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Microbial species and strain heterogeneity affect resistance to high pressure processing

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ABSTRACT

Ten strains of each of *Listeria monocytogenes* (BHI & TSB-D), *Escherichia coli* (TSB-D), *Lactiplantibacillus plantarum* (MRS) and *Saccharomyces cerevisiae* (MEA) were exposed to High Hydrostatic Pressure (HHP; 200, 300 and 400 MPa, 10 min, 20 °C) to investigate the impact of species and strain variability in piezotolerance. *L. monocytogenes* was the most resistant, followed by *E. coli, L. plantarum* and *S. cerevisiae*. *L. monocytogenes* L6 was the most robust and NCTC 10357 the most sensitive strain, while overall survival was better in TSB-D (no glucose) than in BHI (naturally contains glucose) under similar pressures. Strains ranked differently according to their piezotolerance in the two media, while this was serotype-dependent in TSB-D. *E. coli* strain variability was detected under all conditions with O157 VT- and FAM 21843 as most robust and most sensitive respectively. *L. plantarum* FBR04 and ATCC14917 were the most resistant and sensitive (300 MPa), respectively, while for *S. cerevisiae* this corresponded to AD1890 and 028.0315 (200 MPa).

Industrial relevance: This study confirms the importance of species and strain variability in HHP. The results are relevant for the improvement of decontamination efficiency predictions, the design of validation studies and the application of hurdle technology. Knowledge of microbial inactivation and strain variability under mild HHP conditions can allow fine-tuning of hurdle technology and lead to production of safer, more affordable HHP-treated food due to decrease of operating costs.

1. Introduction

For the past few decades, the consumer demand for minimally processed and healthier food has substantially increased leading to consideration of alternative processing technologies for food preservation other than conventional heat treatment. High Pressure Processing (HPP), also known as high hydrostatic pressure processing (HHP) or cold pasteurization, is a non-thermal technology for production of safe and nutritious food with extended shelf life (EFSA, 2022). This process can provide a wide range of applications such as enzyme control, protein denaturation or even meat extraction from mollusks (Ghafoor et al., 2020). One of the main advantages of HPP is the maintenance of the sensory and organoleptic characteristics of the food product which are otherwise affected by heat processing but also the absence of additives or preservatives (clean label foods).

In principle, foods are subjected to isostatic pressures uniformly transmitted through a pressure transmitting fluid (Hugas et al., 2002). The processing parameters, such as pressure, time, and temperature, are chosen with regards to the target application. For foods, inactivation of the most relevant pathogen or spoilage microorganism per product category is targeted to ensure food safety and shelf-life extension (Sevenich & Mathys, 2018) while commercial applications range between 400 and 600 MPa for 1.5–6 min. Most common HPP treated foods include juices and Ready-to-Eat (RTE) meat products that hold 30% of the market share (Aganovic et al., 2021), respectively, however the technology is suitable for most foods. Limitations include processing of low moisture products (<40%), aerated foods and spore inactivation which requires combination of high pressures (>600 MPa) and temperatures above 100 °C.

Microbial inactivation with HPP is achieved through changes in protein conformation and biochemical reactions, DNA synthesis disruption and alterations in fluidity or loss of integrity of the cell membrane (Smelt et al., 2001). HPP efficacy depends on several food intrinsic and extrinsic factors but also factors related to microorganisms (EFSA, 2022). Resistance may vary according to the type, species, and strain of the present microorganism as well as the physiological state (EFSA, 2022). In general, eukaryotes are more susceptible than prokaryotes and Gram-positive bacteria more resistant than Gram-negative

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bacteria. Notably, strain variability can significantly impact resistance while stationary phase cells are more robust than exponential phase ones (Rendueles et al., 2011). Although knowledge of strain variability is well described for thermal processing, limited information is available on its effect at HPP (García-Graells et al., 2000). In addition, data on strain variability of food spoilage microorganisms is generally lacking which necessitates further research (Goh et al., 2007). In a recent opinion by EFSA (2022) the importance of conducting ad-hoc validation studies designed after consideration of the pathogen and product associated variability, was underpinned. These studies should consider pathogen specific variability including strain variability. Serra-Castelló et al. (2021) argued that a pool of strains with different inactivation patterns is relevant for HPP validation studies in RTE meat products to simulate the worst-case scenario on the event of contamination with *Listeria monocytogenes*. Therefore, prior knowledge on the inactivation patterns of different relevant strains is vital.

The aim of this study was to extend the current knowledge on the resistance of foodborne pathogens and spoilage microorganisms relevant to HPP treated foods. *L. monocytogenes* and *E. coli* were chosen as the most relevant targets for ensuring safety of RTE food that are frequently treated with HPP. *L. plantarum* was included in the study as a prominent spoilage organism of different foods and due to a good availability of growth and thermal inactivation data in the literature enabling comparisons (Aryani et al., 2016). *S. cerevisiae* is a good model eukaryotic organism (Bravim et al., 2010), frequently involved in food spoilage and relevant for RTE food (Basak et al., 2002). Targeting these microorganisms will give insight into how decontamination efficiencies of the technologies vary between microorganisms, which is key to determine their product application range and identify the most robust strains and/or species that can be used for optimization and upscaling to achieve optimal quality and safety control.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In this study, ten wild type (WT) strains of four microbial species, namely *Listeria, monocytogenes, Lactiplantibacillus plantarum, Saccharomyces cerevisiae, Escherichia coli,* were used (Table 1). Stock cultures of the strains were kept at −80 °C in 1.2 ml Nalgene™ General Long-Term Storage Cryogenic tubes (Life Technologies Europe, ThermoFisher Scientific) supplemented with 7% dimethyl sulfoxide (DMSO; Sigma Aldrich, Dorset, UK). To prepare the working stock cultures the following media were used: Brain Heart Infusion (BHI; NCM0016A, Neogen, UK), Tryptone Soy Broth without Dextrose (TSB-D, Scharlau, Scharlab S.L, Spain), De Man, Rogosa Sharpe broth (MRS; CM0359, Oxoid, UK) and Malt Extract broth (ME; CM0057, Oxoid, Uk). The stock cultures were streaked on agar plates made from the respective medium (BHI and TSB-D; *L. monocytogenes*, TSB-D; *E. coli*, MRS; *L. plantarum*, ME; *S. cerevisiae*) and bacteriological agar (1.5% wt/wt; Oxoid, UK). *L. monocytogenes* and *E. coli* agar plates were incubated at 37 °C for 24 h. *S. cerevisiae* at 30 °C for 24 h and *L. plantarum* at 30 °C for 48 h under microaerobic conditions. The plates were stored at 5 °C for two months, except for *L. plantarum* (three weeks).

The inoculum preparation differed per microbial species. For L. *monocytogenes* a single colony from the working stock was transferred in 3 ml BHI or TSB-D broth and incubated at 37 °C for 24 h aerobically at 120 rpm (Innova 2300, New Brunswick Scientific, UK). Subsequently, 0.1% (*v*/v) inoculum was transferred to 20 ml BHI or TSB-D broth and incubated under the same conditions for 16–18 h. The preparation of *E. coli* inoculum in TSB-D was conducted as previously described (Millan-Sango et al., 2015). *S. cerevisiae* inoculum was similarly prepared in ME broth but with incubation at 30 °C for 24 h under shaking (160 rpm), while *L. plantarum* cultures were incubated in MRS broth at 30 °C statically. In all cases, stationary cultures were obtained since higher resistance was expected. One lot per medium was used to

Table 1
Microbial species and respective strains used in this study.

Strains	Origin	Serotype/ Information	Reference
Listeria monocytogenes			
ScottA [g]	Human isolate, Massachusetts milk outbreak	4b	Aryani et al., 2015
F2365 [g]	Jalisco cheese	4b	Aryani et al., 2015
EGD-e [g]	Rabbit	1/2a	Aryani et al., 2015
LO28 [g]	Healthy pregnant carrier	1/2c	Aryani et al., 2015
L6 [g]	Milk	1/2b	Aryani et al., 2015
FBR13 [g]	Frozen endive a la creme	1/2a	Aryani et al., 2015
FBR16 [g]	Ham (after cutting machine)	1/2a	Aryani et al., 2015
10403S [f]	Human skin lesion	1/2a	Karatzas et al., 2010
NV8 [h]	Bovine carcass	1/2a	Van Der Veen et al., 2009
NCTC 10357 (DSM20600) [d]	Rabbit	1a	Murray et al., 1926
Lactiplantibacillus plantarum			
SF2A35B [g]	Sour cassava		Aryani et al., 2016
FBR22 [g]	Sausage		Aryani et al., 2016
FBR27 [g]	Sliced cooked ham		Aryani et al., 2016
FBR03 [g]	Salad dressing		Aryani et al., 2016
LMG18035 [g]	Milk		Aryani et al., 2016
FBR23 [g]	Potato salad		Aryani et al., 2016
FBR04 [g]	Cheese with garlic		Aryani et al., 2016
FBR06 [g]	Onion ketchup		Aryani et al., 2016
WCFS1 [g]	Human saliva		Aryani et al., 2016
ATCC 14917 [h]	Pickled cabbage		
Saccharomyces cerevisiae			
CBS 1544 [h]	fermenting fruit juice		Timmermans et al., 2014
AD998 [a]	Fresh cheese		ADRIA Développement
AD999 [a]	Fresh cheese		ADRIA Développement
AD1890 [a]	Fruit		ADRIA Développement
AD2913 [a]	Wine		ADRIA Développement
028.0315 [c]	Industry strain		Arla Foods amda
028.0404 [c]	Industry strain		Arla Foods amda
077.0001 [c]	Industry strain		Arla Foods amda
0106.0004 [c]	Industry strain		Arla Foods amda
130.0014 [c]	Industry strain		Arla Foods amda
Escherichia coli			
ATCC 35218 [h]	canine isolate	Non-Pathogenic, PEF resistant	Timmermans et al., 2014
NCTC 10538 (DSM 11250) [d]	human faeces	K12 O Rough H48, Verotoxin negative	Lederberg, 1951
O157 VT- [f]		VT1, VT2 negative	
K12 (BW25113) [f]			Bulut & Karatzas, 2021
BL21 (DE3) [e]			
NCTC 12900 [e]		O157:H7, Vero toxin negative	

(*continued on next page*)

Table 1 (*continued*)

Strains	Origin	Serotype/ Information	Reference
FAM21805 [b]	soft raw milk cheese	O68:H14, double heat resistance gene clusters	Peng et al., 2013
FAM21843 [b]	semi-hard raw milk	O178:H12, single heat resistance gene clusters	Peng et al., 2013
FAM21845 [b]	semi-hard raw milk cheeses	O68:H14, Multi-drug resistant, biofilm-producer, with single heat resistance gene cluster	Marti et al., 2017
FAM 22082 [b]	Dairy isolate	STEC O9 (stx1-, stx2e+, eae-)	Marti et al., 2017

[a] ADRIA Food Technology Institute, Créac'h Gwen, 29,196 Quimper, France.
[b] Agroscope, Schwarzenburgstrasse 161, 3003 Berne, Switzerland.
[c] Arla Foods amba, Arla Innovation Centre, Agro Food Park 19, Aarhus N, Denmark.
[d] Department of Plasma Biotechnology, Leibniz Institute for Plasma Science and Technology, Greifswald, Germany.
[e] University of Malta, Faculty of Health Sciences, Department of Food Sciences & Nutrition, MSD 2080, Malta.
[f] Department of Food & Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6 CE, UK.
[g] Wageningen Food & Biobased Research, Wageningen University & Research, Wageningen, The Netherlands.
[h] Food Microbiology, Wageningen University & Research, Wageningen, The Netherlands.

minimize the impact of media fluctuations.

2.2. HHP treatment

The effect of HHP treatment on the viability of *L. monocytogenes, L. plantarum, S. cerevisiae* and *E. coli* strains, was investigated. Fixed volume (1 ml) stationary phase bacterial suspensions were placed in sterile plastic stomacher bags (Seward, London, UK) with dimensions 3.5 cm × 4 cm and double sealed before HPP exposure to avoid cross contamination. The bags were submerged in the pressure transmitting medium (20% *v*/v castor oil in alcohol) of the HPP system (Stansted Fluid Power Products Ltd., Harlow, UK) that has an internal diameter of 1.8 cm and a working volume of 20 ml. Three pressure levels were applied (200, 300 and 400 MPa), for a processing time of 10 min at 20 ± 2 °C. The pressure come-up time was 1–2 min depending on the pressure while decompression was almost instant (2–3 s). The temperature in the pressure chamber was monitored using a Digital Thermometer PCE-T 390 (PCE Instruments, Southampton, UK) connected to a K-type thermocouple that was kept in contact with the pressurization liquid. Due to adiabatic heating the temperature increased with a rate of 2 °C per 100 MPa.

2.3. Microbiological analyses

The HPP-treated samples were analysed immediately after the treatment, and decimal dilutions were prepared in Maximum Recovery Diluent (MRD; Fisher Scientific, UK). All experiments were performed in biological triplicates and viability of cultures was determined by spread plating onto the respective medium (Section 2.1) before and after pressure treatment. The average initial concentrations for *L. monocytogenes* before HPP were 9.60 and 8.60 log CFU/ml in BHI and TSB-D, respectively. Similarly, *L. plantarum* reached 9.52 (0.13) log CFU/ml expect for strain FBR27 (9.04; SD 0.16). Lower initial levels of 7.52 (0.31) log CFU/ml were detected for *S. cerevisiae* expect for strains 077.0001 and 028.0315 that had a slightly higher inoculum level of around 8.00 log CFU/ml (SD ± 0.11 and 0.27, respectively). *E. coli* initial concentration was on average 8.80 (0.29) besides strain BL21 with lower initial levels of 8.18 (0.17). The plates were incubated at 30 °C (*S. cerevisiae* & *L. plantarum*) or 37 °C (*L. monocytogenes* & *E. coli*) for 48 h and colony forming units were counted. Counts were log transformed (Log CFU/ml) and the inactivation was calculated as log No – log N, where N is the HPP-treated population and No is the respective initial population. The detection limit of 2.0 log CFU/ml corresponded to 100 CFU/ml.

2.4. Statistical analysis

Statistical comparisons among different species (unpaired two-tailed *t*-test) or strains (one-way analysis of variance and Tukey test) were conducted using GraphPad Prism v 9.5.0 and comparisons were deemed statistically significant when *P-value* was <0.05.

3. Results

3.1. Relevance of strain variability in the HPP resistance of foodborne pathogens Listeria monocytogenes and E. coli

Ten *L. monocytogenes* strains covering a wide range of origins and grown to stationary phase in two different media prior to HPP exposure were assessed (Fig. 1 and **Suppl. Table ST1)**. High hydrostatic pressures of 200 MPa for 10 min had no effect (*P-value* > *0.05*) on the survival of *L. monocytogenes* regardless of the growth medium used (data not shown). Significant variation in survival (2.43 ± 2.13 average log reduction) occurred between strains at 300 MPa/10 min/20 °C when grown in BHI (Fig. 1A), with most resistant strains being L6, FBR16 and F2365 showing no significant reduction (~0.12 log reduction; **Suppl. Table ST1**). In general, a 2–4 log reduction was observed for 6 out of 10 strains, while a significant drop in bacterial numbers was noted for strain NCTC 10357 (~7 log reduction; SD; 0.28). Increase of pressure to 400 MPa resulted in higher overall inactivation with an average log reduction of 7.02 (± 0.5). Under this pressure regime, strain variability was substantially lower and was noted only between L6 and LO28, EGD-e, FBR13 and NV8 (*P-value* < *0.05*). *L. monocytogenes* NCTC 10357 exceeded the detection limit, and it was more sensitive compared to the other 9 strains.

However, when the same strains were grown in TSB-D prior to HPP exposure, the inactivation profile differed significantly at 300 and 400 MPa (*P value* < *0.05*; Fig. 1B, **Suppl. Table ST2 and Suppl. Fig. SF1**). The effect of HPP at 300 MPa was limited for most of the strains (0.35 ± 0.46 av. log reduction), except for strain NCTC 10357 that already showed a 2-log reduction. *L. monocytogenes* strain variability was observed at 400 MPa (3.01 ± 1.44 av. log reduction) with L6 being the most resistant (0.6 log reduction), NCTC 10357 the most sensitive (6 log reduction), while 2–4 log reduction was observed for the other strains. Increase of pressure within the tested range (200–400 MPa) resulted in the decrease of strain variability with BHI, while this was not seen with TSB-D. Serotype 4b strains (F2365, ScottA) had a similar inactivation in contrast to serotype 1/2a strains (FBR16, EGD-e, 10403S, FBR13, NV8) in TSB-D, while there was no serotype association with piezotolerance when strains were grown in BHI. Notably, strains L6 (serotype 1/2b) and NCTC 10357 (serotype 1a) were found to be the most resistant and sensitive, respectively, regardless of the growth conditions or pressure level.

Inactivation of *E. coli* strains grown in TSB-D was detectable at lower pressures of 200 MPa (Fig. 2 and **Suppl. Table ST3**) with the most sensitive dairy isolate FAM 21843 showing a 4.35 (0.17) log reduction followed by BL21 (1.57, SD; 0.29) and NCTC 10538 (1.12, SD; 0.25). Increase by 100 MPa resulted in increase of strain variability in HPP resistance. A difference of >5 log CFU/ml between the most sensitive strain FAM 21843 (~5.6 log reduction) and most resistant (~1 log reduction) strains O157 VT^{-} and FAM 21845 was marked. The rest of the strains showed a moderate 3–4 log reduction. *E. coli* inactivation was

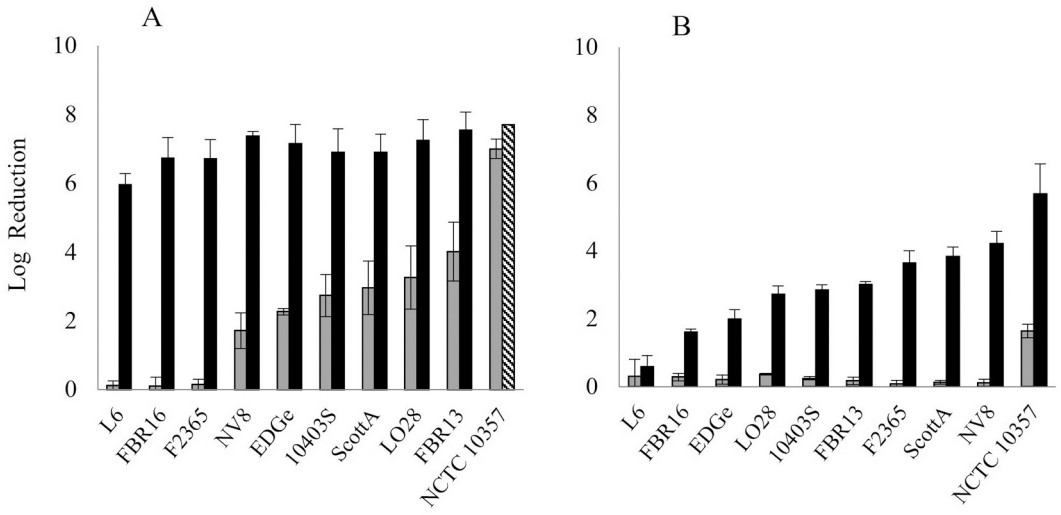

Fig. 1. Inactivation of WT *L. monocytogenes* strains after exposure at 300 MPa (**grey bars**) and 400 MPa (**black bars**) for 10 min at 20 ± 2 °C. Cultures were grown in **A)** BHI and **B)** TSB-D at 37 °C with shaking (120 rpm) until stationary phase before treatment. Experiments were conducted in triplicate, exc. FBR16, L6 and NCTC 10357 in TSB-D at 400 MPa (2). Dashed black bars indicate exceedance of detection limit at 400 MPa. Error bars indicate standard deviations.

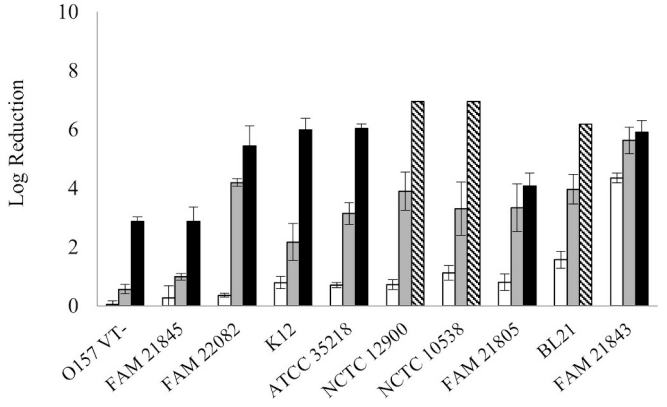

Fig. 2. Log reductions in viable numbers of WT *E. coli* strains after exposure to 200 MPa (**white bars**), 300 MPa (**grey bars**) and 400 MPa (**black bars**) for 10 min at 20 ± °C. Cultures were grown in TSB-D at 37 °C, cells were harvested in stationary phase and resuspended in TSB-D before treatment. Experiments were conducted in triplicate, exc. FAM 21843 and FAM 22082 at 200 MPa (2), BL21 at 300 MPa (2). Dashed black bars indicate exceedance of detection limit at 400 MPa. Error bars indicate standard deviations.

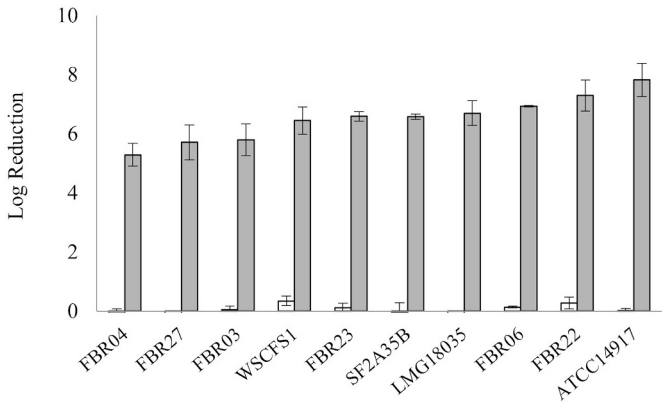

Fig. 3. Log reduction in viable numbers of wild *L. plantarum* strains after exposure to 200 MPa (**white bars**) and 300 MPa (**grey bars**) for 10 min at 20 ± °C. Cultures were grown in MRS at 30 °C, cells were harvested in stationary phase and resuspended in MRS before treatment. Experiments were conducted in triplicate exc. FBR27 at 200 MPa (2). Error bars indicate standard deviations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enhanced by increasing the pressure to 400 MPa for the same holding time but as with *L. monocytogenes* grown in TSB-D (Fig. 1A), heterogeneity in resistance was still detectable. Overall *L. monocytogenes* was significantly (*P-value* < *0.05*) more robust against HPP compared to *E. coli* when both microbial species were grown in TSB-D and subjected at 400 MPa for 10 min at 20 °C. *E. coli* strains O157 VT^{-} and FAM 21845 were again the most resistant and were further reduced with pressure increase by 2 log CFU/ml, while NCTC 10538, NCTC 12900 and BL21 exceeded the respective detection limit (≥ 6 log reduction). Interestingly, increase of pressure to 400 MPa affected less the strains FAM 21805 and FAM 21843, since roughly 1 log further reduction was achieved compared to that reported at 300 MPa.

3.2. Relevance of strain variability in the HPP resistance of food spoilage microorganisms L. plantarum and Saccharomyces cerevisiae

The resistance of *L. plantarum* strains to HPP was examined after 10 min of treatment at 200, 300 and 400 MPa (data not shown) at 20 °C (Fig. 3 and **Suppl. Table ST4)**.

Exposure at the lowest pressure hardly affected the viability of the microorganism, while at 400 MPa the detection limit of the enumeration method was reached by all strains corresponding to >7.5 log reduction (data not shown). Intermediate pressures rendered the microorganism susceptible but with measurable inactivation (6.51 ± 0.76 av. log reduction). Strains FBR03, FBR04 and FBR27 showed around 5.5 $\log_{10}$ reductions differing significantly in their response from FBR22 and ATCC 14917 that exceeded 7 $\log_{10}$ CFU/ml. It is noteworthy that *L. plantarum* strains were able to withstand the stress at 200 MPa significantly better than *E. coli* strains (*P- value* < 0.05; **Suppl. Fig. SF2**). However, the increase of pressure by 100 MPa was significantly more detrimental for *L. plantarum* with an average additional log reduction close to 6.5, in contrast to the 2-log reduction detected for *E. coli.*

Finally, *S. cerevisiae* strains were exposed to the same pressures (Fig. 4 and **Suppl. Table ST5)** and strain variability in resistance to HPP was detected at 200 MPa. Overall, 5 out of 10 strains showed around or lower than 1 log reduction with AD1890 being the most resistant (0.52, SD; 0.24), while strain 028.0315 showed the highest decrease (3.5 log reduction). The counts of AD1890 were further reduced by 2.29 logs at

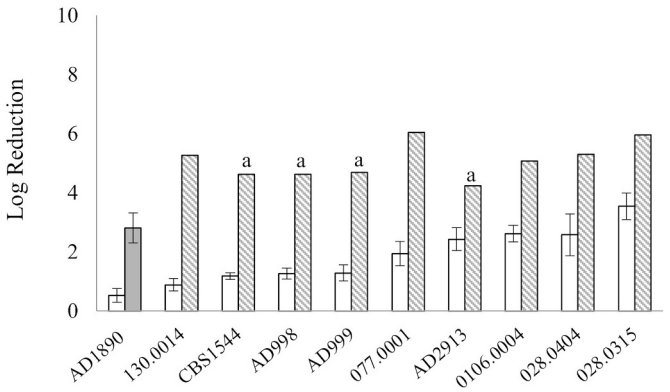

Fig. 4. Log reduction in viable numbers of WT *S. cerevisiae* strains after exposure to 200 MPa (**white bars**), 300 MPa (**grey bars**) for 10 min at 20 ± °C. Cultures were grown in ME at 30 °C, cells were harvested in stationary phase and resuspended in ME before treatment. Experiments were conducted in triplicate and error bars indicate standard deviations. Dashed grey bars indicate exceedance of detection limit at 300 MPa. [a] Indicates exceedance of detection limit for spot plating enumeration method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

300 MPa and the detection limit was reached for the other 9 strains tested that had >4.5 log reduction. At 400 MPa all strains were reduced beyond the detection limit of the respective enumeration method, corresponding to reductions above 5.5 logs. Overall *S. cerevisiae* was found to be the most sensitive (200 MPa) of all foodborne and spoilage microorganisms tested in this study (**Suppl. Fig. SF2**).

With regards to comparison between all species our results indicate that overall *L. monocytogenes* was the most resistant, while *S. cerevisiae* was the most sensitive microorganism. However, the inactivation pattern of *L. monocytogenes* can change with pre-culturing in different media and be equally or less susceptible to that of HPP-treated *E. coli* (e.g. 300 MPa) grown in TSB-D (**Suppl. Fig. SF2 & SF3**). *L. plantarum* was less susceptible compared to *E. coli* at 300 MPa but this reversed with pressure increase to 300 MPa.

4. Discussion

The efficacy of HPP treatment depends on several extrinsic and intrinsic factors, including microbial variability that have not been extensively characterized. Knowledge of microbial species and strain variability is very important for microbiological risk assessment (Den Besten et al., 2017) and can contribute to a better prediction of HPP decontamination efficiency leading to safer food production. In the present study, the variability in piezotolerance between WT strains of 4 different model microbial species was investigated in broth systems. Stationary phase cultures were selected due to higher reported resistance compared to mid-exponential cultures (Rendueles et al., 2011; Karatzas & Bennik, 2002; Tay et al., 2003; Fernandes, 2005) and subjected to relatively mild HPP treatment (200–400 MPa for 10 min). These sublethal conditions were chosen to enable the assessment of microbial species and strain variability but also provide information pertinent to use of HPP in hurdle technology in combination with other antimicrobials/stresses (Huang et al., 2014; Karatzas et al., 2001).

4.1. Variation in resistance to pressure between microbial species

L. monocytogenes was found to be the most resistant microbial species followed by *E. coli* and *L. plantarum,* while *S. cerevisiae* was the most sensitive. The increased susceptibility of *S. cerevisiae* can be explained by the higher structural complexity of eukaryotes that generally leads to increased HPP inactivation compared to prokaryotes (EFSA, 2022; Somolinos et al., 2008). Moreover, *L. monocytogenes* is a Gram-positive bacterium that are often more piezotolerant than the Gram-negative (Datta and Deeth, 2011), probably due to more robust cell envelope and lower membrane fluidity (MacDonald, 1992). This may also explain why *L. monocytogenes* was more piezotolerant in our study compared to *E. coli* when grown in TSB-D. However, we also demonstrate that *L. monocytogenes* can be equally or more susceptible than *E. coli* at 300 MPa and 400 MPa, respectively, when grown in BHI than grown in TSB-D. Several reviews have emphasized the higher piezotolerance of some *E. coli* strains (Borda & Turtoi, 2013; Cebrián et al., 2016; EFSA, 2022) attributed to other factors besides the difference in the cell envelope and membrane, such as the modification in experimental conditions and -as seen in this study- changes in growth history.

4.2. Variation in resistance to pressure between WT L. monocytogenes & E. coli strains

The 10 *L. monocytogenes* strains displayed a wide range of pressure resistance under the HPP conditions tested in this study. The log reductions varied from 0.1 to 7.0 at 300 MPa and 6.0 to 7.7 at 400 MPa in BHI for 10 min. Variability was noted when the same set of strains was subjected to HPP in the presence of TSB-D with log reductions ranging from 0.1 to 1.6 at 300 MPa and 0.6 to 5.7 at 400 MPa. This aligns with prior studies reporting diverse responses and marked variations in log reduction among different *L. monocytogenes* strains exposed to HPP either in buffers or foods (Tay et al., 2003; Alpas et al., 1999; Chen et al., 2009; Van Boeijen et al., 2008; Patterson, 2005). Likewise, Chen et al. (2009) exposed 30 *L. monocytogenes* strains at 400 MPa for 2 min (21 °C) revealing a 5.2 log difference between the most sensitive and most resistant strain and Patterson (2005) observed significant variation between 13 *L. monocytogenes* strains at 600 MPa (2 min at 20 °C) on cooked chicken, with the most resistant strains showing ~1 log reduction and the most sensitive ~5.6 log reduction. Tay et al. (2003) found substantial variability among 9 *L. monocytogenes* strains in tryptose broth, with 3 to over 4 log differences in strain inactivation at 400 MPa (1.4 to 4.3 log reduction) and 500 MPa (3.9 to 8 log reduction) for 1 min at 30 °C. Alpas et al. (1999) demonstrated viability loss variation among 9 pressurized *L. monocytogenes* at 345 MPa (5 min, 25 °C), ranging from 0.9 to 3.5 log reduction, while Van Boeijen et al. (2008) showed variability in HPP inactivation of L. *monocytogenes* strains LO28, Scott A, and EGD-e at 350 MPa (20 °C).

Strain variability in piezotolerance was noted for *E. coli* strains under all conditions tested in this study. Variability ranged from 0.6 to 5.6 log reduction and 2.8 to above 7 log reductions between the most and least robust strain at 300 and 400 MPa, respectively. A study from Alpas et al. (1999) investigating the inactivation of 6 pathogenic *E. coli* STEC (0157:H7) strains in 1% peptone solution at 345 MPa for 5 min (25 °C), showed a variation in HPP resistance ranging from 2.8 to 5.6 log reduction. Similarly, 15 min treatment of inoculated buffer with 3 different STEC strains at 600 MPa, yielded 4 and 6 log reduction, respectively (Patterson et al., 1995). Benito et al. (1999) found profound variations in the pressure resistance of 6 *E. coli* O157:H7 strains, as well as Sheen et al. (2015) that reported varying D values (0.9–25.7 min) between 39 STEC strains when treated at 350 MPa (4 °C) on ground beef. González-Angulo et al. (2021) exposed 34 *E. coli* isolates to HPP (500 MPa, 1 min) in acidified media simulating fruit juices and found variable inactivation ranging from 0.7 to above 5.0 log CFU/ml.

Differences in inactivation levels and strain variability between studies (at ambient temperatures) can be attributed to differences in the pressure/time combinations, the physiological state and history of cells, the strain selection, the inoculum preparation prior to HPP, the food matrix/medium but also the HPP equipment (**C**ome **U**p **T**ime and **C**ome **D**own **T**ime) (Valdramidis et al., 2007; Possas et al., 2017; Bucur et al., 2018; Aganovic et al., 2021; Huang et al., 2014; this study). Genetic factors might also influence resistance to HPP for some strains due to emergence of mutations (Karatzas & Bennik, 2002), presence of

prophage/inhibited prophage systems (Duru et al., 2020), presence of σ^B (Wemekamp-Kamphuis et al., 2002), or a Generalised Stress Response system conferring resistance to multiple types of stress. An important finding of this study is the high and low piezotolerance of L6 and NCTC 10357, respectively, regardless of the growth medium used. Aryani et al. (2015) found that strain L6 is the most heat resistant strain among a considerable pool of strains. Thus, the presence of a global stress response system playing a role in both technologies can be inferred, although further research (e.g. comparative genomics) needs to be conducted. Moreover, the piezotolerant strain L6 may be a relevant candidate for validation studies in food matrices (animal vs plant-based origin) as such or in a cocktail with other strains (EFSA, 2022; Bergis et al., 2021; ISO 20976-2:2022).

Variations among different strains of target pathogens should be considered, especially when designing or assessing the efficacy of HPP for food safety applications. Selection of appropriate pressure/time combination for HPP of a particular food should be based on the most relevant piezotolerant pathogen but also strain (EFSA, 2022). This study extends the knowledge on the effect of strain variability on HPP efficacy and suggests one relevant candidate for use in challenge growth and inactivation studies.

4.3. Strain variability of L. monocytogenes is affected by HPP intensity & pre-culturing conditions

L. monocytogenes strain variability in BHI significantly decreased with increase of pressure from 300 MPa to 400 MPa and most of the strains did not differ in their inactivation level. These results are in accordance with Chen et al. (2009) where variations were more profound at lower pressure levels. In general, increase of pressure causes higher inactivation and cell damage masking the strain effect, while very low pressures might not provide sufficient reduction to detect strain variability (EFSA, 2022). However, this was not the case for *E. coli* strains used in this study since substantial variability was detected under all pressure levels under the conditions tested.

Notably, pre-culturing of *L. monocytogenes* in different media can influence overall resistance but also intra-species variability as evidenced by this study, indicating that growth history is very relevant for the survival of this microbial species. Strain variability was only detected at 400 MPa when the strains were grown in TSB-D and probably higher pressures are needed to achieve comparable reductions between strains as mentioned above. Varying resistance due to the growth medium might be explained by the different constituents of the preculturing media (Patterson, 2005). Moreover, recent studies report the protective effect of *L. monocytogenes* acid adaptation before HPP exposure to an acidic buffer matrix (Rolfe et al., 2023). Inclusion of BHI in the experiments was decided on the basis that glucose fermentation during growth of *L. monocytogenes* and *E. coli* in the presence of the carbohydrate leads to pH drop and cells can be pre-adapted to mildly acidic conditions (acid habituation) that might confer increased tolerance to a subsequent similar or different type of stress (cross-protection; Ryan, Hill, & Gahan, 2008; Koutsoumanis et al., 2003; Álvarez-Ordóñez et al., 2012). Interestingly, our study demonstrated that *L. monocytogenes* had a higher overall resistance when grown in TSB-D compared to BHI and the inactivation pattern between strains differed when strain variability was detectable. For instance, strain NV8 was more resistant than FBR13 in BHI at 300 MPa but this reversed when in TSB-D and exposed at 400 MPa. The absence of glucose cannot solely explain the increased resistance since the two media differ in the origin of other constituents (e.g tryptose vs casein in BHI & soy peptones in TSB-D). However, a probable explanation could be that absence of glucose in TSB-D resulted in a higher pH value when cultures were grown overnight (7.3) compared to when grown in BHI (6.1). HPP causes electrostriction and intracellular pH imbalances leading to further drop of pH, thus cells already suspended in lower pH (here in BHI) might experience additional stress. Reduction in the pH of suspending media has been shown to greatly influence HPP inactivation (Koseki & Yamamoto, 2006) under mild HPP but also pH levels here were not low enough (~5.0) to elicit acid adaptation phenomena as previously seen with stationary phase cells (Davis et al., 1996). Therefore, further research needs to be conducted to verify whether pH alone is the determining factor in HPP inactivation after growth in different substrates. Quantitative data of this research can form the basis for selecting appropriate growth media and laboratory protocols to study microbial inactivation and induction of resistance phenomena.

4.4. Serotype is not associated with resistance to HPP

It has been documented that STEC *E. coli* strains (serotype O157:H7) have higher piezotolerance compared to other pathogenic vegetative microorganisms (Huang et al., 2014). We demonstrated that this depends on strain variability which is affected by cell history. For example, STEC 0157VT$^-$ grown in TSB-D, survived significantly better than the most resistant *L. monocytogenes* L6 at 400 MPa in BHI, however growth of L6 in TSB-D rendered the strain more resistant than STEC 0157VT$^-$ under the same conditions. Moreover, piezotolerance varied between STEC strains with 0157 VT$^-$ being the most resistant followed by NCTC 12900 and FAM 22082, while non-pathogenic *E. coli* strains can also be equally or more resistant. This is evidenced by the similar survival of FAM 21845 with O157 VT$^-$ at 300 and 400 MPa, but also lower susceptibility of K12 and FAM21805 compared to STEC NCTC 12900 and FAM 22082 at 300 and 400 MPa, respectively. This underpins the importance of careful strain selection and pre-culturing conditions in validation studies or when HPP parameters are defined. Huang et al. (2014) suggested that the required 5 log reduction objective (USDA-FSIS, 2012) to achieve a margin of safety (adulterated raw or RTE products) should be based on *E. coli* O157:H7 strains and Torres et al. (2016) mentioned *E. coli* K12 in orange juice as a good alternative indicator. We propose a HHP pre-screening with relevant strains for the investigated food matrix and selection of suitable strains for the respective validation study.

4.5. Variation in HPP resistance of food spoilage microorganisms

The food spoilage microorganisms *S. cerevisiae* and *L. plantarum* showed overall lower strain variability to HPP compared to the other microbial species under the conditions tested while variability decreased with increasing pressure as expected (Fernandes, 2005). The effect of HPP to *L. plantarum* was detrimental at 400 MPa and reduction above 6.5 $\log_{10}$ CFU/ml was detected at 300 MPa apart from strains FBR03, FBR04 and FBR27 that decreased by 5.5 $\log_{10}$ CFU/ml. Milder HPP at 200 MPa had limited effect on all strains, in line with Perrier-Cornet et al. (2005) that reported similar low inactivation levels of pressurized *L. plantarum* 103151T at 200 MPa and exceedance of 8 log reduction at 300 MPa. Similarly, *L. plantarum* LA 10–11 was resistant (<1 log reduction) at 200 MPa for 2 to 240 min of HPP (Wouters & Glaasker, 1998). Treatments at 200 MPa and pH 6.5 in milk buffer inoculated with *L. plantarum* TMW1.460 did not reduced the counts within 20 min, while additional 100 min were needed to achieve 5 log reduction at pH 6.5 according to Molina-gutierrez et al. (2002).

S. cerevisiae piezotolerance varied after exposure to 200 MPa for 10 min with the fruit isolate AD1890 showing the highest resistance (0.5 log reduction) and the dairy isolate 028.0315 being the most sensitive (3.5 log reduction). The different origin of the isolates did not correlate with the observed phenotype. At 300 MPa, almost all strains reached the detection limit (>4 log reductions), except for AD1890, which reduced by around 2.8 log CFU/ml. The lack of variability detection at higher pressures may be caused by lower initial inoculum compared to the other microbial species (around 7.5 log CFU/ml), or different enumeration methods. Scarce data in *S. cerevisiae* strain variability in the literature also makes it hard to draw a conclusion. Paniagua-Martínez et al. (2018) demonstrated ~5 log reduction at 300–400 MPa when

different *S. cerevisiae* were treated in liquids (fruit juice or media) aligning with the results reported by Sokołowska et al. (2013) (5 log reduction of *S. cerevisiae* NCFB 3191 strain in PBS at 300 MPa for 10 min or 3 log reduction in beetroot suspension). HPP of *S. cerevisiae* ATCC 2373 in meat sauce at 300 MPa led to complete inactivation in some studies (Pandya et al., 1995), while 600 MPa were needed for a similar outcome for ATCC 4113 in apple juice (Marx et al., 2011). Exposure of strain CBS 1171 at 250 MPa at 25 °C for 10 min resulted in 4 log inactivation (Perrier-Cornet et al., 2005) and Basak et al. (2002) demonstrated a 3 log reduction for *S. cerevisiae* ATCC 38618 after exposure at 250 MPa for 18 min in single strength orange juice or 2.5 log reduction after 40 min at 400 MPa in concentrated orange juice.

These results highlight the diverse piezotolerance of *S. cerevisiae* strains and underscore the importance of tailored HPP conditions for specific strains and food matrices. Therefore, the effect on viability of the two food spoilage microorganisms highly depends on the strain, the suspension medium and pressure/time combinations as described above. In general, a combination of 300–400 MPa for 10 min processing time should be sufficient to reduce the majority of the spoilage strains, however further research is needed.

Knowledge of different species and strain pressure characteristics relevant for ensuring the safety of RTE products can be a useful tool for improvement of validation studies. Incorporation of strain variability in models for prediction of microbial inactivation kinetics can be of relevance for food industry, but also for risk assessment studies. A promising piezotolerant candidate strain found here was *L. monocytogenes* L6 for which information on inactivation parameters (D value), origin, serotype, and the maximum growth rate as function of different variables (Aryani et al., 2015, 2016) have been previously described. Future research in food matrices under the same HPP conditions is important to verify whether this strain is fit for purpose, since extrapolation of results obtained in broth systems to real food systems can become a complex issue. For example, it has been observed that *E. coli* inactivation in juices was always higher than in media regardless of the HPP conditions applied (Whitney et al., 2007), while food components (fat, protein, carbohydrates) conferred protection to *L. monocytogenes* in milk and meat compared to laboratory media (Rendueles et al., 2011). Thus, microbial inactivation can be influenced by the interplay of food intrinsic factors (EFSA, 2022; Serra-Castelló et al., 2021) necessitating the use of the suggested piezotolerant strain in process validation or challenge tests of the studied food matrix.

5. Conclusions

This study confirms the importance of species and strain variability in HPP and adds to the current knowledge on the response of microorganisms upon HPP. Strain variability was influenced by pre-culturing conditions as evidenced by this study among other well-known factors. Moreover, resistant, and sensitive strains to HPP were identified which can facilitate the selection of marker strains to achieve the HPP target but can also form the basis for molecular research to understand underlying mechanisms of resistance or susceptibility. Discoveries on the function of such mechanisms governing HPP inactivation and their interaction under different matrices/conditions can lay the foundation for improvement of decontamination efficiency predictions, the better design of inactivation strategies and for the advancement of hurdle technology.

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CRediT authorship contribution statement

Theocharia Tsagkaropoulou: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Kimon Andreas G. Karatzas:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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