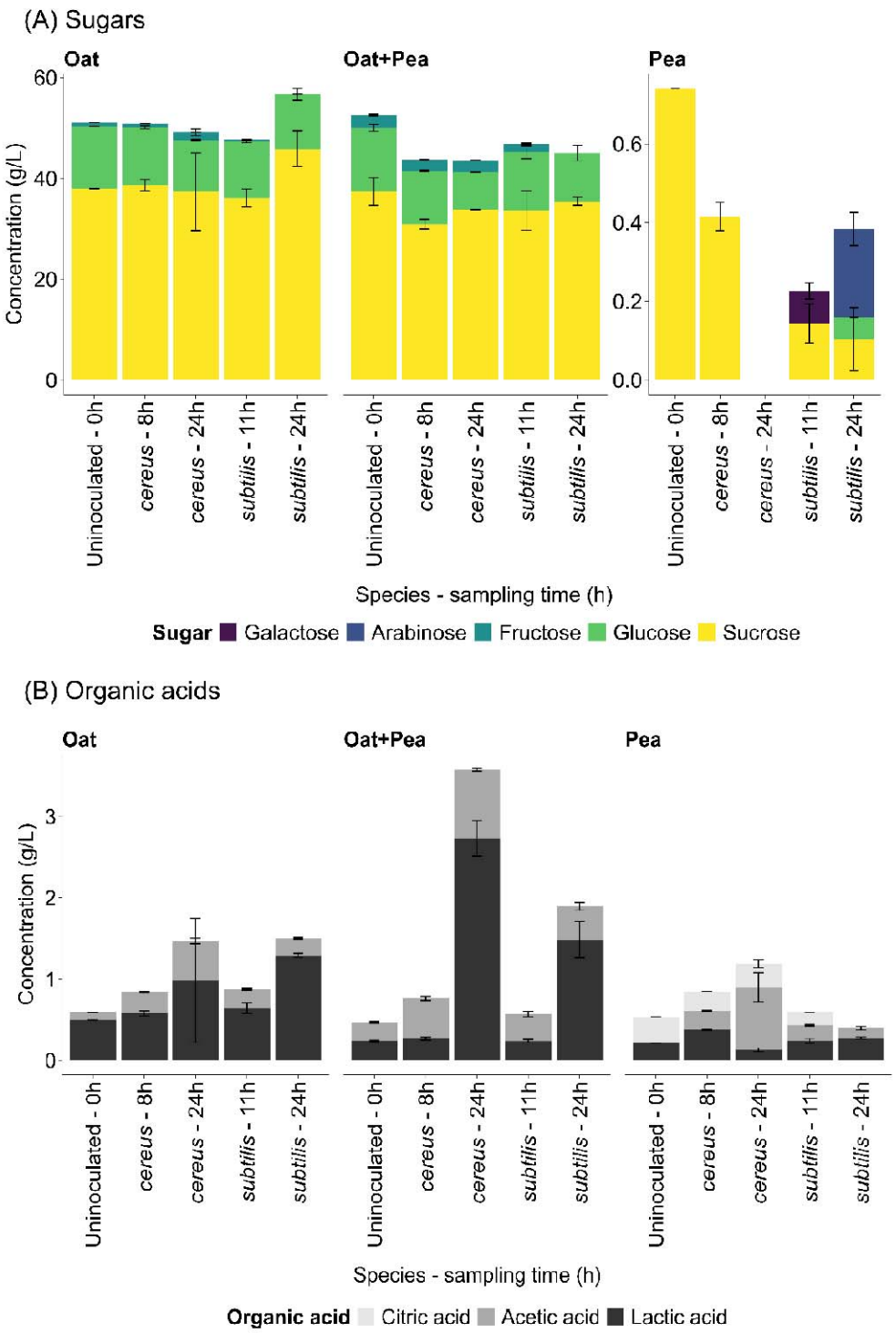

Figure 6: Metabolic profiling of *Bacillus cereus* Y48H10 and *Bacillus subtilis* DSM21393 cultivated in oat, pea, and oat-pea matrices. Error bars are based on two biological replicates, each of which consists of four pooled technical replicates. The stacked bars represent the total sugars (A) and organic acids (B), with each segment showing the contribution of individual compounds.

Free amino acids were observed in all uninoculated matrices, with the highest levels detected in the (non-autoclaved) oat drink (Figure 7). *B. cereus* and *B. subtilis* had distinct effects on the free amino acid profiles depending on the matrix. The most pronounced effects were observed during *B. subtilis* growth on oat and *B. cereus* growth on pea, both increasing free amino acid levels, as also reflected in the PCA (Figure 5). These effects differed in the types of free amino acids released and the timing of their accumulation. For *B. cereus* in pea, total free amino acids increased from 59 µmol/g ± 1 (8 hours) to 219 µmol/g ± 54 (24 hours) ($p = 0.003$, EMM), primarily due to accumulation of Gly, Phe, Ala, Ser, Leu and Tyr. In contrast, *B. subtilis* displayed higher total free amino acid levels in oat than in pea ($p = 10^{-4}$, EMM), peaking at 227 µmol/g ± 38 at maximum metabolic activity after 11 hours, with elevated levels of Asp, Gly and Ala, and a reduction in Asn. In the oat-pea matrix, *Bacillus* did not significantly alter total free amino acid levels over time ($p = 1$, EMM).

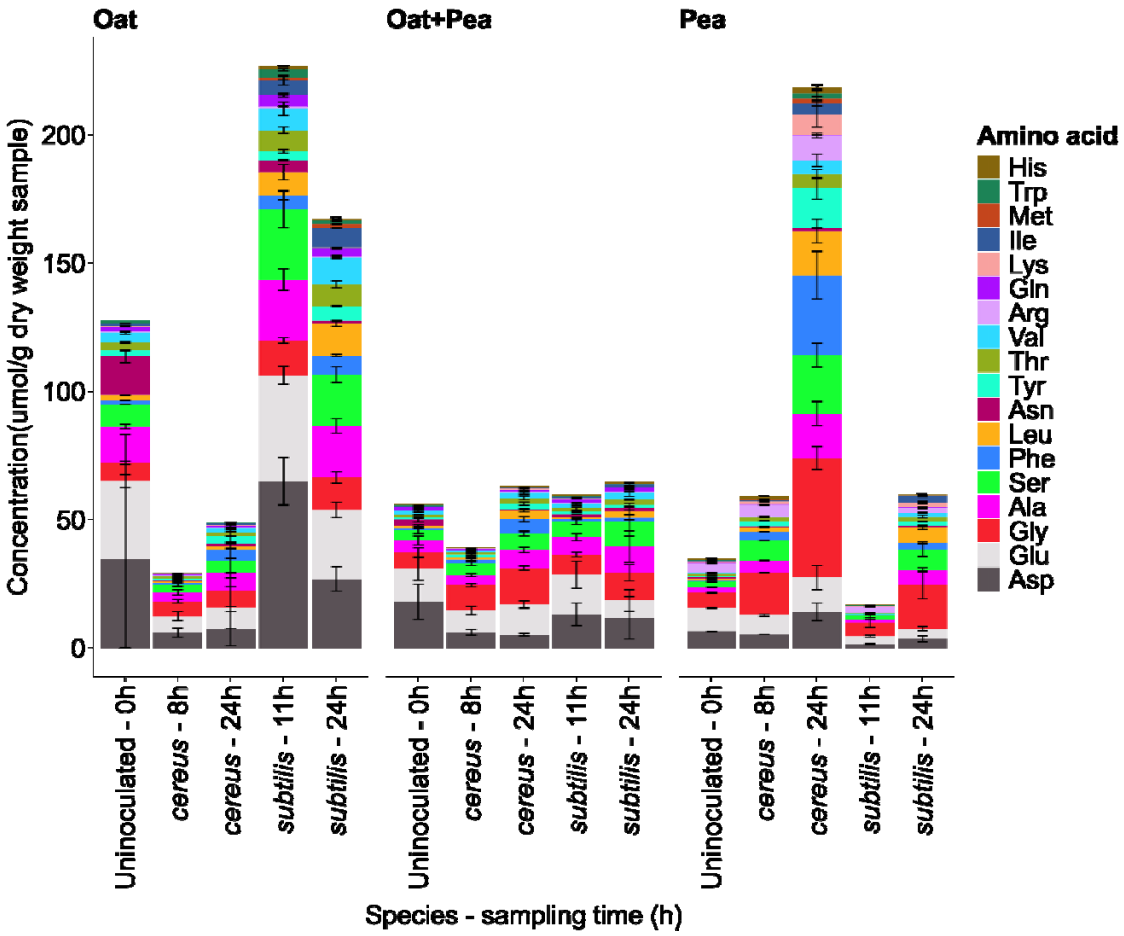

Figure 7: Free amino acid profile of *Bacillus cereus* Y48H10 and *Bacillus subtilis* DSM21393 cultivated in oat, pea, and oat-pea matrices. Error bars are based on two biological replicates, each consisting of four pooled technical replicates. The stacked bars represent the total free amino acids, with each segment showing the contribution of individual amino acids.

Cereulide was exclusively quantified in the uninoculated matrices and in the *B. cereus* samples, since it was assumed that *B. subtilis* is incapable of producing cereulide. Cereulide was below the detection limit (0.1 ng/mL) in the pea matrix. In contrast, concentrations of 3.0 and 2.9 ng/mL were detected in the oat-pea matrix, while the oat drink exhibited substantially higher concentrations of 46 and 48 ng/mL in the two biological replicates.

4. Discussion

To examine the metabolic heat production of *B. cereus* and *B. subtilis*, we analyzed 24 *Bacillus* strains representing diverse origins, pathogenic potential, and genetic backgrounds. The pathogenic potential - reflected in the ability to produce enterotoxins and/or emetic toxins (Table 1) - partially aligned with phylogenetic clustering, suggesting a link between genetic lineage and toxin capacity. All emetic strains showed closest ANIm resemblance to *B. paranthracis*, except the emetic psychrotolerant strain *B. mycoides* M67. Enterotoxin-producing strains were identified in all three *B. cereus* clades and were not distinguishable by phylogeny or IMC-derived metabolic profiles. Strain classification within the *B. cereus* group (Table 1) reflects recent taxonomic revisions. *B. wiedmannii* was proposed as a new species in 2016 (Miller et al., 2016). *B. paranthracis, B. pacificus, and B. tropicus* were proposed in 2017 (Liu et al., 2017), and *B. weihenstephanensis* was merged with *B. mycoides* in 2018 (Liu et al., 2018). Although a formal species-level taxonomic framework is still needed for the *B. cereus* group, whole-genome phylogeny supports inference of clade-dependent metabolic behaviour.

Clade-specific growth dynamics were evident across all Gompertz parameters during cultivation in BHI at 30°C. *B. subtilis* exhibited a higher model-estimated total heat output (2745 mJ) than the observed value (2473 mJ), indicating sustained metabolic activity beyond the 20-hour endpoint. In contrast, *B. cereus* reached an earlier plateau, with modelled (1941 mJ) and measured (2000 mJ) values closely aligned. Both species displayed a shoulder peak following the main heat flow peak (Figure 3B), likely reflecting a shift in substrate utilization. Calorimetric lag phase differed markedly: *B. cereus* averaged 4.3 h ± 0.8, significantly shorter than *B. subtilis* (7.9 h ± 1.0), with no overlap.

These metabolic growth profiles likely reflect distinct ecological strategies in nutrient-rich media such as BHI. *B. subtilis* may sustain metabolic activity and produce more heat in BHI via a broader peptide/carbohydrate catabolism supported by a diverse enzymatic repertoire. In contrast, *B. cereus* showed rapid onset and early saturation of metabolic activity, which may reflect a faster

environmental adaptation and a less energetically intensive growth strategy in nutrient-rich conditions. Phylogenetic variation within each group further influenced metabolic heat production. *B. mycoides* (clade B) strains released significantly less heat than other *B. cereus* clades. One member of clade B, MC67, is psychrotolerant (Thorsen et al., 2006), which may indicate that cardinal temperatures are skewed towards lower ranges, resulting in less effective metabolic kinetics. Clade-specific metabolic differences, including calorimetric lag time, may depend on initial inoculum size. Lankford et al (1966) demonstrated that increasing *Bacillus* inoculum size shortens the cell-based lag phase, presumably because a larger proportion of cells are already in a physiologically active state (Lankford et al., 1966). Importantly, calorimetric lag time does not necessarily equal cell-based lag time, as IMC detection depends on reaching the signal threshold. IMC detection limits vary widely, e.g. *B. subtilis* has been detected from <□10□CFU per vial (Brueckner et al., 2016), whereas *Proteus mirabilis* required ~10□ cells per vial (Braissant, Keiser, et al., 2015). In *Lactobacillus plantarum*, IMC detected growth faster than optical density at high inocula (>10^2□CFU/mL), but similarly at low inocula (<10^2□CFU/mL) (Fricke et al., 2019). Thus, the IMC-based lag time we observed (*B. cereus* 4.3 h ± 0.8, *B. subtilis* 7.9 h ± 1.0) may be dependent on the inoculum size (OD_{600} 0.2, ~10^6 CFU/mL in this study).

The influence of substrate composition on *Bacillus* metabolic kinetics became more apparent when strains were cultivated in food matrices. To test persistence of clade-specific metabolic traits across matrices, three *B. cereus* strains and three *B. subtilis* strains were cultivated in milk, oat, pea, and combined oat-pea matrix. As in BHI medium, *B. subtilis* generally exhibited longer lag times than *B. cereus*, most notably in milk (12.4□hours vs. 7.4□hours). However, species-level distinctions were less pronounced in the food matrices compared to BHI, suggesting that substrate composition exerted a stronger influence on physiological responses than phylogenetic background.

To elucidate the biochemical mechanisms underlying matrix-dependent growth, we profiled sugar, organic acids, and free amino acids during cultivation of one representative *B. cereus* and *B. subtilis* strain in oat, pea, and oat-pea. Direct comparison of metabolic profiles across matrices was complicated by differential thermal processing. The oat drink had already undergone UHT processing and was not autoclaved, as autoclaving inhibited *B. cereus* growth. Autoclaving of oat drink likely degraded heat-sensitive nutrients such as free amino acids (Evans & Butts, 1949), which are known to support *B. cereus* growth (Canoy et al., 2025; Agata et al., 1999). However, autoclaving the matrices containing pea was necessary to solubilize the proteins and eliminate contaminants. This discrepancy introduces a confounding variable, as thermal processing can trigger Maillard reactions, deplete key nutrients, and alter the redox balance (Lund & Ray, 2017). These effects likely contributed to the elevated free amino acid levels observed in the non-autoclaved oat matrix compared to the autoclaved pea-containing matrices.

Despite these limitations, clear matrix-dependent metabolic trends emerged. The oat-pea matrix supported the highest *Bacillus* activity, with the highest maximum calorimetric growth rate for both *Bacillus* species observed among all tested matrices, and substrates. This suggests that the combination of oat and pea provides a more favourable nutrient environment than either substrate alone. *Bacillus spp* showed the most pronounced accumulation of organic acids, including lactic acid and acetic acid. This suggests that complementary nutrient interactions between peptides and free amino acids from pea and readily fermentable sugars from oat could promote more efficient carbohydrate metabolism, possibly reducing metabolic stress, and thereby contribute to higher calorimetric growth.

The autoclaved oat-pea matrix had a lower initial pH (5.8) than autoclaved pea (pH 6.9) and non-autoclaved oat matrix (pH 7.1). Autoclaving likely contributed to these pH differences, since we observed a similar pH decrease when UHT oat drink was subjected to autoclaving, from pH 7.1 to 5.3. Given comparable organic acid levels among the non-inoculated matrices (Figure 6B), the pH drop likely reflects abiotic heat-induced glucose degradation (Leitzen et al., 2021), especially in the carbohydrate-rich oat matrices. However, moderately lower pH alone is unlikely to explain enhanced metabolic activity in the oat-pea matrix. In *B. subtilis*, moderate acidic conditions (pH 6.0) upregulate acid-stress pathways rather than promoting further acidification (Wilks et al., 2009). Similarly, in *B. cereus*, low pH has been associated with reduced production of acid fermentation end products and shifts toward alternative metabolites under stress, rather than increased acidogenesis (Le Lay et al., 2015). Together, these results suggest that enhanced *Bacillus* metabolism and organic acid accumulation in the oat–pea matrix is primarily nutrient driven, although pH-nutrient interactions cannot be entirely ruled out.

To further dissect matrix compositional effects, we focused on the pea matrix, characterised by low sugar content, the unique presence of citrate, and clear PCA separation from oat and oat-pea. During cultivation on pea, both *B. cereus* and *B. subtilis* produced and consumed organic acids, but their metabolic patterns diverged. *B. cereus* produced more acetic acid than *B. subtilis* at the end of metabolism (24 hours). *B. subtilis* significantly reduced citrate, consistent with its ability to take up citrate via Mg^{2+}-citrate transporters CitM/CitH and use it as a carbon source (Schilling et al., 2007; Warner & Lolkema, 2002). In contrast, citrate levels remained unchanged during *B. cereus* growth in pea. Citrate is rarely reported as a carbon source for *B. cereus*, although specific strains can metabolize it (Venkateswaran et al., 2017). These species-specific responses suggest that sugar limitation in pea may drive differential organic acid turnover.

Species-specific strategies also emerged in free amino acid dynamics, revealing differential nutrient interactions of *B. cereus* and *B. subtilis*. *B. subtilis* showed transient free amino acid release in oat, consistent with its capacity to metabolize complex carbohydrates and synthesize amino acids under

nutrient-rich conditions. In contrast, *B. cereus* accumulated free amino acids in pea, likely reflecting proteolytic activity and amino acid catabolism under nutrient or oxygen stress. Limited changes of free amino acid levels in the oat-pea matrix, despite high organic acid production, suggests a shift away from amino acid turnover toward carbohydrate fermentation. These observations align with previous findings that *B. cereus* preferentially utilizes proteinaceous substrates under limited oxygen conditions (Chang et al., 2021).

Cereulide, the heat- and acid-stable emetic toxin produced by *B. cereus*, was quantified across food matrices. The toxin was detected in oat and oat-pea matrices, but not in pea, indicating that composition influences toxin production. Interestingly, although *B. cereus* Y48H10 exhibited the highest heat production in the oat-pea matrix, cereulide levels were lower than in oat alone. Previous studies have similarly shown that *B. cereus* can exhibit comparable growth profiles in a large variety of foods, including bechamel sauce, pasta, and potato puree, but that cereulide production is highly variable (Rajkovic et al., 2006). For example, Ellouze et al. (2021), using *B. cereus* F4810/72, observed faster cereulide formation in a cereal-based matrix (rice) than in a vegetable matrix (vegetable purée) despite slower bacterial growth in rice, supporting the notion that cereulide production is highly matrix-dependent. In contrast to their vegetable matrix, we detected no quantifiable cereulide in pea at 30°C within 24 hours.

Cereulide expression is highly sensitive to environmental factors, including pH, nutrient profile, and redox state (Dommel et al., 2010; Ehling-Schulz et al., 2015). High sugar in oats may enhance ATP availability, thereby favouring cereulide production (Marxen et al., 2015). Several studies report stronger cereulide activity in carbohydrate-rich matrices versus carbohydrate-poor foods (Agata et al., 2002; Messelhäusser et al., 2014; Rajkovic et al., 2006). The lack of detected cereulide in pea and lower levels in oat-pea, despite microbial activity, may reflect indirect inhibitory effects of pea compounds on regulatory networks controlling cereulide biosynthesis. Whether components in pea actively suppress biosynthesis or limit precursor availability remains unresolved and warrants further investigation.

While cereulide is typically associated with starchy foods (Messelhäusser et al., 2014), outbreaks also occur in legume-based products such as fermented soybeans (Inatsu et al., 2020) and soaked navy beans (Nicholls et al., 2016), indicating that low-carbohydrate environments can support toxin synthesis under permissive conditions. Thorsen et al. (2011) found no emetic strains in a survey of African bread snacks made from legumes, whereas Kyrylenko et al. (2023) reported that 9% of *B. cereus* isolates from plant-based dairy alternative ingredients carried cereulide-producing genes. These findings underscore substrate-dependent risk and the need for matrix-specific evaluations of cereulide hazards.

Metabolic heat profiling in monoculture provided complementary insights into *Bacillus* physiology, distinguishing species- and clade-level behaviour under controlled conditions. Our findings reveal that *B. subtilis* consistently produces more metabolic heat than *B. cereus*. Lag time and growth rate differences were matrix-dependent, highlighting the limitations of extrapolating from defined media such as BHI. Although calorimetry alone cannot resolve metabolic pathways, its integration with metabolomic profiling offers a robust framework to assess microbial activity, adaptation, and potential safety risks in complex food systems. While the metabolic capacities of *B. subtilis* and *B. cereus* are well-characterized in defined media, their proteomic responses in food matrices remain underexplored (Yeo et al., 2012). Future studies integrating calorimetry, metabolomics, and proteomics could help reveal matrix-specific regulatory mechanisms that govern spoilage activity and beneficial fermentation traits in *B. subtilis*, as well as toxin production in *B. cereus*. Such approaches are essential to understand *Bacillus* behaviour across diverse food environments.

5. Conclusion

This study demonstrates that *Bacillus cereus* and *Bacillus subtilis* exhibit distinct metabolic behaviour across food-relevant matrices. In BHI at 30°C with an initial inoculum of 10^6 CFU/mL, *B. subtilis* showed longer calorimetric lag times and higher total heat production than *B. cereus*. However, in food matrices, substrate composition had a more pronounced effect on metabolic activity than species identity, although *B. subtilis* consistently generated more heat.

The combined oat-pea matrix supported the highest calorimetric growth rates, likely driven by enhanced carbohydrate metabolism, as evidenced by reduced sugar levels and increased organic acids. In contrast, the pea matrix, characterised by low sugar levels and the unique presence of citrate, triggered post-metabolic consumption of citrate by *B. subtilis*. Free amino acid profiles reflected matrix-specific strategies, *B. subtilis* released free amino acids in oat, while *B. cereus* predominantly did so in pea. Interestingly, despite high calorimetric activity, the oat-pea matrix showed limited change in free amino acid levels, suggesting a shift toward carbohydrate-driven metabolism.

Cereulide production by *B. cereus* was strongly matrix-dependent, with high levels detected in oat, moderate levels in oat-pea, and none in pea, highlighting the role of substrate composition in toxin synthesis. Together, these findings underscore the need for matrix-specific microbial risk assessment tools. They also demonstrate that integration of isothermal microcalorimetry with metabolomics provides a more nuanced understanding of microbial behaviour in complex food environments. Similar heat profiles can mask divergent metabolic strategies; thus, this combined approach offers a valuable tool for evaluating spoilage and contamination risks, particularly in the development of safer plant-based food products.

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Conflict of interest

The authors declare that they have no known competing financial interests or other conflicts of interest.

Author contribution

Emilie V. Skriver: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review and editing; Tessa S. Canoy: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review and editing; Yuandong Sha: Formal analysis, Investigation, Visualization; Morten A. Rasmussen: Software, Formal analysis; Bekzod Khakimov: Methodology, Formal analysis, Supervision; Susanne Knøchel: Writing – review and editing, Resources, Supervision, Project administration, Funding acquisition; Henriette L. Røder; Conceptualization, Methodology, Resources, Writing – review and editing, Supervision, Project administration, Funding acquisition.

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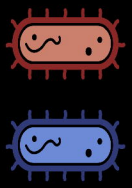
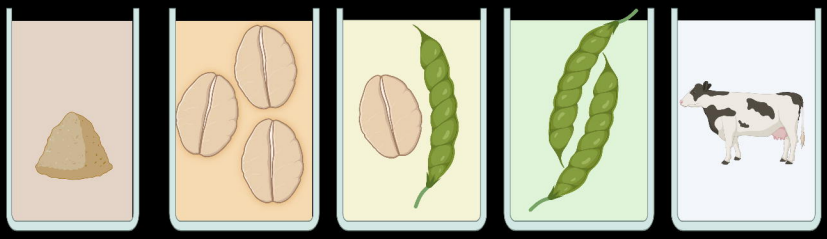
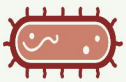




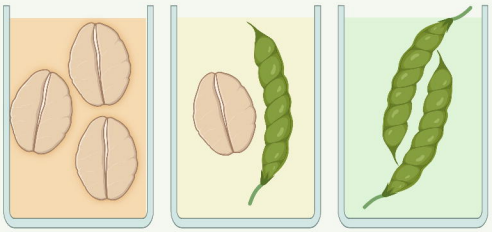
μ Maximum calorimetric growth rate
A Maximum heat production
λ Calorimetric lag time

B Single-strain combined IMC with metabolomics analysis



B. cereus Y48H10

B. subtilis DSM21393



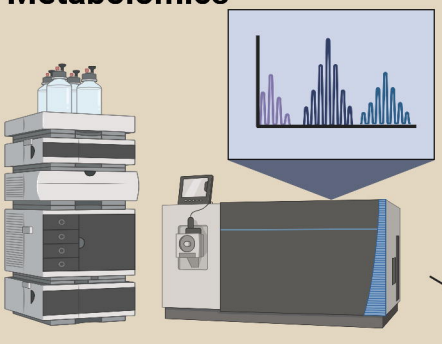
Oat drink Oat + Pea Pea protein

Isothermal microcalorimetry (IMC) + **Metabolomics**

Sampling

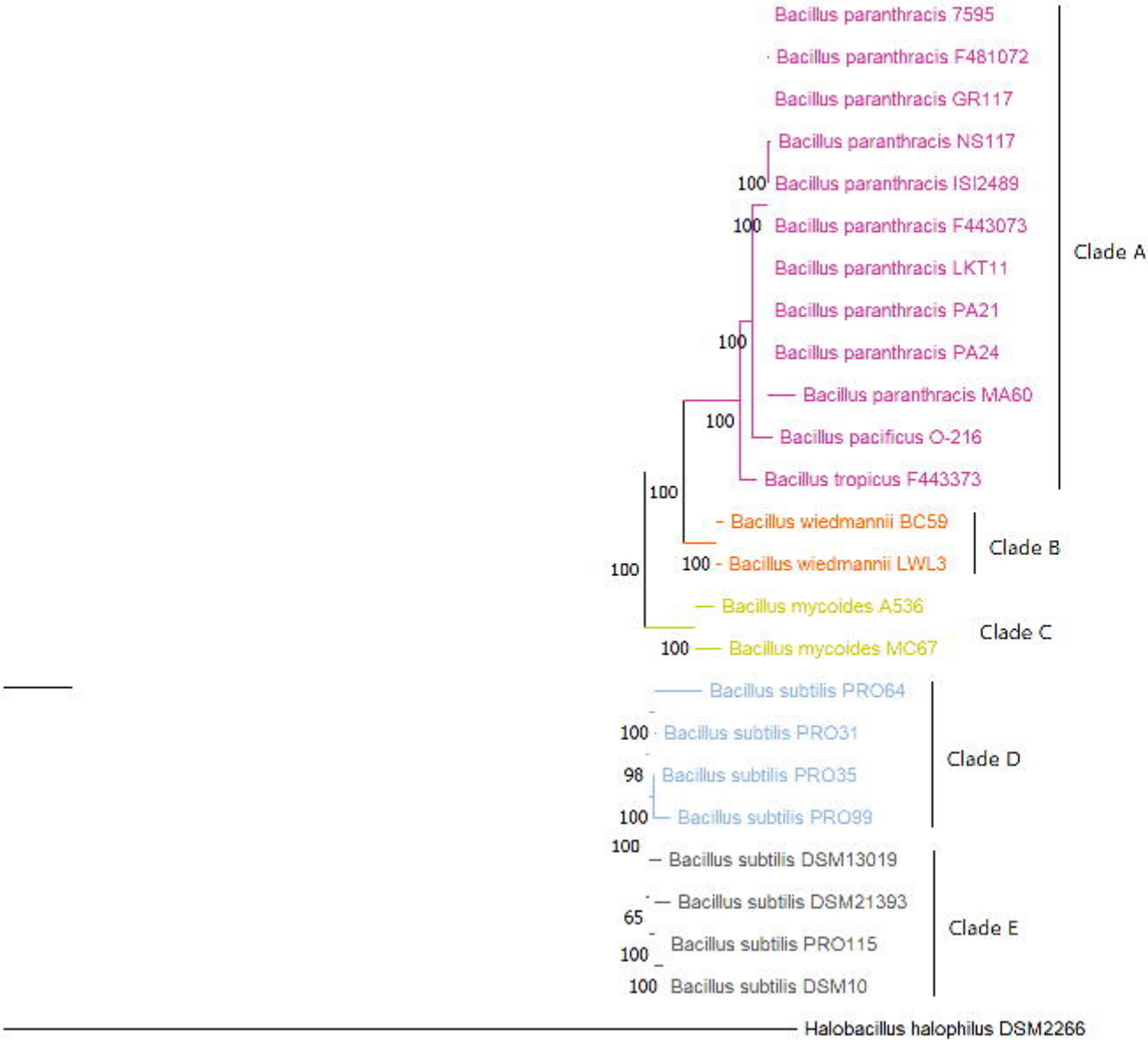
Metabolic peak (max μ) - at 8h or 11h

End of metabolism - at 24h



Organic acids, sugars, free amino acids

Cereulide (only *B. cereus*)


Bacillus paranthracis 7595
Bacillus paranthracis F481072
Bacillus paranthracis GR117
Bacillus paranthracis NS117
100
Bacillus paranthracis ISI2489
100
Bacillus paranthracis F443073
Bacillus paranthracis LKT11
Bacillus paranthracis PA21
100
Bacillus paranthracis PA24
Bacillus paranthracis MA60
100
Bacillus pacificus O-216
Bacillus tropicus F443373
Clade A
100
Bacillus wiedmannii BC59
100
Bacillus wiedmannii LWL3
Clade B
100
Bacillus mycoides A536
100
Bacillus mycoides MC67
Clade C
Bacillus subtilis PRO64
100
Bacillus subtilis PRO31
98
Bacillus subtilis PRO35
100
Bacillus subtilis PRO99
Clade D
100
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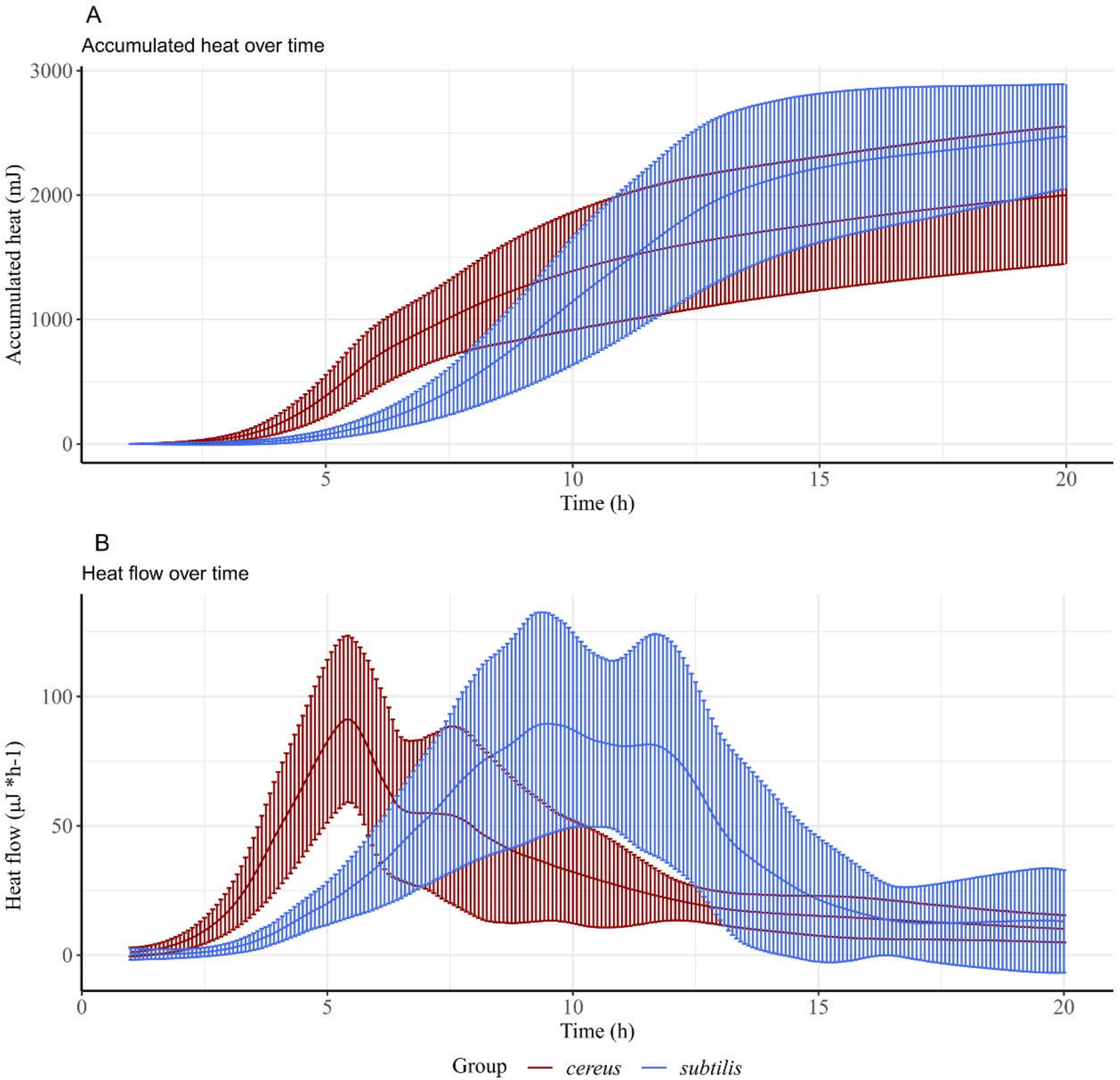
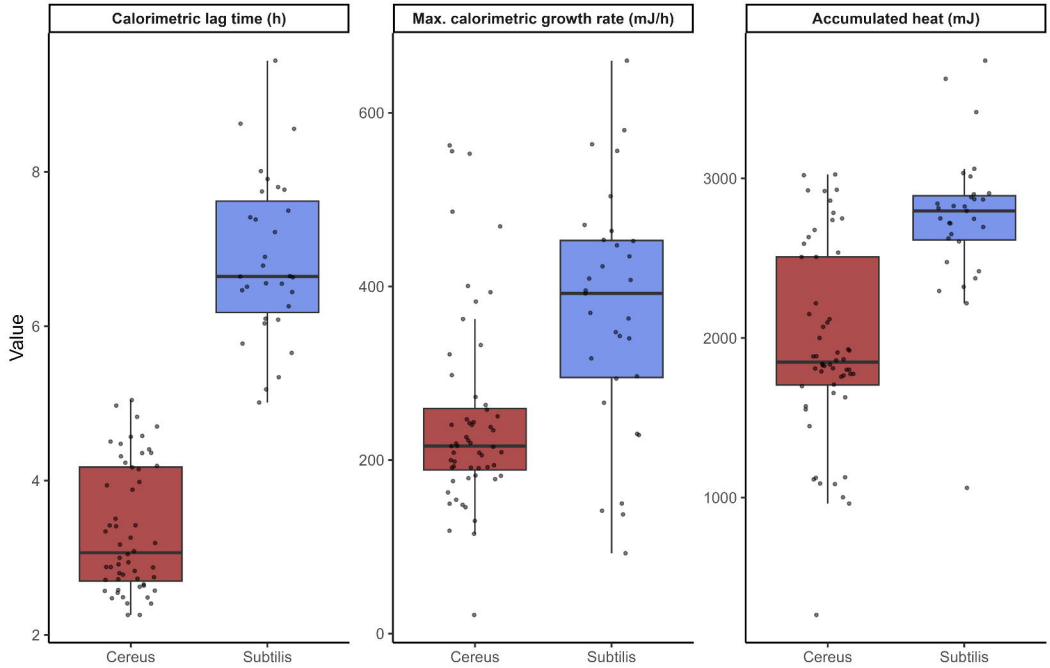
100
Bacillus subtilis DSM10
Clade E
Halobacillus halophilus DSM2266


A
Accumulated heat over time
Accumulated heat (mJ)
3000
2000
1000
0
5
10
15
20
Time (h)
B
Heat flow over time
Heat flow (µJ *h-1)
100
50
0
0
5
10
15
20
Time (h)
Group
cereus
subtilis

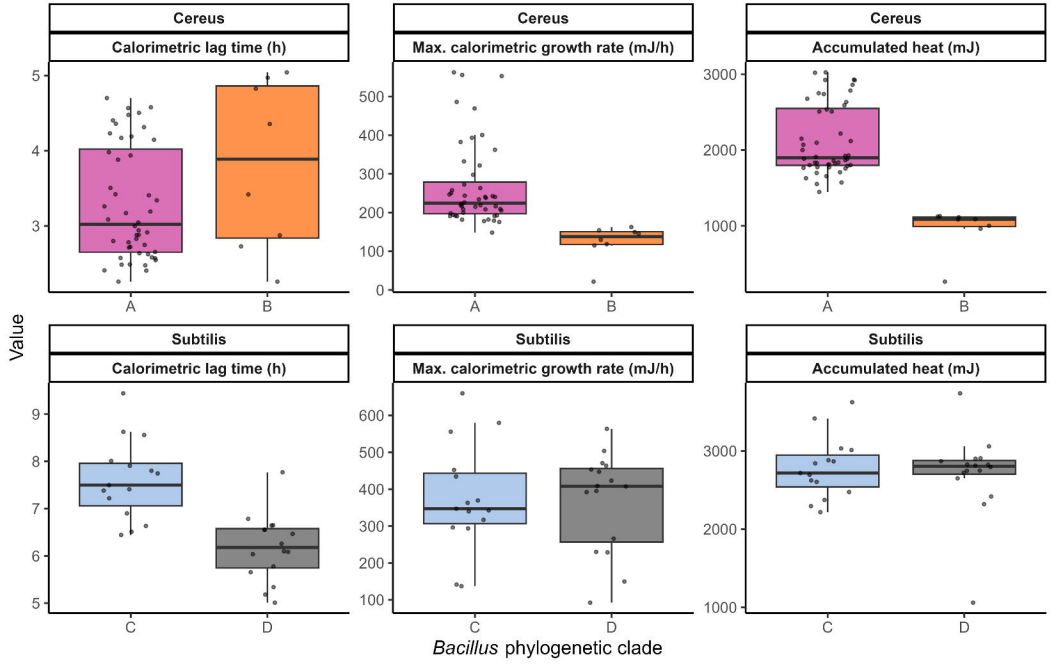
A

Comparison of Gompertz heat parameters between *Bacillus cereus* and *Bacillus subtilis*

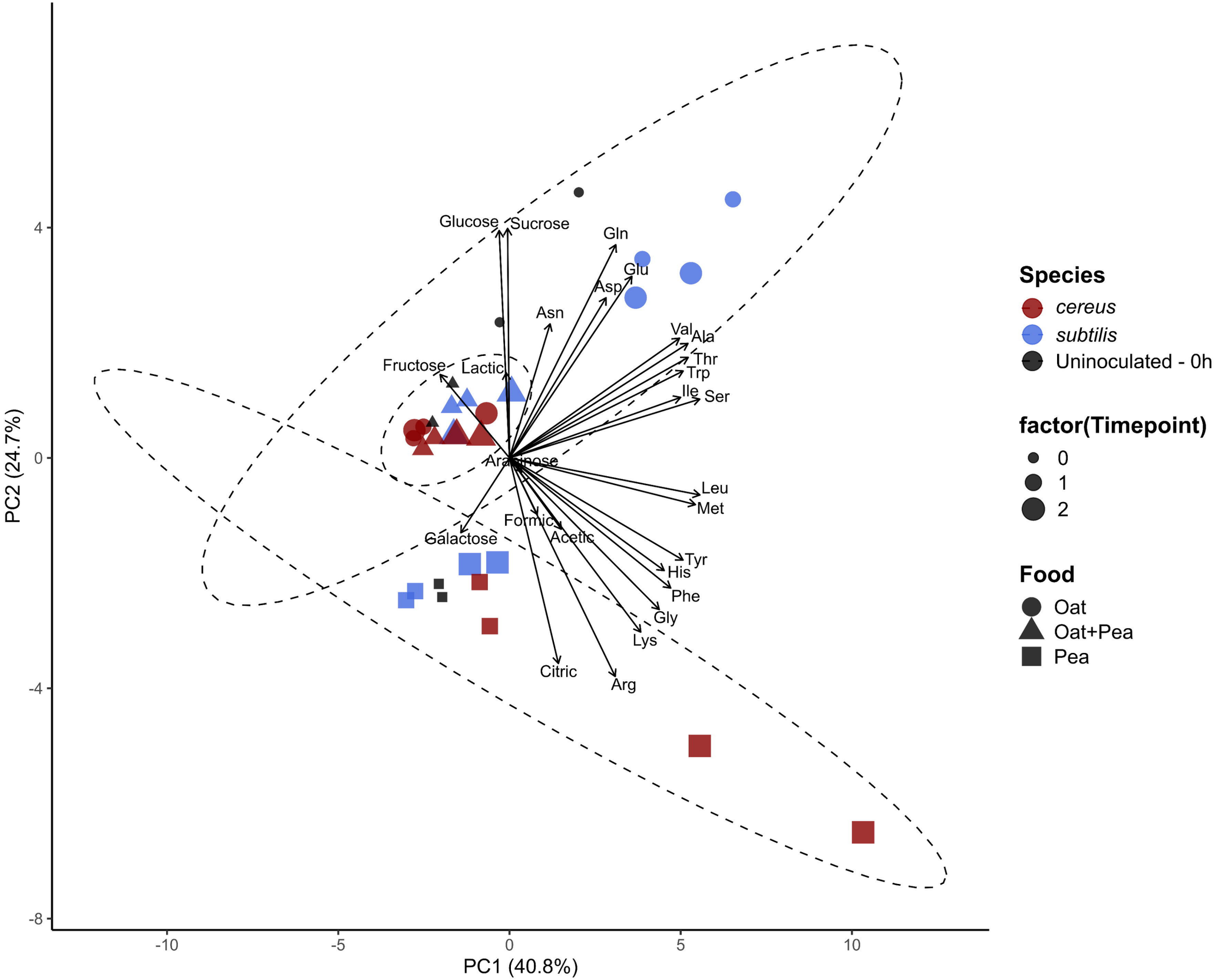


B

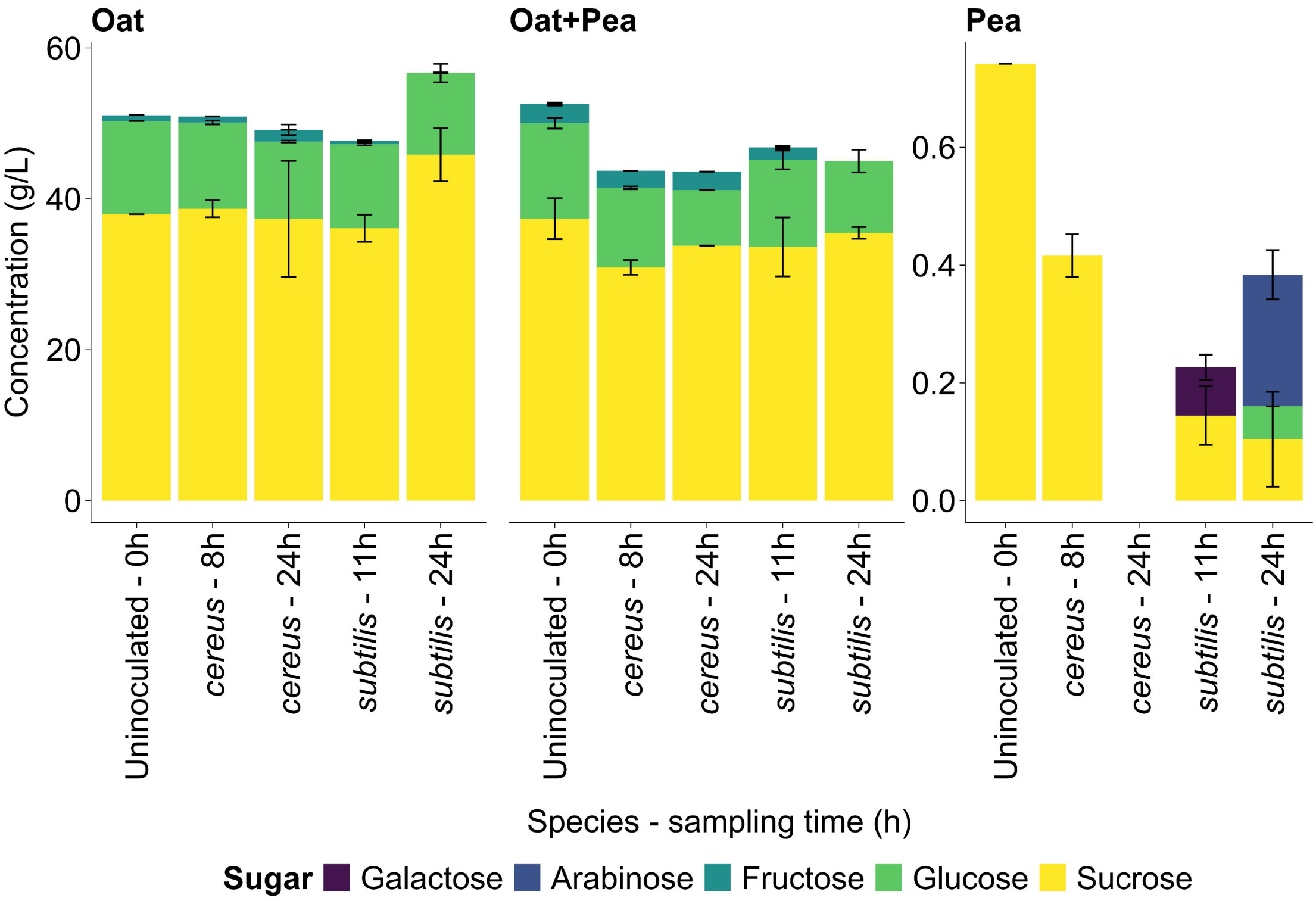
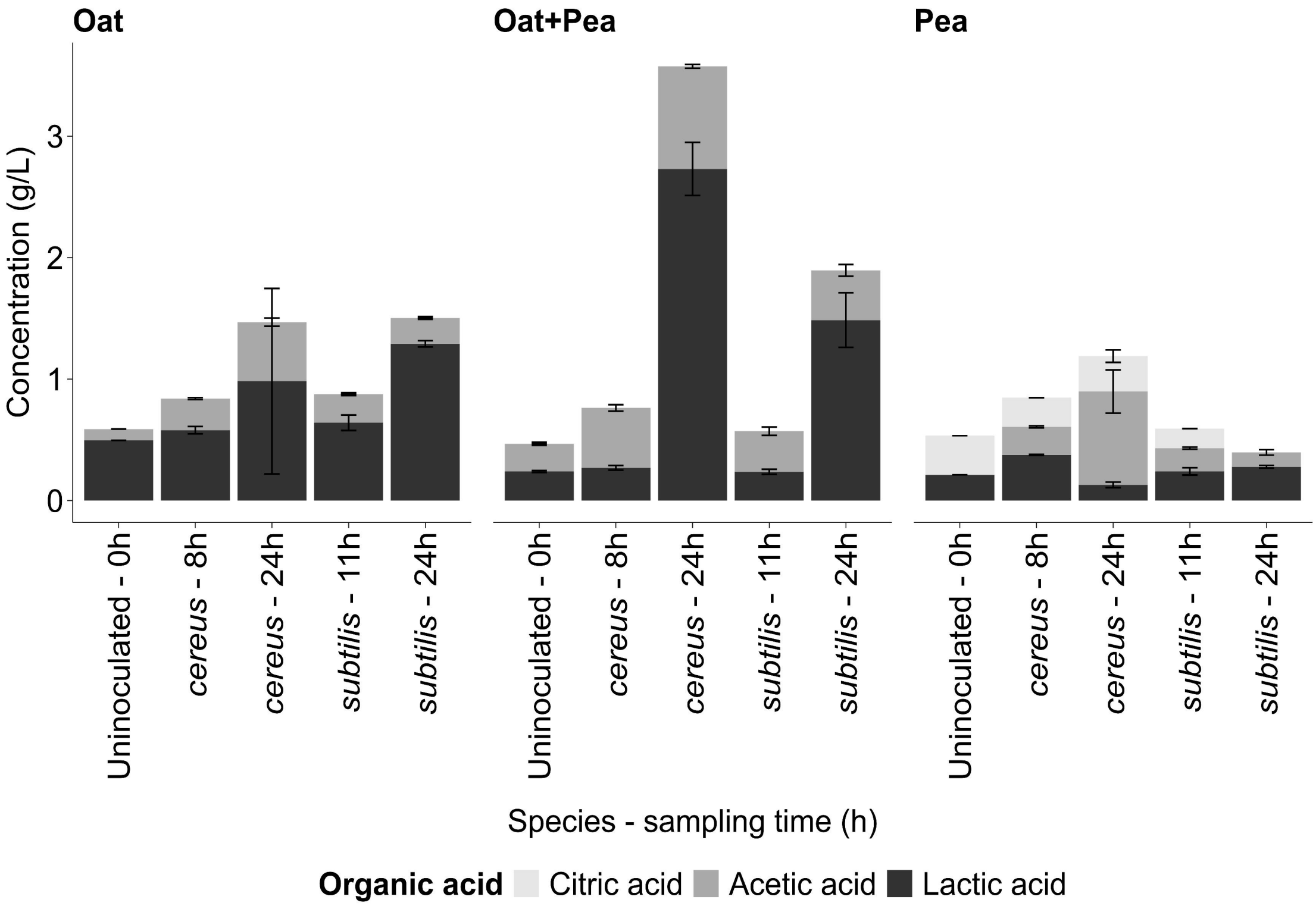
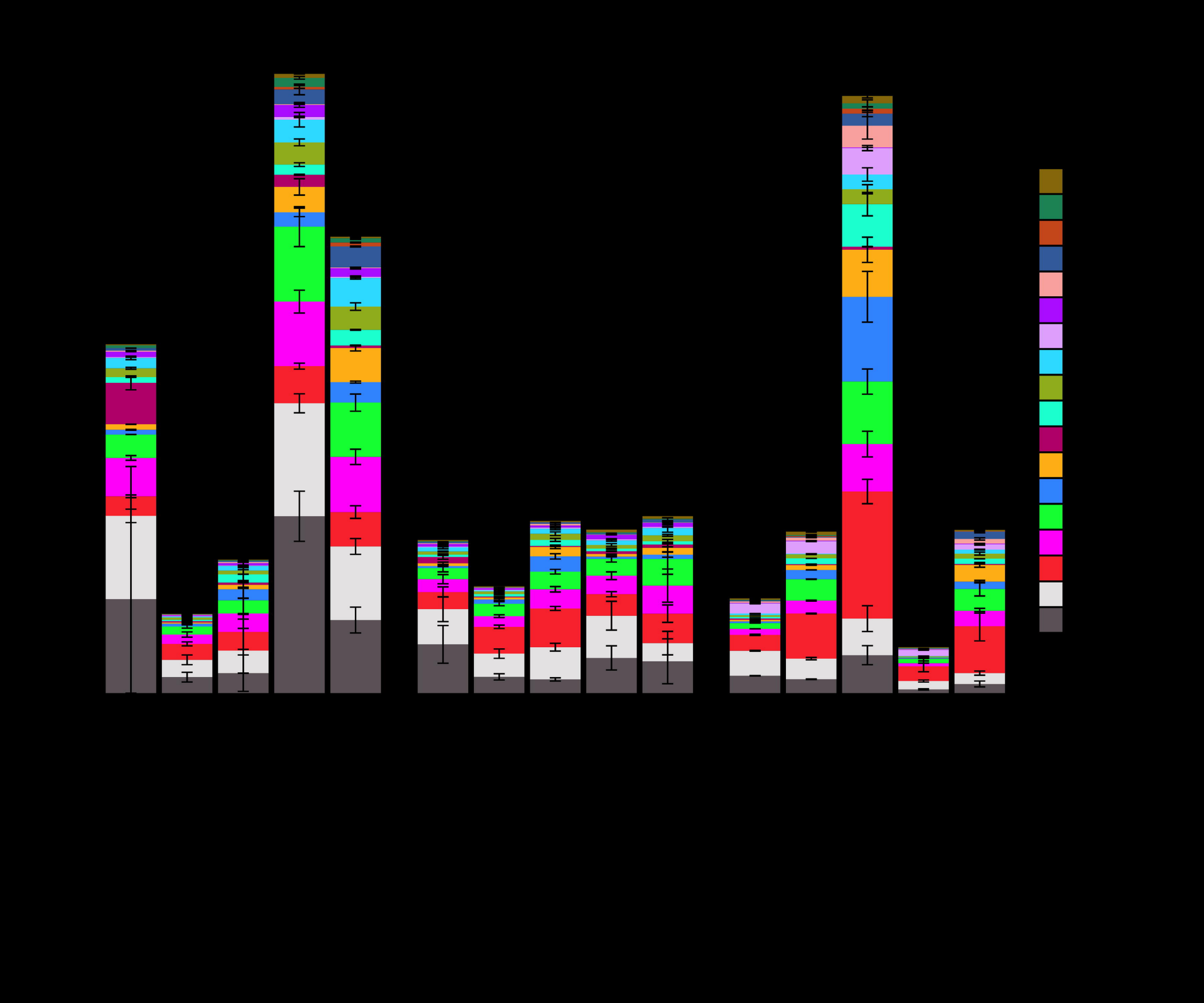
Comparison of Gompertz heat parameters within *Bacillus cereus* and within *Bacillus subtilis* clades



Combined Metabolite Analysis



(A) Sugars



(B) Organic acids