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Evaluation of strain variability of food microorganisms in response to decontamination by pulsed electric fields and thermal treatments

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ABSTRACT

The effect of pulsed electric fields (PEF) and thermal treatments on the inactivation of the population of 40 strains of 4 model microorganisms (*Escherichia coli*, *Listeria monocytogenes*, *Lactiplantibacillus plantarum*, *Saccharomyces cerevisiae*) were investigated. Microbial samples of McIlvaine buffer pH 7.0 were subjected to pulses with electric field strength 20 kV/cm and total specific energies (88, 136, and 184 kJ/kg). Depending on the species and strain, microorganisms exhibited various resistances. PEF microbial resistance and strain variability data were correlated to the total specific energy used. *E. coli* strains showed statistical $\log_{10}$ inactivation differences under the 88 and 136 kJ/kg but not under the 184 kJ/kg PEF treatment. In contrast, *L. monocytogenes* strains showed statistical $\log_{10}$ inactivation differences only under the 184 kJ/kg treatment. *L. monocytogenes* L6 strain was identified as the most resistant strain at PEF treatment (184 kJ/kg). This result was in accordance with the resistance under thermal treatment (62.8 °C, 30 min).

Industrial relevance: The identification of target microorganisms related to their resistance in one or more technologies can help at establishing treatment conditions that reassure food safety. Data obtained in this research show that species and strain behaviours vary and are dependent on the technology and the applied treatment conditions. Thus, the resistance exhibited by microorganisms of public health importance may be dependent on the used technology and the applied treatment.

1. Introduction

Thermal processing, with typical examples the industrial pasteurization of milk, Low Temperature Long Time (LTLT) (63 °C for 30 min) or the equivalent of High Temperature Short Time (HTST) (72 °C for 15 s) aims at reassuring food safety by inactivating pathogenic target microorganisms while minimizing any related health risks at various points of the supply chain (Golberg, Fischer, & Rubinsky, 2010; Peng et al., 2017; Tucker, 2015). Although proper application of thermal treatments can ensure the safety and preservation of food, they could also lead to loss of nutrients including vitamins and bioactive phytochemicals (Fernandez et al., 2018). Nowadays, novel processing technologies such as high hydrostatic pressure (HPP), pulsed electric fields (PEF), ultrasound (US), and non-thermal /cold plasma (NTP) have emerged as alternatives to classical pasteurization processes (Valdramidis & Koutsoumanis, 2016) However, different parameters need to be addressed including regulation, technology and cost for scaling up (Masotti et al., 2023). Up to date PEF and HPP can be found commercially in the food industry as alternative to thermal treatment for food preservation (Food Safety Authority of Ireland, 2020).The application of PEF for microbial inactivation is based on high electric field pulses of a short duration (from micro - to milli-seconds) with an electric field strength of 15–40 kV/cm (Raso et al., 2016). PEF is based on the electroporation phenomenon and to the ability of providing adequate cellular stress which leads to inactivation

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of microorganism targets (Barba et al., 2018; Peng et al., 2020), over lower temperatures than conventional treatments, shorter processing times, and the potential to be applied in both batch and continuous flow treatments (Bhat et al., 2019).

The efficiency of microbial inactivation by PEF is proven to be multifactorial and is dependent on the microbial characteristics (type of microorganism, the species, the strain, size and shape of the microorganism (Heinz et al., 2001; Lado & Yousef, 2003; Saldaña et al., 2009), growth phase of the microorganism (Álvarez et al., 2002; Rodrigo, Rodrigo, & Ruı, 2003), environmental factors (pH, electrical conductivity, a_w) (García et al., 2005; Saldaña et al., 2009) and processing parameters (such as electric field strength, treatment time, total specific energy etc.) (Saldaña et al., 2009, 2010). (Saldaña et al., 2009, 2010). For example, a study from Lee et al. (2015) on various species including *Lactobacillus brevis, Escherichia coli,* and *Saccharomyces cerevisiae*, exhibited different levels of sensitivity to PEF treatment when applied in low fat milk. More specifically, PEF treatment at a total specific energy of 200 kJ/L showed reductions of 4.4 $\log_{10}$, 4.5 $\log_{10}$, and 6.0 $\log_{10}$, respectively (Lee et al., 2015). Additionally, *L. monocytogenes* strains showed higher resistance to a treatment medium of pH 7.0 compared to *E. coli*, where opposite effects were identified for a medium of pH 4.0 (Saldaña et al., 2009). Furthermore, *L. monocytogenes* showed higher resistance to the PEF treatments (25 and 28 kV/cm) at stationary than in exponential phase (and two different incubation temperatures (4 and 35 °C) (Álvarez et al., 2002). Finally, the efficiency of microbial inactivation by PEF appeared higher after application of high electric field strength (and consequently temperature raise) or long treatment times (Wouters, Alvarez, & Raso, 2001). The increase in temperature of the medium due to the increase of the total specific energy is known as Joule's or Ohmic heating which is of great importance due to the thermal effects that can cause (Schottroff et al., 2018).

Various studies have reported the microbial inactivation of vegetative cells by PEF treatments at different food products such as high acid juices (pH < 4.6) (Timmermans et al., 2014), low acid juices (pH > 4.6) (Timmermans et al., 2014), milk (Alirezalu et al., 2020; Lee et al., 2015), and liquid whole egg (Jin et al., 2009). However, from an industrial perspective, the use of PEF has been only regulated for commercial pasteurization of juices by the Food and Drug Administration (FDA), setting a 5 $\log_{10}$ reductions requirement for the most resistant food pathogens (Food & Administation, 2000). Pasteurization processing for a specific product is mainly based on challenge tests, legislation and experience (Van Asselt & Zwietering, 2006). For challenge tests, the use of multiple target strains (i.e., a mixture of up to 5) or a selected robust target strain following screening inactivation studies is suggested (National Advisory Committee on Microbiological Criteria for Foods, 2010). Thus, the selection of a microbial target strain is crucial for designing and conducting a specific challenge test study and establishing a successful heat treatment, e.g., pasteurization process, for a given food product (Lianou & Koutsoumanis, 2013b).

Various studies have been reported on strain variability in thermal resistance for different microbial species including *E. coli* (Whiting & Golden, 2002), *Salmonella enterica* (Lianou & Koutsoumanis, 2013a), *Listeria monocytogenes* (Aryani et al., 2015a), *Lactiplantibacillus plantarum* (Aryani, den Besten, & Zwietering, 2016), and *S. cerevisiae* (Montanari et al., 2019). Similarly, PEF treatment microbial variability resistance studies have been carried out for L. *monocytogenes* (Lado & Yousef, 2003; Saldaña et al., 2009), *Staphylococcus aureus* (Rodríguez-Calleja et al., 2006; Saldaña et al., 2009), *E. coli*, and *Salmonella Typhimurium* (Saldaña et al., 2009). However, there is lack of information regarding the strain resistance variability of similar species in different PEF conditions and how this compares with thermal treatments.

In view of this, the current study aims to evaluate the strain resistance variability of different microbial species when present in a liquid system of pH 7.0 under different PEF conditions. Furthermore, the resistance variability of the same strains when subjected to thermal treatment will also be assessed in an attempt to compare the efficacy of PEF and thermal technologies. In order to achieve this, strains from two pathogenic (*E. coli, Listeria monocytogenes*) and two spoilage (*L. plantarum*, and *S. cerevisiae*) microorganisms will be utilised.

2. Material & Methods

2.1. Strains, media & culture conditions

Ten strains of each species of four model food microorganisms including: *E. coli*, *L. monocytogenes, L. plantarum,* and, *S. cerevisiae* (coming from different origins (Table 1) were used during this study. Microbial strains were sticked at −80 °C in glycerol (30:70 *v*/v) (Merck, Germany) . *E. coli* and L. *monocytogenes* stock cultures were re-activated by inoculation onto solid media of Tryptic Soya Agar (Scharlab Spain) plates, whereas L. *plantarum* by inoculation onto de Man Rogosa Sharpe Agar (MRSA, Oxoid, United Kingdom) sealed plates and *S. cerevisiae* onto Malt Extract Agar (MEA,Oxoid, United Kingdom) plates. The plates were incubated for 24 ± 2 h at 37 °C, 48 ± 2 h at 30 °C, and 36–48 h at 30 °C, respectively, to obtain single colonies. Microbial cultures were stored at 4 °C and were refreshed monthly.

2.1.1. E. coli

The primary inoculum was prepared by selecting a single colony from the re-activated plate and incubating it in Tryptic Soya Broth without dextrose TSB-D (Scharlab, Spain) at 37 °C for 24 ± 2 h. A subculture was also prepared in TSB-D (1% *v*/v) at the same temperature and for 17–18 h allowing the bacteria to reach the stationary phase (10^8–10^9 $\log_{10}$ CFU/mL). After incubation, the culture was centrifuged (3000 x*g*) for 20 min and washed with Phosphate Buffer Saline solution (PBS, Oxoid United Kingdom). Hereafter, the pellet of each strain was resuspended: a) in citrate-phosphate Mcilvaine buffer (combination of citric acid and disodium hydroxide phosphate), for the PEF experiments, of pH 7.0 ± 0.1 (Dawson, Elliot, Elliot, & Jones, 1974),(measured at 20–25 °C); with an adjusted conductivity of 2 ± 0.05 mS/cm (measured at 20–25 °C) which was checked by a conductivity probe which was checked Alhborn conductivity probe (Alhborn, Almemo, Germany), and b) in TSB-D for the thermal treatments.

2.1.2. L. monocytogenes

The primary inoculum was prepared by selecting a single colony from a stock culture which was incubating in (TSB-D) at 37 °C with shaking at 120 rpm for 24 ± 2 h. A subculture was then prepared in TSB-D (1% *v*/v) at the same temperature and shaken for 17 ± 1 h allowing the bacteria to reach the stationary phase (10^8–10^9 CFU/mL). After incubation, the culture was centrifuged (3000 ×*g*) for 20 min and washed with (PBS). Similarly, harvested pellets were resuspended: (as above 2.1.1) for PEF and thermal treatments.

2.1.3. L. plantarum

The primary inoculum was prepared by selecting a single colony from the stock culture which was incubated in de Man Rogosa Sharpe Broth (MRS Broth, Oxoid, United Kingdom) at 30 °C for 24 ± 2 h. A subculture was also prepared in MRS Broth (0.5% v/v) at the same temperature for 17 ± 1 h allowing the microorganisms to reach the stationary phase (10^8–10^9 CFU/mL) as previously described by (Aryani et al., 2016). After incubation, the culture was centrifuged (3000 ×*g*) for 20 min and was washed with (PBS). The pellet of each strain was resuspended: a) (as above 2.1.1) for the PEF experiments, and b) in de Man Rogosa Sharpe Broth (MRS Broth, Oxoid, United Kingdom) for the thermal treatment treatments.

2.1.4. S. cerevisiae

The primary inoculum was prepared by selecting a single colony from the stock culture which was incubating it in Malt Extract Broth (MEB, Oxoid United Kingdom) at 30 °C with shaking at 160 rpm for 24 ± 2 h. A subculture was then prepared in MEB (1% v/v) at the same

Table 1
Escherichia coli, Listeria monocytogenes, Lactiplantibacillus plantarum and Saccharomyces cerevisiae strains.

E. coli

Strain	Serotype	Origin/Information	Source	Reference
NCTC 12900	(O157:H7)	ATCC 700728; WDCM 00014, E81186, NCTC library	UoM	(Millan-sango et al., 2015)
BL21 (DE3)	–	New England Biolabs	UoM	(New England Biolabs, 2022)
K12	(OR:H48:K-)	NBRP *E. coli* Japan	UoR	(Bulut & Karatzas, 2021)
O157 VT-	(O157 VT-)	CPHL E1567630 Chapman P21432	UoR	–
NCTC 10538 (DSM 11250)	(K12 O rough H48)	Human, human faeces	INP	(UK Health Security Agency, 2024)
ATCC35218	(O157:H7)	PEF-resistant surrogate from *E. coli* O157:H7	WR	(Timmermans et al., 2014)
FAM 21805	(O68:H14)	Raw milk cheese (double heat resistance gene clusters)	AG	(Peng et al., 2013)
FAM 21843	(O178:H12)	Raw milk cheese (single heat resistance gene cluster)	AG	(Peng et al., 2013)
FAM 21845	(O68:H14)	Raw milk cheese (Multi-drug resistant, biofilm-producer, with single heat resistance gene cluster)	AG	(Marti et al., 2017)
FAM 22082	(STEC O9) (stx1-, stx2e+, eae-)	Dairy isolate	AG	–

L. monocytogenes

Strain	Serotype	Origin/Information	Source	Reference
Scott A	4b	Human isolate from Massachusetts milk outbreak	WU	(Aryani et al., 2015a)
F2365	4b	Jalisco cheese	WU	(Aryani et al., 2015b)
EGD-e	1/2a	Rabbit	WU	(Aryani et al., 2015a)
LO28	1/2c	Healthy pregnant carrier	WU	(Aryani et al., 2015b)
L6	1/2b	Milk	WU	(Aryani et al., 2015a)
FBR13	1/2a	Frozen endive a la creme	WU	(Aryani et al., 2015b)
FBR16	1/2a	Ham (after cutting machine)	WU	(Aryani et al., 2015a)
10403S	1/2a	Human skin lesion	UoR	(Boura et al., 2016)
NV8	1/2a	Bovine carcass	WR	–
NCTC 10357 (DSM 20600)	1a	Rabbit	INP	(UK Health Security Agency, 2023)

L. plantarum

Strain	Serotype	Origin/Information	Source	Reference
SF2A35B	–	Sour cassava	WU	(Aryani et al., 2016)
FBR22	–	Sausage	WU	(Aryani et al., 2016)
FBR27	–	Sliced cooked ham	WU	(Aryani et al., 2016)
FBR03	–	Salad dressing	WU	(Aryani et al., 2016)
LMG18035	–	Milk	WU	(Aryani et al., 2016)
FBR23	–	Potato salad	WU	(Aryani et al., 2016)
FBR04	–	Cheese with garlic	WU	(Aryani et al., 2016)
FBR06	–	Onion ketchup	WU	(Aryani et al., 2016)
WCFS1	–	Human saliva	WU	(Aryani et al., 2016)
ATCC14917	–	Pickled cabbage	WR	–

S. cerevisiae

Strain	Serotype	Origin/Information	Source	Reference
AD998	–	Fresh cheese	ADRIA	–
AD999	–	Fresh cheese	ADRIA	–
AD1890	–	Fruit	ADRIA	–
AD2913	–	Wine	ADRIA	–
CBS1544	–	Fermenting fruit juice	WR	(Timmermans et al., 2014)
0106.0004	–	Industry strain	ARLA	–
028.0404	–	Industry strain	ARLA	–
028.0315	–	Industry strain	ARLA	–
130.0014	–	Industry strain	ARLA	–
077.0001	–	Industry strain	ARLA	–

ADRIA: ADRIA Food Technology Institute, Créac'h Gwen, 29,196 Quimper, France.
AG: Agroscope, Schwarzenburgstrasse 161, 3003 Berne, Switzerland.
ARLA: Arla Foods amba, Arla Innovation Centre, Agro Food Park 19, Aarhus N, Denmark.
INP: Department of Plasma Biotechnology, Leibniz Institute for Plasma Science and Technology, Greifswald, Germany.
UoM: University of Malta, Faculty of Health Sciences, Department of Food Sciences & Nutrition, MSD 2080, Malta.
UoR: Department of Food & Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6 CE, UK.
WR: Wageningen Food & Biobased Research, Wageningen University & Research, Wageningen, The Netherlands.
WU: Food Microbiology, Wageningen University & Research, Wageningen, The Netherlands.

temperature and shaking (24 ± 2 h) allowing the microorganisms to reach the stationary phase (10^7–10^8 CFU/mL). After incubation, the culture was centrifuged (3000 ×*g*) for 20 min and washed with PBS. Similarly, with above, the pellet of each strain was resuspended: Hereafter, the pellet of each strain was resuspended a) (as above 2.1.1) for the PEF experiments, and b) Malt Extract Broth, for the thermal treatment treatments.

2.2. PEF treatments

The EPULSUS-PM-10 (2 kW, Energy Pulse System Lisbon, Portugal) pulse generator used in this work is capable of providing monopolar square wave pulses (1–200 μs) a maximum output voltage of 10 kV, a current of 200 A and frequencies of up to 200 Hz. The system was used in a continuous mode with a flow rate of 5 L/h, pumping the sample with a peristaltic pump equipped with an 8-roller rotor through a parallel titanium electrodes chamber of 0.4 cm gap (3 cm length, 0.5 cm width) (See Fig. S1) leading to a residence time of 0.43 s. Samples were tempered to 20 °C (inlet temperature) using coil immersed in a water bath before treatment and cooled down on cooling coil to ambient temperature after PEF treatment. The residence time within the pipeline connecting the treatment chamber's exit to the cooling heat exchanger was 0.5 s. During this short interval, the sample's temperature decreased by several degrees (ranging from 4.5 to 7.5 °C), subject to the exit temperature from the treatment chamber. The sample's residence time in the cooling heat exchanger was 4.38 s, and in all instances, the exit temperature remained below 20 °C (see Fig.S2).The actual voltage during processing was measured with a high-voltage probe (Tektronix, P6015A, Wilsonville, Oregon, United States) connected to an oscilloscope (Tektronix, TDS 220). Inlet and outlet temperature measurements of the chamber as well as inlet and outlet temperatures of the heat exchanger that was used for the cooling of the sample were monitored with thermocouples type K (Ahlborn, Holzkirchen, Germany). Square pulses of 5 μs width at an electric field strength of 20 kV/cm and 3 different repetition rates (30, 47, 64 Hz) were applied, corresponding to outlet temperatures of 41 ± 0.3 °C, 52.5 ± 0.5 °C and 64 ± 0.7 °C (see Fig. S2), respectively. The duration of the treatments derived from theoretical values (residence time x frequency x pulse width) leading to 64.5, 101, 137.5 μs, respectively. The total specific energy for the three conditions tested ((88,136, 184 kJ/kg) was estimated by calculating the temperature increase during pulses under presumed adiabatic conditions (Heinz et al., 2001) according to the following equation:

$$W = (T_{outlet} - T_{inlet}) \times C_p \quad (1)$$

where T_{outlet} is the temperature of the sample after the PEF treatment, T_{inlet} is the temperature of the sample just before entering the treatment chamber, and C_p is the specific heating capacity (C_p water: 4.186 kJ /kg in 20 °C).

2.3. Thermal treatment

In this study, a combination of temperature and time close to the classical pasteurization process (63 °C, 30 min) (Tucker, 2015) was used on an experimental set-up similar to a domestic batch approach (Tembo, Holmes, & Marshall, 2017). Thermal treatments were applied on 6 mL samples in falcon tubes (15 mL) using a digital thermostatic water bath (601/3, Nahita, United Kingdom) at 63 °C. The sample took 8 min to reach the 62.8 ± 0.1 °C temperature, and was subjected to the treatment for 30 min. Temperatures were constantly monitored with a thermocouple (OM-HL-EH-TC, OMEGA). Following the applied treatments samples were immediately cooled to ambient conditions.

2.4. Enumeration of viable cells

The untreated and treated cell suspensions were diluted in (PBS) and 0.1 mL of the diluted sample was used for plating (pour plating method for PEF experiments and surface spread plating for thermal experiments). Three biological replicates were performed for each strain and for each biological replicate two technical replicates were performed. The media used for the enumeration of the viable cells were (TSA) for *E. coli* and L. *monocytogenes*, (MRSA) for L. *plantarum* and (MEA) for *S. cerevisiae*. Nutrient agars for enumerating *E. coli* and *L. monocytogenes* samples were incubated for 24 ± 2 h at 37 °C while incubation for L. *plantarum* and *S. cerevisiae* samples was for 48 ± 2 h at 30 °C. After incubation, plates were counted with an improved image analyser automatic counter (Protos, Analytical Measuring Systems, Cambridge, UK) as previously described (Condón et al., 1996) for PEF treatment experiments, while 30–300 colonies were counted for thermal treatment experiments. Colony counts corresponded to the viable microorganisms expressed as colony form unit per millilitre (CFU/mL) or its decimal logarithm ($\log_{10}$ CFU/mL). The survival fraction was determined by dividing the number of microorganisms that persist after the treatment (N_t) with the initial count of viable cells (N_0).

$$log_{10}reduction = log_{10}\left(\frac{N_t}{N_0}\right) \quad (2)$$

2.5. Statistical analysis

Three biological samples were analysed for each condition. The data values were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey tests were performed to compare the mean of $\log_{10}$ cycles reduction of each microorganism using GraphPad Prism 10.0.2 (GraphPad Software, San Diego, California, United States). *Multiplicity adjusted P value* was calculated for each comparison as described by (S.Paul Wright, 1992; Peter & Randall, 2000). Differences between $\log_{10}$ cycles reduction values lower than $p < 0.05$ were considered as significant. Similar analysis was performed for comparing the mean of $\log_{10}$ reduction of all strains for each microorganism under the different total PEF specific energies.

3. Results and discussion

The inactivation data of the different studied microbial strains following the application of PEF treatments (refer to section 2.2) were collected. In this investigation, a continuous system was deliberately chosen to simulate an industrial process similar to previous studies (Gurtler et al., 2010; Waite-Cusic, Diono, & Yousef, 2011). This approach deviates from prior examinations of strain variability resistance under PEF treatments, which predominantly employed batch mode systems (Lado & Yousef, 2003; Saldaña et al., 2009). Differences in microbial resistance were apparent, contingent upon the microbial characteristics (e.g., microorganism types, strains) and processing parameters (specifically total specific energy input). Notably, as the strain $\log_{10}$ reductions were contingent upon processing parameters, the resistance ranking of the strains and the statistical differences between the strains exhibited variability (Fig. 1).

3.1. Microbial strain variability under PEF treatments

E. coli strains K12 and BL21(DE3) were identified as the most resistant strains for PEF treatments of 88 kJ/kg ($\log_{10}$ 1.68 and 1.83) and 136 kJ/kg ($\log_{10}$ 2.34 and 2.19), respectively. The statistical significance between the $\log_{10}$ reductions ofresistant strains (K12 and BL21) and the most sensitive strains (see Table S1) were identified *($p \leq 0.0001$)* for the aforementioned conditions (88 and 136 kJ/kg) . However, a prior study conducted on 15 strains of *E. coli* K12 (ATCC 23716) under PEF treatments (22 kV/cm, 45 °C, 59 μs and 20 kV/cm, 55 °C, 70 μs) in orange juice at pH 3.4 (±0.1) revealed that *E. coli* K12 may not be the suitable microbial target for acidic pH conditions due to its high sensitivity under low pH and PEF treatments when compared with *E. coli* O157:H7 and surrogate *E. coli* ATCC 35218 (Gurtler et al., 2010). Under

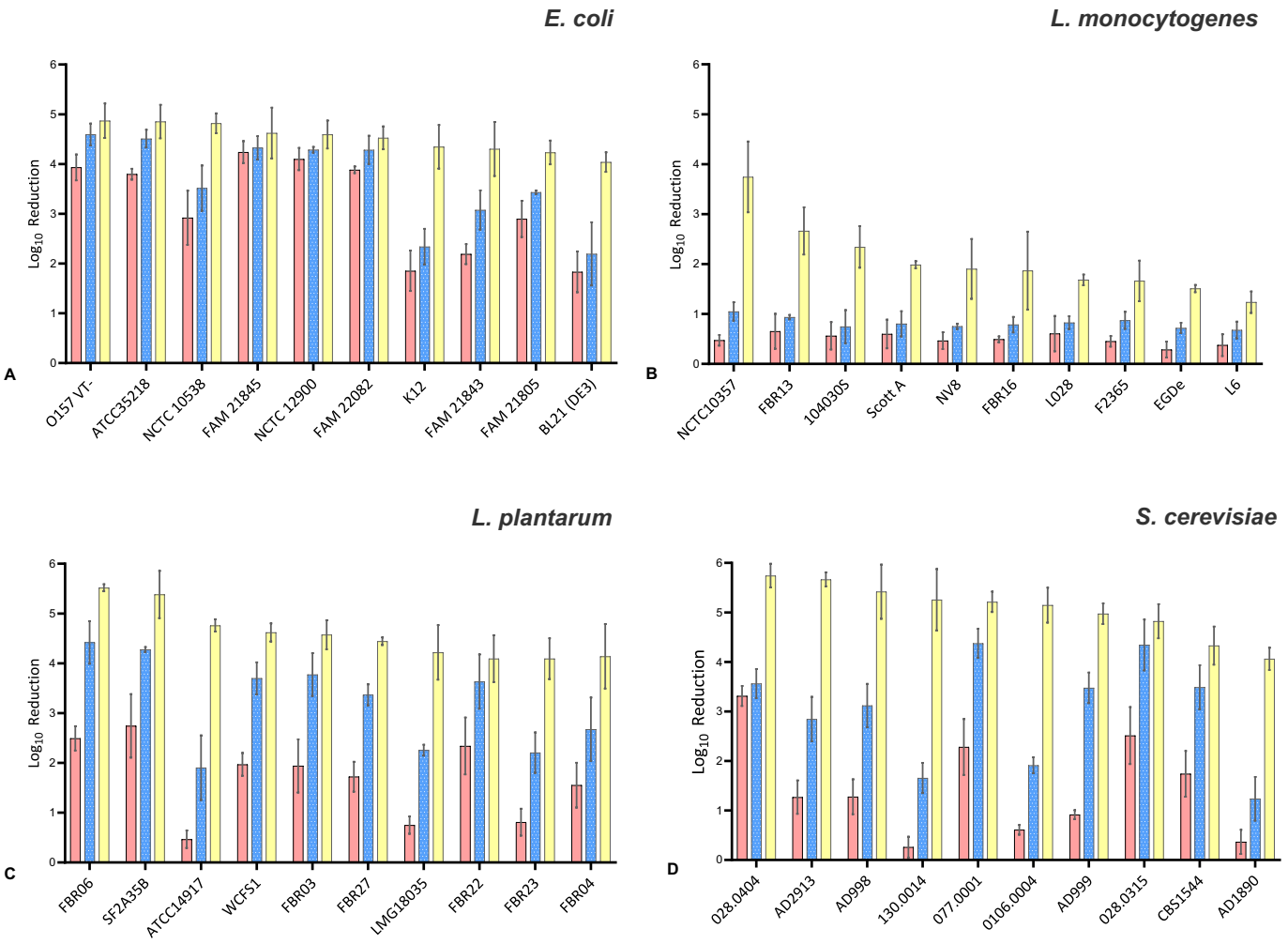

Fig. 1. Log_{10} reductions of (A) *E. coli*, (B) *L. monocytogenes*, (C) *L. plantarum* and (D) *S. cerevisiae* strains under 3 different PEF total specific energies: a) 88 kJ/kg b) 136 kJ/kg c) 184 kJ/kg. Experiments were performed in 3 biological replicates. Bars represent the standard deviations of these measurements.

the most intensive PEF treatment (184 kJ/kg), there was no statistical difference between the different *E. coli* strains (see Table S1), and all showed a log_{10} reduction >4.04. Additionally, *E. coli* sensitive strains (FAM 21845, NCTC 12900, O157 VT-, FAM22082, ATCC35218) showed a difference on the inactivation of ≤1.06 log_{10} at 184 kJ/kg PEF (20 kV/cm,137.5 μs) when compared to 88 kJ/kg PEF (20 kV/cm, 64.5 μs). Similarly, an investigation for *S. typhimurium* 878, showed a 2.0 log_{10} reduction during PEF treatment at 20 kV/cm, 45 μs with pH 7.0 while after 150 μs the inactivation was higher only by 1 log_{10} (Delso et al., 2020). This result highlights the non-linear nature of log_{10} reduction during PEF treatments (Delso et al., 2020), and suggests that a similar pattern may apply to the previously mentioned sensitive strains of *E. coli*.

L. monocytogenes was identified as the most resistant microorganism under PEF treatments. All L. *monocytogenes* strains for the first two parameters (88 and 136 kJ/kg), showed a log_{10} reduction <1.05 and no statistical difference was identified between the strains. In general, Gram positive microorganisms are considered more resistant to the technology as a result of their thicker cell wall and stiffening lipoteichoic acids (Lado & Yousef, 2002). Additionally, this resistance may be a result of the size and shape of L. *monocytogenes* in comparison with the other aforementioned microorganisms (Heinz et al., 2001). More specifically under PEF treatment (20 kV/cm, 184 kJ/kg), the most sensitive L. *monocytogenes* strain, i.e. NCTC 10357, resulted in a log_{10} reduction of 3.74 which was on average lower than the log_{10} reduction of the most sensitive strains of *E. coli, L. plantarum* and *S. cerevisiae* (> 4 log_{10} reduction). The strain of L. *monocytogenes* L6 was identified as the most resistant strain (log_{10} reduction of 1.23) (See Table 2), at a treatment of 184 kJ/kg PEF, and showed a statistical difference ($p \leq 0.0001$) in comparison with the NCTC 10357 (see Table S2). In addition to membrane disruption, PEF may induce localized overheating via the conductivity of the formed pores (Simpson et al., 1999), potentially leading to denaturation of membrane-bound proteins and affecting chaperones *in* L. *monocytogenes'* response. As evidenced by a study of Lado et al. (2004) a transient reduction in the expression of the levels of molecular chaperones GroEL, GroES, and DnaJ when sensitive and resistance *Listeria* strains were compared.

In agreement with our observations, a study including 20 different strains of both Gram positive and negative bacteria (*L. monocytogenes*, *S aureus*, *E. coli*, *Salmonella*) has shown a low inactivation (<1 log_{10}) and highest PEF resistance, for three L. *monocytogenes* strains: 4031 (the most resistant, log_{10} reduction of 0.4 ± 0.1), 672, 4032 in McIlvaine medium of pH 7.0 and after PEF treatment in static conditions of 30 kV/cm and 180 kJ/kg (Saldaña et al., 2009). Additionally, studies on low acid-watermelon (pH:5.46 ± 0.11) and melon (pH:5.82 ± 0.04) juices, underlined the resistance of L. *monocytogenes* in comparison to *E. coli* and *S. enteritidis* (Mosqueda-Melgar, Raybaudi-Massilia, & Martín-Belloso, 2007). In contrast, Saldaña et al. (2009), reported that L. *monocytogenes* strains were more sensitive (log_{10} reductions between 1.5 and 3.6) than *E. coli* strains (log_{10} reductions between 0.1 and 2.9) at PEF treatment in static conditions of 30 kV/cm and 180 kJ/kg, at pH 4.0 indicating that L. *monocytogenes* is more sensitive to acidic environments

Table 2
The most resistant and sensitive strains of *E. coli*, *L. monocytogenes*, *L. plantarum* and *S. cerevisiae* after PEF and thermal treatments. The categorization of the resistant and sensitive strains is independent for each microorganism and condition and dependent on the highest statistical difference between the strains.

Microorganism	Strains	PEF (88 kJ/kg)	PEF (136 kJ/kg)	PEF (184 kJ/kg)	Thermal treatment
E. coli	Resistant	K12 BL21 (DE3)	BL21 (DE3) K12	–	ATCC35218
	Sensitive	FAM 21845 NCTC 12900 O157 VT- FAM22082 ATCC35218	O157 VT- ATCC35218 FAM 21845 FAM22082 NCTC 12900	–	BL21 (DE3) FAM 21843 FAM22082
L.monocytogenes	Resistant	–	–	L6	L6
	Sensitive	–	–	NCTC 10537	NCTC 10537
L. plantarum	Resistant	ATCC14917	ATCC14917	FBR23 FBR22 FBR04	–
	Sensitive	SF2A35B FBR06	FBR06 SF2A35B	FBR06 SF2A35B	–
S. cerevisiae	Resistant	AD1890 130.0014	AD1890	AD1890	028.0315 130.0014
	Sensitive	028.0404	077.0001 028.0315	028.0404	0106.004 AD998

which indicates that acidic environments can be a hurdle for the microorganism. In harmony with that, an investigation at PEF treatment (25 kV/cm for 300 exponential decay pulses) showed that at pH 4.0 *E. coli* O157:H7 was the most resistant microorganism, when L. *plantarum* was more resistant than L. *monocytogenes* (García et al., 2005). However, after 2 h holding time L. *plantarum* showed higher sensitivity than L. *monocytogenes* which indicates the effect on the recovery of the microorganism (García et al., 2005).

L. plantarum resulted in microbial reductions which were statistically different among all the PEF conditions. ATCC14917 was the most resistant strain for 88 kJ/kg ($\log_{10}$ reduction of 0.46) and 136 kJ/kg ($\log_{10}$ reduction of 1.90). Under a PEF treatment at 184 kJ/kg FBR23, FBR22 and FBR04, strains exhibited the highest resistance with $\log_{10}$ reductions of 4.09, 4.09, 4.14, respectively. FBR06 and SF2A35B were the most sensitive strains for all the processing conditions exhibiting $\log_{10}$ reductions of 2.49, 2.74 (in 88 kJ/kg), of 4.42, 4.27 (in 136 kJ/kg), and of 5.51, 5.36 (in 184 kJ/kg). For 80 and 136 kJ/kg, differences between the resistant and sensitive strains were more significant ($p \leq 0.0001$, see Table S3) in comparison with the184 kJ/kg treatment differences. The *S. cerevisiae* AD1890 and 130.0014 were the most resistant strains under the 88 kJ/kg treatment ($\log_{10}$ reduction of 0.36, 0.26, respectively), while the *S. cerevisiae* AD1890 strain emerged as the most resistant strain for the 136 kJ/kg ($\log_{10}$ reduction of 1.23) and for the 184 kJ/kg ($\log_{10}$ reduction of 4.06) treatments. In contrast, the *S. cerevisiae* 028.0404 was identified as the most sensitive strain under the 88 kJ/kg ($\log_{10}$ reduction of 3.31) and 184 kJ/kg ($\log_{10}$ reduction of 5.74) treatments. For the 136 kJ/kg, *S. cerevisiae* 077.0001 and 028.0315 were the most sensitive strains with $\log_{10}$ reductions of 4.37 and 4.34, respectively. Resistant and sensitive strains exhibited stronger statistical differences under the 88 and 136 kJ/kg ($p \leq 0.0001$, see Table S4) than the 184 kJ/kg ($p \leq 0.001$) treatment.

3.2. Influence of total specific energy input on the inactivation of microorganisms under PEF treatments

Observed microbial reductions for all strains of the studied species (*E. coli*, *L. monocytogenes*, *L. plantarum* and *S. cerevisiae*) were averaged for each PEF total specific energy applied i.e., 88, 136 and 184 kJ/kg and compared (Fig. 2). For *E. coli*, a statistical difference ($p \leq 0.01$) was identified between the $\log_{10}$ reductions of 88 and 184 kJ/kg while there was no statistical difference between the $\log_{10}$ reductions of 136 kJ/kg in comparison with the other two aforementioned parameters. Previous studies have shown that PEF is not "an all or nothing event", and that there is a sublethal damage (for studies of both acidic or neutral pH) which is proportional to the PEF treatment intensity (Gurtler et al., 2010; Saldaña et al., 2009; Zhao et al., 2011) and the outlet temperature (Gurtler et al., 2010). Thus, this may indicate that the sublethal population of *E. coli* was inactivated only after application of 184 kJ/kg. The

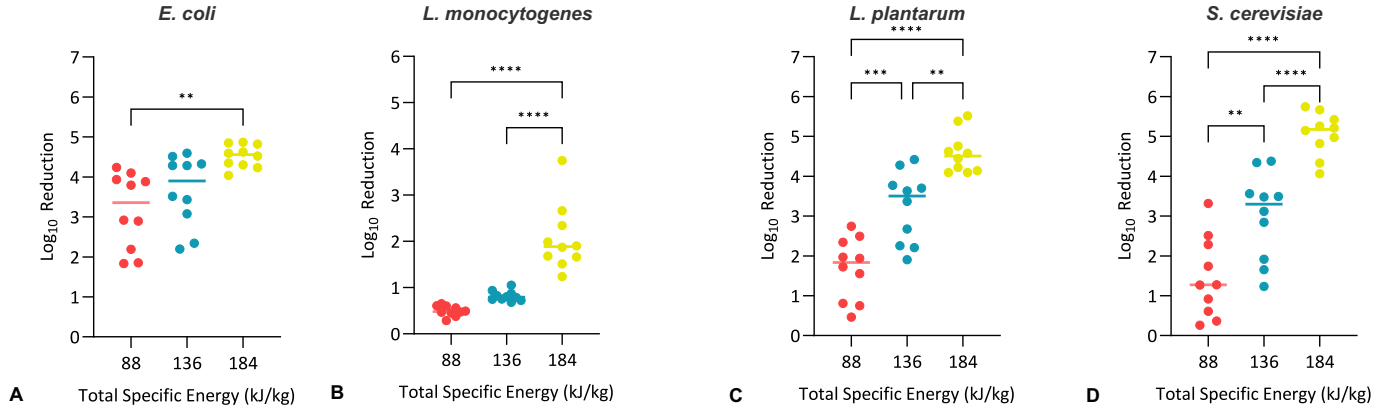

Fig. 2. Ordinary one-way ANOVA multiple comparisons between the Log_{10} reductions achieved for three different PEF total specific energies. (A) *E. coli strains,* (B) *L. monocytogenes strains,* (C) *L. plantarum strains.* (D) *S. cerevisiae* strains. Specific energies are: 88 kJ/kg, 136 kJ/kg, and 184 kJ/kg. Statistical analysis included all the biological replicates for all the different strains of each species. * $p \leq 0.05$, ** $p \leq 0.01$,*** $p \leq 0.001$,;**** $p \leq 0.0001$).

average $\log_{10}$reductions of L. *monocytogenes* at 184 kJ/kg showed a statistical difference ($p \leq 0.0001$) when compared with the $\log_{10}$ reduction at 88 and 136 kJ/kg, when there was no statistical difference between the $\log_{10}$ reduction at 88 and 136 kJ/kg. Thus, it is evident that the strain variability in the resistance as shown in (Fig. 2), as well as the inactivation efficiency becomes significant only at 184 kJ/kg. Both the average $\log_{10}$ reductions of L. *plantarum* and *S. cerevisiae* showed statistical differences between the different treatments. More specifically, *L. plantarum* $\log_{10}$ reduction at 88 kJ/kg showed a statistical difference ($p \leq 0.001$) when compared with the treatments at 136 kJ/kg and at 181 kJ/kg ($p \leq 0.0001$). The $\log_{10}$ reduction at 136 kJ/kg showed a statistical difference ($p \leq 0.01$) with that of 184 kJ/kg. Finally, for *S. cerevisiae* there was a statistical difference on the $\log_{10}$ reduction at treatments of 88 and 136 kJ/kg ($p \leq 0.01$), while it had a $p \leq 0.0001$ at 88 when compared with 136 and 184 kJ/kg.

3.3. Microbial strain variability under thermal treatments

The same strains of the aforemntioned microorganisms (i.e., *E. coli, L. monocytogenes*, *L. plantarum*, and *S. cerevisiae*) were subjected to thermal treatment (refer to Section 2.3). The impact of this process on microbial reductions is illustrated in Fig. 3. *E. coli* has resulted in a statistical difference ($p \leq 0.05$) between the resistant strain ATCC35218 ($\log_{10}$ reduction of 3.20) and the sensitive strains of BL21 (DE3) ($\log_{10}$ reduction of4.16), FAM 21843 ($\log_{10}$ reduction of 4.13) and FAM 22082 ($\log_{10}$ reduction of 4.08). BL21 (DE3) which was identified as one of the most sensitive bacteria after thermal treatment showed high resistance to PEF treatments (88 and 136 kJ/kg).

L. monocytogenes L6 strain (resulted in 2.74 $\log_{10}$ reduction) was identified as the most resistant and NCTC10537 the most sensitive ($\log_{10}$ reduction of 3.81) ($p \leq 0.05$) for the applied thermal treatment of 30 min at 62.8 °C as well as the PEF treatment (20 kV/cm, 184 kJ/kg). In agreement with these results, *L. monocytogenes* L6 strain was also the most thermal resistant strain from a selection of 20 stains of different origins under thermal treatment (55, 60, 65 °C) (Aryani et al., 2015a). In general, *L. monocytogenes* strains showed higher microbial reduction and significant differences under PEF treatment (184 kJ/kg). A comparison between PEF and thermal strain variability experiments also indicated higher strain differences under PEF treatment (25 kV/cm, 144 μs) and it was suggested that this difference may be related to the mechanism of action of the technology under these conditions (Lado & Yousef, 2003) .

L. plantarum strains under thermal treatment showed no statistical differences between $\log_{10}$ reductions (between 3.03 and 3.39) which maybe is related to the end point used for this study. However, in another kinetic study by Aryani et al. (2016) including the majority of the strains used in this research, has shown high strain variability between the strains. *L. plantarum* SF2A35B was identified as the most sensitive strain under various thermal treatments (55, 58, 60, 63 °C), having the lowest *D* values (min)). In agreement with this work, SF2A35B was identified as one of the two most sensitive strains for all PEF conditions investigated which indicates a relation in the resistance between the two technologies. However, FBR06 which was also identified as sensitive to all PEF treatments did not appear having the same sensitivity to thermal treatments for both the above-mentioned experimental designs.

S. cerevisiae 028.0315 and 130.0014 were identified as the most resistant and showed a $\log_{10}$ reduction of 3.06, while 0106.004 and AD998 were identified as the most sensitive with $\log_{10}$ cycles reductions

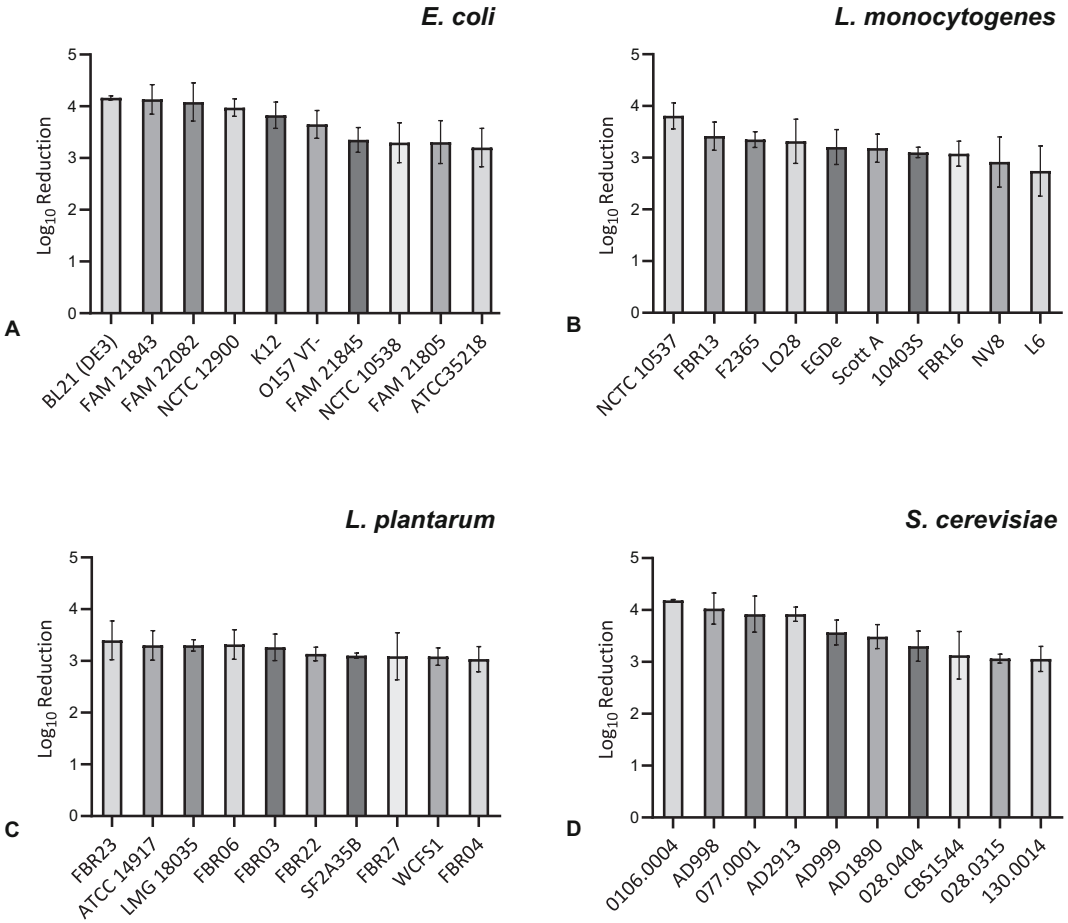

Fig. 3. $\log_{10}$ reductions of (A) *E. coli*, (B) *L. monocytogenes*, (C) *L. plantarum* and (D) *S. cerevisiae* strains of thermal treatment (62.8 ± 0.1 °C, 30 min). Experiments were performed in 3 biological replicates. Bars represent the standard deviations of these measurements.

of 4.18 and 4.02, respectively. A relation with PEF treatments was identified only for 130.0014 strain under PEF treatment (20 kV/cm,88 kJ/kg) which shows also a resistance under this condition. However, AD1890 was identified as the most resistant strain in the other PEF conditions. The stress response of *S. cerevisiae* was different under thermal and PEF treatment conditions. The stress response under PEF treatment has been reported to be more related to oxidation (*GLR1*) gene while under thermal treatment was related to heat shock (*HSP104*) gene (Tanino et al., 2012). Thus, this indicates that a strain/microorganism identified resistant under PEF treatment may not show the same resistance under thermal treatment.

3.4. Microbial targets under PEF and thermal treatments

The most resistant and sensitive strains of 4 indicative model microorganisms (*E. coli*, *L. monocytogenes*, *L. plantarum* and *S. cerevisiae*) under 3 different PEF treatments (88, 136 and 184 kJ/kg) in pH 7.0 and thermal treatment in TSB-D were identified. The knowledge of PEF and thermal resistance of each microorganism and strain is important for the selection of microbial strain targets when the technology is applied alone or in combination.

In general, high inactivation level ($\log_{10}$ reductions) differences were identified for *E. coli* (1.68–4.24, 2.19–4.59), *L. plantarum* (0.46–2.74, 1.90–4.42), and *S. cerevisiae* (0.26–3.31,1.23–4.37) at 88 and 136 kJ/kg in comparison with 184 kJ/kg (i.e., 4.04–4.87, 4.09–5.51, 4.06–5.74, respectively). On the contrary, for L. *monocytogenes* higher differences (1.23–3.74) were only reported at 184 kJ/kg. For thermal treatments, low inactivation differences ($\log_{10}$ reductions) were identified for *E. coli* (3.20–4.16), *L. monocytogenes* (2.74–3.81), *L. plantarum* (3.03–3.39) and *S. cerevisiae* (3.05–4.18). Thus, the fewer statistical different results between the strains in the high PEF conditions may stem from the higher $\log_{10}$ reductions achieved due to the intensity of the treatment and potentially the higher thermal effect.

Further investigations focusing on the inactivation kinetics of the most robust microorganisms could further be developed to optimize the efficacy of PEF processes alone or in combination with other technologies. Studies on application of PEF-based hybrid schemes (exploring the benefits of physical or chemical hurdles) could further enhance the PEF effectiveness and broaden its applicability at an industrial level. The combination of different hurdle technologies, used at sublethal levels, may unleash the effects that compromise the microbial stability whilst maintaining the nutritional and organoleptic characteristics of the processed food (Caminiti et al., 2011; Pataro et al., 2014). For example, Walter et al. (2016) reported an inactivation of >6 $\log_{10}$ for *P. fluorescens* in whole milk (4% fat *w*/w) after a combination of PEF (35 kV/cm,50 μs) and thermal treatment of 54.4 °C for 40 s. Additionally, research on understanding the main mechanisms of resistance with studies focusing on transcriptomics (understanding of stress responses, identification of resistance genes), proteomics (identification and characterization of stress response proteins) and metabolomics (metabolic profiling and identification of the resistance related metabolites) could also help at identifying those molecular components that are critical in the microbial decontamination. Finally, these results should be considered for selecting target microorganism/s for modelling PEF microbial inactivation kinetics and developing optimized processes. Based on that, food safety will be enhanced and health risks will be reduced across various conditions and procedures. Establishing optimized processes that will reassure the food safety and minimize quality deterioration. However, the aforementioned $\log_{10}$ reductions and PEF resistance ranking cannot directly be compared with results of other laboratories due to the different apparatus and strains and can only be indicative for future researchers.

4. Conclusions

In conclusion, PEF resistance may vary between species and also between strains of the same microorganism, with the variability between strains being dependent of the total specific energy. For *E. coli*, *L. plantarum* and *S. cerevisiae* strains showed higher statistical differences at low PEF intensities, i.e., 88 and 136 kJ/kg, in comparison with the highest PEF intensity (184 kJ/kg). *E. coli* strains were identified to have no statistical differences after the highest intensity PEF treatment. On the contrary, *L. monocytogenes* strains showed statistical differences on the $\log_{10}$ inactivation only under the 184 kJ/kg PEF treatment). Considering the PEF resistance of the different microorganisms at pH 7.0, *L. monocytogenes* showed low $\log_{10}$ reductions (<1.05) over the 88 and 136 kJ/kg PEF treatments for all the strains. However, only at the highest intensity PEF treatment (184 kJ/kg) *L. monocytogenes* strains emerged as the most PEF resistant, with strain L6 appearing as the most PEF insensitive, which was in accordance with its behaviour under thermal treatments.

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CRediT authorship contribution statement

Fotios Lytras: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Georgios Psakis:** Writing – review & editing, Supervision, Methodology. **Ruben Gatt:** Writing – review & editing, Supervision, Funding acquisition. **Joerg Hummerjohann:** Writing – review & editing. **Javier Raso:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Vasilis Valdramidis:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103731.

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