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Heat resistance of *Lactobacillus brevis*, *Pediococcus acidilactici* and *Enterococcus faecium* in buffer (pH 4), alcoholic and alcohol-free beer

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Abstract

Why was the work done: To measure the heat resistance of three vegetative bacteria in buffer (pH 4), alcoholic and alcohol-free beer. To verify that *Pediococcus acidilactici* ATCC 8042 and *Lactobacillus brevis* BSO 566 are as heat resistant as previously reported and to establish if *Enterococcus faecium* NRRL B-2354 could have application in validation studies for the pasteurisation of beer.
How was the work done: : The heat resistance of *L. brevis*, *P. acidilactici* and *E. faecium* in buffer, alcoholic and alcohol-free beer was determined using two approaches - capillary test tubes and flask method.
What are the main findings: *E. faecium* was the most heat resistant microorganism in all three liquids. D values were significantly greater, and z-values were similar or significantly greater than the corresponding values of *L. brevis* and *P. acidilactic*i.
Why is the work important: *E. faecium* is used in the food industry as a pathogenic surrogate for the validation of thermal and non-thermal processes. The work reported here suggests that *E. faecium* can also be used for the validation of pasteurisation of beer. Its high z-value suggests that at higher pasteurisation temperatures (>65°C) it may be more resistant than yeast ascospores and could therefore be used as an indicator for flash pasteurisation.

Keywords

Non-alcoholic beer, heat resistance, beer spoilage, *Pediococcus acidilactici* ATCC 8042, *Lactobacillus brevis* BSO 566, *Enterococcus faecium* NRRL B-2354, surrogate

Introduction

Although the heat resistance of beer spoilage bacteria has been investigated (Adams et al. 1989; L'Anthoën and Ingledew 1996; Zufall and Wackerbauer 2000; Reveron et al. 2005; Rachon et al. 2018), there are some inconclusive results suggesting that beer spoilage bacteria can survive mild pasteurisation. While most research reports (Table 1) suggest that the heat resistance of vegetative bacteria in beers is relatively low with a D_{60} = 0.08-0.87 minutes in alcoholic beers (Rachon et al. 2018; Tsang and Ingledew 2018) and D_{60} = 0.45-2.6 minutes in non-alcoholic beers (Adams et al. 1989; Rachon et al. 2021) other reports suggest that the heat resistance of these microorganisms is significantly greater. Accordingly, the D_{60} of *P. acidilactici* ATCC 8042 in non-alcoholic beer was 7.6 minutes with z value of 49.3°C with a D_{60} = 1.33 minutes with z value of 24.6°C in alcoholic beer (L'Anthoën and Ingledew 1996). Similarly, the D_{60} of *Lactobacillus brevis* and *Pediococcus damnosus* in alcoholic beer was respectively 2.0 minutes and 2.07 minutes (Zufall and Wackerbauer 2000). Further, Ohkochi and Takahashi (1982) reported the D_{60} for Lactobacilli as 4.4 minutes with z = 8°C although no information was given about beer style, ABV, pH or IBU. These studies suggest that *L. brevis* BSO 566 (Rachon et al. 2018) is one of the most heat resistant beer spoilage vegetative bacteria.

The heat resistance of microorganisms is described by two values: D and z. The D-value is the time required at a specific temperature for a decimal (i.e. 1 log or 90%) reduction in the population of a microorganism; the z-value is defined as the change in temperature required for a 10-fold change in the D-value (EBC Technology and Engineering Forum 1995; Gaze 2006). D values are determined experimentally by performing heat inactivation trials. There are different approaches for performing these trials including the capillary tube method, flask method, test tube/vial method, pouches (Sorqvist 2003), other (thermal chambers or thermoresistometer) (Condón et al. 1993; Martinez et al. 2003; Rachon et al. 2016).

The most used aprroach uses glass or stainless-steel capillary tubes, where heat transfer is efficient, and the sample reaches the target temperature within a few seconds. Although the method is the most precise, it is time consuming as the glass capillary tubes must be filled and then heat sealed one by one before each heat inactivation trial followed by retrieval and analysis. A second approch is the flask method, where the liquid under test is heated to the target temperature. Typically, this involves placing a small (100 mL) conical flask, containing the liquid and stirrer into a water bath. Once the target temperature is reached, the liquid is inoculated and mixed, with samples taken for the quantification of viable microorganisms. The method benefits from ease of use, with a detection limit of 1 mL per timepoint. However, disadvantages include losses through evaporation during heating and the time for mixing. The tube/vial method is similar to the capillary tube method except that the volume tested is much greater. This increases ramp-up time which, in some cases compromises trials at higher temperatures, as the microorganisms can be killed. Pouches are easy to use but achieving the target temperature is not as rapid as with capillary tubes. Other options include thermal chambers (thin sealed chambers), the slug-flow method and submerged coil heating apparatus or thermoresistometer. Studies can involve different methods determined by time (capillary tubes method are the most time consuming) or by the availability of equipment. In this study, testing was conducted using two methods: capillary tubes and the flask method.

The heat resistance of two beer spoilage microorganisms; *L. brevis* BSO 566 - one of the most heat resistant beer spoilage microorganisms (Rachon et al. 2018) - and *P. acidilactici* ATCC 8042 was measured. In addition, the heat resistance was determined of *E. faecium* NRRL B-2354, a surrogate microorganism used in the food industry for mild heat treatment of foods. *E. faecium* is used for the validation of thermal processes where the elimination of pathogens must be verified (Almond Board of California 2014; Bianchini et al. 2014; Ceylan and Bautista 2015; Rachon et al. 2016). The similarity of *E. faecium* to pathogenic microorganisms has made it a surrogate for vegetative pathogenic bacteria such as *Escherichia coli*, *Salmonella* or *Listeria monocytogenes*. Accordingly, the aim of this study was to determine if *P. acidilactici* ATCC 8042 is as heat resistant as previously reported (L'Anthoën and Ingledew 1996) and to measure the D_{60} value as the heat resistance was only measured in the lower temperature range.

Table 1.

D and z-values of spoilage vegetative bacteria in premium and alcohol-free beers

Organism	Heating substrate	Temp (°C)	D value (min)	z value (°C)	Reference
Lactobacillus brevis BSO566	0% lager pH 4.4, IBU 25.9	60	0.7	N/A	(Rachon et al. 2021)
Lactobacillus lindneri BSO943	0% lager pH 4.4, IBU 25.9	60	0.5	N/A	(Rachon et al. 2021)
Lactobacillus brevis BSO566	0% lager, pH 4.4, IBU 5.6	60	1.3	N/A	(Rachon et al. 2021)
Lactobacillus lindneri BSO943	0% lager, pH 4.4, IBU 5.6	60	0.6	N/A	(Rachon et al. 2021)
Lactobacillus brevis BSO566	0% lager, pH 4.4, IBU 8.6	60	0.98	N/A	(Rachon et al. 2022)
Lactobacillus brevis BSO566	0% lager, pH 4.4, IBU 25	60	0.76	N/A	(Rachon et al. 2022)
Lactobacillus brevis BSO566	0% lager, pH 4.4, IBU 50	60	0.62	N/A	(Rachon et al. 2022)
Lactobacillus E93	<0.05% lager, pH 4.2	60	2.6	12.1	(Adams et al. 1989)
Pediococcus acidilactici ATCC8042	0.5% lager, pH 5.2	55	9.56	49.3	(L'Anthoën and Ingledew 1996)
Pediococcus acidilactici ATCC8042	0.5% lager, pH 5.2	60	7.7[a]	49.3	(L'Anthoën and Ingledew 1996)
Lactobacillus delbruecki	0.5% lager, pH 5.25	57	6.63	15.5	(L'Anthoën and Ingledew 1996)
Lactobacillus delbruecki	0.5% lager, pH 5.25	60	4.01[b]	15.5	(L'Anthoën and Ingledew 1996)
Lactobacillus E93	0.9% lager, pH 4.0	60	1.6	9.2	(Adams et al. 1989)
Lactobacillus E93	3.3% synthetic lager, pH 4.2	60	1	10	(Adams et al. 1989)
Lactobacillus E93	4.4% synthetic lager, pH 4.2	60	0.3	10.6	(Adams et al. 1989)
Lactobacillus brevis BSO566	4.5% lager pH 4.3, IBU 6.9	60	0.17	N/A	(Rachon et al. 2021)
Lactobacillus lindneri BSO943	4.5% lager pH 4.3, IBU 6.9	60	0.15	N/A	(Rachon et al. 2021)
Acetobacter pasteurianus BSO547	4.6% ale, pH 3.7, IBU 22.1	60	0.09	5.2	(Rachon et al. 2018)
Lactobacillus brevis BSO566	4.6% ale, pH 3.7, IBU 22.1	60	0.2	9.5	(Rachon et al. 2018)
Lactobacillus hilgardii BSO600	4.6% ale, pH 3.7, IBU 22.1	60	0.07	7.7	(Rachon et al. 2018)
Acetobacter pasteurianus BSO547	4.8% stout, pH 3.9, IBU 23.5	60	0.14	6.7	(Rachon et al. 2018)
Lactobacillus brevis BSO566	4.8% stout, pH 3.9, IBU 23.5	60	0.15	8.7	(Rachon et al. 2018)
Lactobacillus hilgardii BSO600	4.8% stout, pH 3.9, IBU 23.5	60	0.08	8.5	(Rachon et al. 2018)
Lactobacillus brevis BSO566	4.9% lager pH 4.3, IBU 24.9	60	0.1	N/A	(Rachon et al. 2021)
Lactobacillus lindneri BSO943	4.9% lager pH 4.3, IBU 24.9	60	0.08	N/A	(Rachon et al. 2021)
Lactobacillus delbrueckii	5% lager, pH 4.0	47	1.1	12.3	(Tsang and Ingledew 2018)
Lactobacillus frigidus	5% lager, pH 4.0	53	1.2	14.4	(Tsang and Ingledew 2018)
Pediococcus acidilactici	5% lager, pH 4.0	53	3.3	11.2	(Tsang and Ingledew 2018)
Pediococcus acidilactici ATCC8042	5% lager, pH 4.26	53.3	2.54	24.6	(L'Anthoën and Ingledew 1996)
Pediococcus acidilactici ATCC8042	5% lager, pH 4.26	60	1.33	24.6	(L'Anthoën and Ingledew 1996)
Lactobacillus delbruecki	5% lager, pH 4.26	57	1.86	7.23	(L'Anthoën and Ingledew 1996)
Lactobacillus delbruecki	5% lager, pH 4.26	60	0.637	7.23	(L'Anthoën and Ingledew 1996)
Lactobacillus paracasei (C1096)	5% Pilsen beer pH 4.28, IBU 20.5	50	0.65	6.5	(Reveron et al. 2005)
Lactobacillus brevis	Wheat beer	60	2	7.2[c]	(Zufall and Wackerbauer 2000)
Pediococcus damnosus	Wheat beer	60	2.07	7.2[c]	(Zufall and Wackerbauer 2000)

[a] - D_{60} extrapolated from z-value of 49.3°C calculated from D_{49} - D_{55} min

[b] - D_{60} extrapolated from z-value of 24.6°C calculated from D_{47} - D_{53} min

[c] - D and z-values calculated from 2 and 3 time points respectively

In addition, the heat resistance of the surrogate pathogen, *E. faecium* NRRL B-2354 was measured.

Materials and methods

Beers and buffer

The heat resistance of microorganisms was measured in McIlvine buffer (pH 4) and in two American style lagers of the same brand; one alcoholic (4.5% ABV), and the other alcohol free (0.05% ABV). The McIlvaine buffer (pH 4) was prepared by mixing 7.71 mL of 0.2 M disodium phosphate (Sigma-Aldrich UK) with 12.29 mL of 0.1 M of citric acid (Sigma-Aldrich, UK) (McIlvaine 1921). The buffer was kept chilled (2-4°C) and used within one month. The pH of the buffer was confirmed before each trial (AR15 pH meter, Accumet Research, USA). The beers contained barley malt, rice, malt extract, hops, hop extract and natural flavours. The bitterness, pH and ABV of the beers was measured and the concentration of carbohydrates, sugars and protein was obtained from the package label (Table 2). Bitterness expressed as IBU (International Bitterness Units) was measured using the EBC Analytica method 9.8. The ABV was measured by gas chromatography following the EBC Analytica method 9.3.2. The pH of the beers was measured as described above.

Table 2.

Beer analysis

Beer	Alcohol-free beer	Alcoholic beer
ABV (%) - label	0.0	4.5
ABV (%) - analysed	0.02	4.68
pH	4.3	4.2
Bitterness (IBU)	8.2	8.2
Carbohydrates (g/100mL)	3.3	3.1
Sugars (g/100mL)	0.1	0.1
Protein (g/100mL)	0.1	0.3

Microorganisms

Lactobacillus brevis BSO 566 (fermented beverage isolate), *Pediococcus acidilactici* ATCC 8042 (NCIMB6990) and *Enterococcus faecium* NRRL B-2354 (ATCC 8459, NCIMB 2699) were used in this study. All microorganisms were recovered from storage (liquid nitrogen - *L. brevis* BSO 566 or from -70°C freezer - *P. acidilactici* ATCC 8042 and grown anaerobically in MRSB (de Man, Rogosa and Sharpe broth – Oxoid, UK) and MRSA (de Man, Rogosa and Sharpe agar - Oxoid, UK) for 5 days (broth) and 7 days (agar) at 27°C. *P. acidilactici* ATCC 8042 was grown aerobically in MRS broth and MRS agar for 48 h at 37°C. *E. faecium* NRRL B-2354 was grown aerobically in TSB (Tryptone Soya broth, Oxoid, UK) and TSA (Tryptone Soya agar, Oxoid, UK) for 2 days at 37°C. Following incubation and verifying purity, the broths were centrifuged at 3,000 x *g* for 5 min and the pellets were resuspended in sterile distilled water (SDW). Microorganisms were stored at 2-4°C and used (within 5 hours) in heat inactivation experiments on the day of preparation.

Heat inactivation trials

Capillary tubes method

Glass capillary tubes were filled with 50 µL of liquid inoculated with 10^7-10^8 CFU/mL of microorganisms (Jordan et al. 2011; Rachon et al. 2018). The tube ends were heat sealed and placed in a water bath (T100-ST5, Grant Instruments Ltd, UK) at the test temperatures (52, 54, 56, 58, 60 and 62°C) and held for the required time. At each heat interval, three capillary tubes (three replicates) were removed from the water bath and cooled in ice water. The test suspension was recovered from the capillary tubes with Maximum Recovery Diluent (Oxoid, UK) and the number of inoculated microorganisms (N_0) and surviving microorganisms after each interval (N_t) enumerated by spread plating. D- and z-value calculations and statistical analysis (one-way ANOVA) were performed using Minitab 20 software.

Flask method

Three 100 mL Erlenmeyer flasks filled with 50 mL of liquid were placed into a water bath (T100-ST26, Grant Instruments Ltd, UK) tempered to the target temperature (Menegazzi and Ingledew 1980; L'Anthoën and Ingledew 1996; Tsang and Ingledew 2018). Magnetic stirrers (MIXdrive 1 XS, MIXcontrol eco - 2mag AG, Germany) were used to mix the liquid in the flasks. Once reaching the target temperature, 0.5 mL of inoculum was added, and the trial started. Preliminary work established that 10 minutes was required to equilibrate the Erlenmeyer flasks. The number of inoculated microorganisms (N_0) was enumerated from the parallel unheated (three) Erlenmeyer flasks and the number of surviving

microorganisms at each heating interval was enumerated. At each timepoint, 0.5 mL of sample was pipetted into cooled ice water with 4.5 mL aliquots of Ringer's solution (-1 decimal dilution). These solutions were decimally diluted, and the number of surviving microorganisms enumerated by spread plating. D- and z-value, 95% confidence interval (CI), standard error (SE) and coefficient of determination (R2) were calculated using the MiniTab 20 software.

The D and z-values of *L. brevis* BSO 566, *P. acidilactici* ATCC 8042 and *E. faecium* NRRL B-2354 were compared with the D and z-values of *S. cerevisiae* BRYC 501 determined using the same beers (Rachon et al. 2022).

Results

The heat inactivation experiments showed that *Enterococcus faecium* was the most heat resistant bacteria using capillary test tubes (Table 3) and the flask method (Table 4). Most heat inactivation curves (71 out of 78) showed a high fit to linear regression ($R^2 > 0.95$) but in few cases a lower R^2 was recorded (0.816 – 0.938). These were not associated with microorganisms, liquid matrix and occurred at various temperatures.

On comparing the two methods, the majority (26 of 39) of D values from the two methods were significantly different ($p < 0.05$) with the capillary test tube method producing higher D values reflecting possible adaptation of microorganisms. For *Pediococcus acidilactici* (Figure 1), seven of 15 D values were significantly different and, (in five of seven) the capillary test tube method resulted in higher D values. Similarly for *L. brevis* (Figure 2) nine of 12 D values were significantly different and, in the majority, (seven of nine) the capillary test tube method produced higher D-values. For *E. faecium* (Figure 3), 10 out of 12 D values were significantly different, and all D-values determined by the capillary test tube method were higher than these of the flask method.

However, the z-values determined by the two methods were not significantly different. The p-values for the z-values in this study were greater than 0.05, except for *E. faecium* in 4.5% ABV lager (p=0.002). The D-values for *E. faecium* were significantly higher than the D-values of *P. acidilactici* and *L. brevis*. This identifies *E. faecium* as the more heat resistant vegetative bacteria which can represent a worst-case scenario during pasteurisation.

However, when extrapolating data and estimating D values for higher temperatures, (Figure 4, red line representing a typical z-value curve for 0.0% and 4.5% ABV lager; Rachon et al. 2022), there is a possibility that at higher temperatures, *E. faecium* is more heat resistant than ascospores of *Saccharomyces cerevisiae* BRYC 501 used to identify optimal pasteurisation regimes. Therefore, in such studies, it is recommended that *E. faecium* is used in parallel with *S. cerevisiae*.

The breaking point for the heat resistance 'switch' for the liquids was calculated from z-value curves (crossing point of two curves) as 65.1 for alcohol free lager and 66.7 for 4.5% ABV lager (Figure 4).

Figure 1.

Heat Resistance (D values) of *P. acidilactici* in a) buffer (pH 4), b) 0.0% ABV and c) 4.5% ABV lager using capillary tubes (purple) and the flask (grey) method.

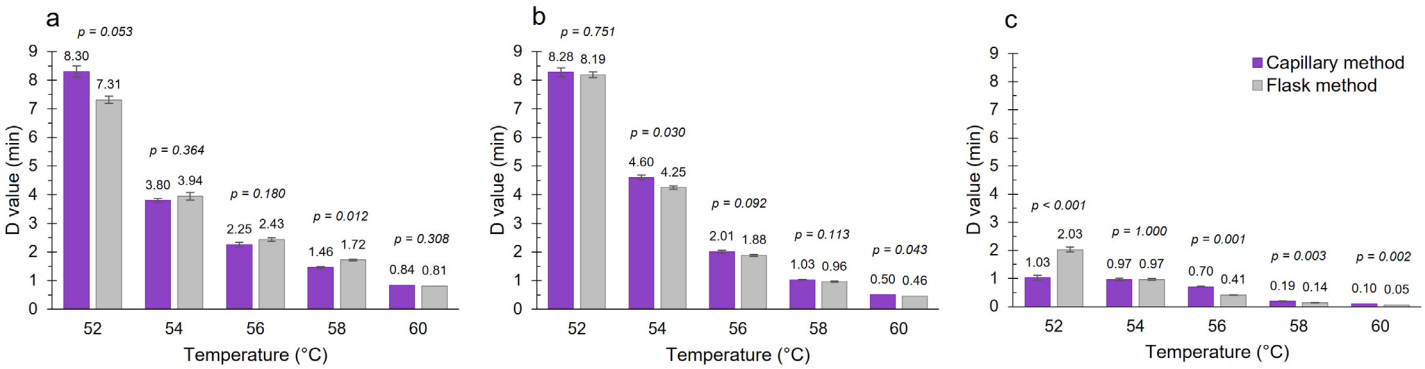

Figure 2.

Heat Resistance (D values) of *L. brevis* in a) buffer (pH 4), b) 0.0% ABV and c) 4.5% ABV lager using capillary tubes (purple) and the flask (grey) method.

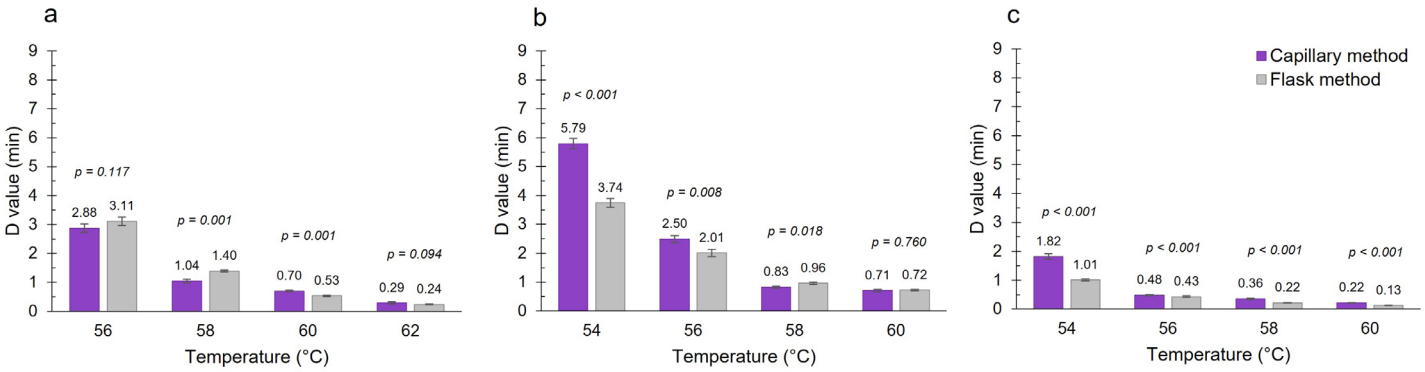

Figure 3.

Heat Resistance (D values) of *E. faecium* in a) buffer (pH 4), b) 0.0% ABV and c) 4.5% ABV lager using capillary tubes (purple) and the flask (grey) method.

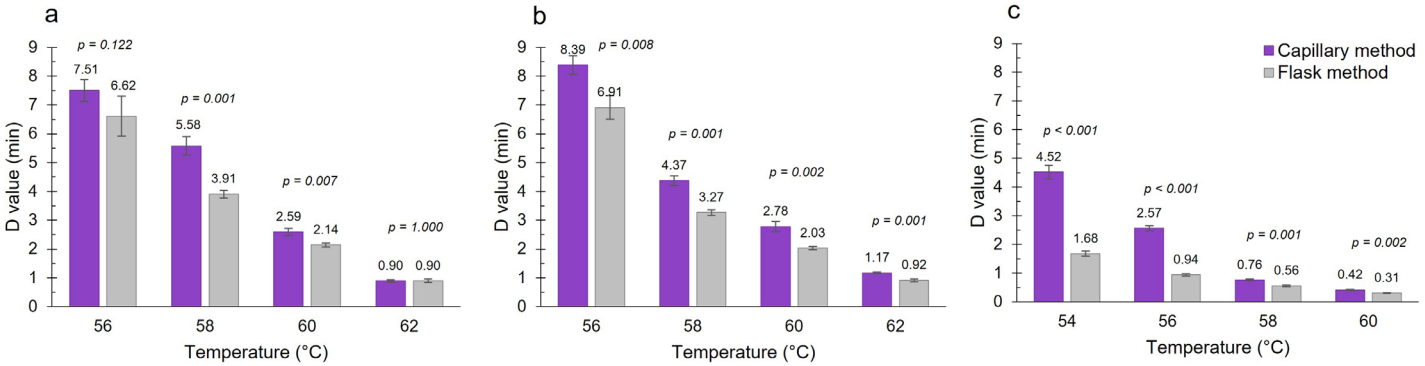

Figure 4.

z-values of *L. brevis*, *P. acidilactici* and *E. faecium* in a) buffer (pH 4), b) 0.0% ABV and c) 4.5% ABV lager

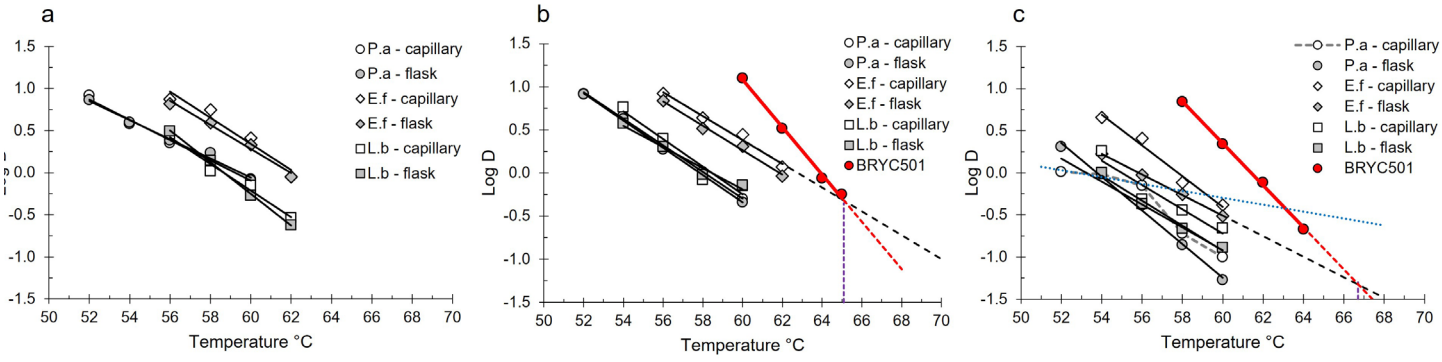

Table 3.

D and z-values of *P. acidilactici*, *L. brevis* and *E. faecium* using the capillary test tube method.

***Pediococcus acidilactici* ATCC8042**

Liquid	Temp (°C)	D (min)	95%CI	SE	R^2	z-value	SE	R^2
Buffer	52	**8.30**	(7.44, 9.39)	0.465	0.949			
	54	**3.80**	(3.42, 4.27)	0.198	0.961			
	56	**2.25**	(2.10, 2.41)	0.072	0.987	**8.3**	0.521	0.988
	58	**1.46**	(1.30, 1.67)	0.084	0.972			
	60	**0.84**	(0.78, 0.91)	0.032	0.983			
0.0% ABV Lager	52	**8.28**	(7.80, 8.82)	0.246	0.983			
	54	**4.60**	(4.30, 4.94)	0.154	0.979			
	56	**2.01**	(1.84, 2.21)	0.087	0.971	**6.48**	0.163	0.999
	58	**1.03**	(0.93, 1.14)	0.048	0.959			
	60	**0.50**	(0.48, 0.53)	0.014	0.990			
4.5% ABV Lager	52	**1.03**	(0.93, 1.14)	0.047	0.979			
	54	**0.97**	(0.81, 1.20)	0.087	0.927			
	56	**0.70**	(0.62, 0.82)	0.045	0.965	**7.40**	1.520	0.888
	58	**0.19**	(0.17, 0.22)	0.011	0.973			
	60	**0.10**	(0.09, 0.13)	0.009	0.922			

***Lactobacillus brevis* BSO 566**

Liquid	Temp (°C)	D (min)	95%CI	SE	R^2	z-value	SE	R^2
Buffer	56	**2.88**	(2.60, 3.22)	0.142	0.979			
	58	**1.04**	(0.93, 1.20)	0.062	0.961	**6.32**	0.694	0.977
	60	**0.70**	(0.65, 0.76)	0.025	0.995			
	62	**0.29**	(0.23, 0.40)	0.037	0.816			
0.0% ABV Lager	54	**5.79**	(5.43, 6.20)	0.183	0.985			
	56	**2.50**	(2.27, 2.77)	0.119	0.962	**6.22**	1.159	0.956
	58	**0.83**	(0.76, 0.92)	0.036	0.990			
	60	**0.71**	(0.62, 0.83)	0.046	0.969			
4.5% ABV Lager	54	**1.82**	(1.66, 2.04)	0.089	0.979			
	56	**0.48**	(0.45, 0.53)	0.019	0.979	**6.90**	1.652	0.880
	58	**0.36**	(0.32, 0.41)	0.020	0.993			
	60	**0.22**	(0.20, 0.24)	0.008	0.984			

***Enterococcus faecium* NRRL B-2354**

Liquid	Temp (°C)	D (min)	95%CI	SE	R^2	z-value	SE	R^2
Buffer	56	**7.51**	(6.78, 8.41)	0.378	0.976			
	58	**5.58**	(4.97, 6.35)	0.324	0.945	**6.45**	1.097	0.942
	60	**2.59**	(2.35, 2.90)	0.132	0.954			
	62	**0.90**	(0.82, 1.00)	0.043	0.972			
0.0% ABV Lager	56	**8.39**	(7.75, 9.14)	0.328	0.951			
	58	**4.37**	(4.06, 4.75)	0.165	0.980	**7.24**	0.625	0.996
	60	**2.78**	(2.46, 3.20)	0.174	0.938			
	62	**1.17**	(1.12, 1.23)	0.026	0.993			
4.5% ABV Lager	54	**4.52**	(4.06, 5.11)	0.243	0.973			
	56	**2.57**	(2.40, 2.76)	0.087	0.959	**5.50**	0.582	0.988
	58	**0.76**	(0.70, 0.85)	0.035	0.964			
	60	**0.42**	(0.38, 0.46)	0.020	0.957			

Table 4.

D and z-values of *P. acidilactici*, *L. brevis* and *E. faecium* using the flask method.

***Pediococcus acidilactici* ATCC8042**

Liquid	Temp (°C)	D (min)	95%CI	SE	R^2	z-value	SE	R^2
Buffer	52	**7.31**	(6.53, 8.32)	0.425	0.948			
	54	**3.94**	(3.69, 4.23)	0.130	0.980			
	56	**2.43**	(2.18, 2.76)	0.135	0.957	**8.8**	0.570	0.988
	58	**1.72**	(1.60, 1.85)	0.058	0.986			
	60	**0.81**	(0.75, 0.88)	0.031	0.983			
0.0% ABV Lager	52	**8.19**	(7.46, 9.08)	0.386	0.968			
	54	**4.25**	(4.06, 4.47)	0.099	0.992			
	56	**1.88**	(1.78, 2.00)	0.053	0.991	**6.34**	0.107	0.999
	58	**0.96**	(0.89, 1.04)	0.036	0.973			
	60	**0.46**	(0.42, 0.50)	0.019	0.963			
4.5% ABV Lager	52	**2.03**	(1.84, 2.26)	0.099	0.954			
	54	**0.97**	(0.81, 1.20)	0.087	0.927			
	56	**0.41**	(0.37, 0.47)	0.024	0.956	**5.00**	0.199	0.994
	58	**0.14**	(0.12, 0.16)	0.008	0.962			
	60	**0.05**	(0.04, 0.08)	0.008	0.876			

***Lactobacillus brevis* BSO 566**

Liquid	Temp (°C)	D (min)	95%CI	SE	R^2	z-value	SE	R^2
Buffer	56	**3.11**	(2.84, 3.44)	0.141	0.967			
	58	**1.40**	(1.33, 1.47)	0.032	0.996	**5.31**	0.137	0.999
	60	**0.53**	(0.49, 0.59)	0.022	0.993			
	62	**0.24**	(0.21, 0.28)	0.014	0.996			
0.0% ABV Lager	54	**3.74**	(3.45, 4.09)	0.149	0.982			
	56	**2.01**	(1.77, 2.32)	0.122	0.984	**8.12**	0.951	0.973
	58	**0.96**	(0.87, 1.08)	0.046	0.994			
	60	**0.72**	(0.67, 0.78)	0.026	0.985			
4.5% ABV Lager	54	**1.01**	(0.94, 1.09)	0.035	0.987			
	56	**0.43**	(0.38, 0.49)	0.026	0.960	**6.72**	0.512	0.989
	58	**0.22**	(0.20, 0.24)	0.010	0.982			
	60	**0.13**	(0.11, 0.15)	0.008	0.983			

***Enterococcus faecium* NRRL B-2354**

Liquid	Temp (°C)	D (min)	95%CI	SE	R^2	z-value	SE	R^2
Buffer	56	**6.62**	(5.43, 8.47)	0.692	0.894			
	58	**3.91**	(3.64, 4.21)	0.136	0.984	**6.98**	0.596	0.986
	60	**2.14**	(2.00, 2.31)	0.073	0.978			
	62	**0.90**	(0.78, 1.05)	0.062	0.921			
0.0% ABV Lager	56	**6.91**	(6.14, 7.90)	0.408	0.955			
	58	**3.27**	(3.07, 3.49)	0.098	0.991	**7.06**	0.455	0.992
	60	**2.03**	(1.92, 2.15)	0.056	0.986			
	62	**0.92**	(0.83, 1.03)	0.048	0.961			
4.5% ABV Lager	54	**1.68**	(1.50, 1.91)	0.094	0.955			
	56	**0.94**	(0.85, 1.05)	0.046	0.968	**8.19**	0.138	0.995
	58	**0.56**	(0.51, 0.62)	0.027	0.953			
	60	**0.31**	(0.28, 0.34)	0.015	0.977			

Taking all this into account, D and 6D (6 log reduction time) values for a range of temperatures were calculated (Table 5). At lower temperatures (<65.1 for 0.0% ABV and <66.7°C 4.5% ABV lager), D values for *E. faecium* were lower than those of *S. cerevisiae* BRYC 501 ascospores. At higher temperatures (>65.1/0.0% ABV and >66.7°C/4.5% ABV lager) D values for *E. faecium* are higher than those of *S. cerevisiae* BRYC 501 ascospores. Consequently, the time required for a 6-log reduction for both microorganisms was calculated. With the 0.0% ABV lager at 65.1°C, the $D_{65.1}$ value for both microorganisms will be the same as the time required for the 6-log reduction of both microorganisms at 170 seconds. At higher temperatures (>70°C) the extrapolated D_{70} value for *E. faecium* will be greater (D_{70} = 0.10 minutes) than the D_{70} value for *S. cerevisiae* BRYC 501 ascospores (D_{70} = 0.02 minutes). Consequently a 6-log reduction of *E. faecium* would be achieved after 36 seconds compared to 8 seconds for *S. cerevisiae* ascospores.

Discussion

The results show that *Pediococcus acidilactici* ATCC 8042 was not as heat resistant as reported by L'Anthoën and Ingledew (1996), who used a low range of temperatures (49.2-55.0°C for alcohol free lager, 47-53.3°C for beer). The authors reported Z-values for *P. acidilactici* which were extremely high (49.3°C for alcohol-free and 24.6°C standard beer). In their study, L'Anthoën and Ingledew (1996) reported that D_{60} could not be experimentally established and noted that 'heat killing experiments cannot be conducted at 60°C because the cells die too quickly to be sampled and enumerated. Therefore, phantom thermal death time data are extrapolated to 60°C'. However, in the same study, the authors reported that $D_{50.8}$ = 0.55 min.

Presumably, the D_{60} value could not be extrapolated as the z-value curve was not linear beyond the maximum temperature of 55°C used here. Interestingly in this work, the z-value curve of *P. acidilactici* (Figure 4c; 4.5% ABV lager) was not linear (R^2 = 0.888). If the z-value for the lower temperatures (52, 54 and 56°C) was used, the z-value would be 24.4°C (Figure 4, blue dotted trendline). The heat resistance of *P. acidilactici* was also investigated by Tsang and Ingledew (2018) who used the flask method in degassed lager (5% ABV, pH 4.0). This was closer to the results reported here, where D_{53} = 3.3 minutes with z = 11.2°C. Although there is no published data of the heat resistance of this microorganism in beer, there are reports of this microorganism in low moisture pet food (Ceylan and Bautista 2015), toasted oats cereal and peanut butter (Deen and Diez-Gonzalez 2019) or ground

Table 5.

Calculated and extrapolated D and 6D-values for *E. faecium* and *S. cerevisiae* ascospores at temperatures used for tunnel and flash pasteurisation.

Beer	Temp (°C)	*E. faecium* NRRL B-2354			Yeast ascospores BRYC501		
		D (min)	6D (min)	6D (s)	D (min)	6D (min)	6D (s)
0.0% ABV Lager	60	2.41	14.4	867	12.02	72.1	4328
	65	0.49	2.95	177	0.51	3.06	184
	65.1	0.47	2.84	170	0.47	2.84	170
	70	0.10	0.60	36	0.02	0.13	8
	75	0.02	0.12	7	0.00	0.01	0.3
	80	0.004	0.02	1	0.000	0.00	0.0
	85	0.001	0.01	0.3	0.000	0.00	0.0
4.5% ABV Lager	60	0.311	1.87	111.9	2.234	13.40	804.1
	65	0.076	0.46	27.4	0.126	0.75	45.3
	66.7	0.047	0.28	17.0	0.047	0.28	17.0
	70	0.019	0.11	6.7	0.007	0.04	2.5
	75	0.005	0.03	1.6	0.000	0.00	0.1
	80	0.001	0.01	0.4	0.000	0.00	0.0
	85	0.000	0.00	0.1	0.000	0.00	0.0

beef (Ma et al. 2007).

The heat resistance of *Lactobacillus brevis* BSO 566 agreed with previous work (Rachon et al. 2018, 2021, 2022). D_{60} values of beer spoilage *Lactobacillus* E93 from unpasteurised beer (Adams et al. 1989), are similar to those reported here for standard (alcoholic/premium) beer; D_{60} = 0.31 vs D_{60} = 0.22 and D_{60} = 0.13 minutes. However, the results are different for non-alcoholic beers; D_{60} = 2.56 vs D_{60} = 0.71 minutes and D_{60} = 0.72 minutes. As the same methodology was used, these results reflect differences in the production and composition of alcohol-free beers over the last 35 years.

L'Anthoën and Ingledew (1996) investigated the heat resistance of *Lactobacillus dulbrueckii* in premium and alcohol-free beer. The highest temperature used in their study was 56.9°C so their results are difficult to compare. Nevertheless, their results for 54 and 56°C were significantly higher which may be explained by differences in the products. Similarly, Tsang and Ingledew investigated the heat resistance of *L. delbrueckii*, *L. frigidus* in 5% ABV lager (pH 4.0) but the highest temperature at which resistance was measured was lower at 47 and 53°C (Tsang and Ingledew 2018). As with *Pediococcus*, the heat resistance of *Lactobacillus* has been investigated in different products. Tajchakavit et al (1998) reported D_{60} = 22 seconds for *Lactobacillus plantarum* ATCC 14917 in apple juice. Jordan and Cogan (1999) reported heat resistance data for *L. plantarum* (D_{57} = 1.35 minutes and D_{57} = 0.56 minutes) and *L. paracasei* (D_{60} = 22.5 minutes and D_{60} = 14.7 minutes) in respectively reconstituted skim milk and MRS broth. The z-values for these strains were like those reported here (z = 5.3-6.7°C) (Tables 3 and 4).

The heat resistance of *Enterococcus faecium* NRRL B-2354 in beer has not been assessed, as the published data relates to low moisture foods. Annous and Kozempel (1998) reported D_{60} values in a range of low pH products; D_{60} = 0.14 and 0.70 minutes in pineapple juice (pH = 3.70), D_{60} = 0.33 and 1.05 minutes in apple juice (pH = 3.92) and a D_{60} = 0.65 and 1.36 minutes in tomato juice (pH = 4.45). The D_{60} values reported in this study; $D_{60\text{-buffer}}$ = 2.59 and 2.14 minutes, $D_{60\text{-0.0\%Lager}}$ = 2.78 and 2.03 minutes, $D_{60\text{-4.5\%Lager}}$ = 0.42 and 0.31 minutes (Tables 3 and 4) were similar especially for those products at comparable pH. Martinez et al (2003) reported a D_{70} between 0.32-1.73 minutes in Sorensen buffer at pH 7 and showed the impact of growth temperature and the age of cultures. This strain has been used in the food industry for the validation of heat treatment of low moisture products. There is no relevent data in fermented beverages. The Almond Board of California (2014) published guidelines for using this microorganism as a pathogenic surrogate for the validation of commercial almond processing. Generally, the heat resistance of microorganisms increases by decreasing the moisture (Gu et al. 2022), so such results cannot be compared to those from fermented low pH beverages. The D-values of *E. faecium* in low moisture foods are measured at much higher temperatures. Ceylan and Bautista (2015) measured the heat resistance of *E. faecium* in pet food between 76.7-87.8°C and reported D-values between 13.8-1.1 minutes. For cornmeal, Gu et al (2022) reported a D_{60} of 3.3, 18.9 and 140 min at moisture values of 28, 22 and 16%.

It was anticipated that the results obtained using the capillary test tubes and the flask method would be similar as factors contributing potential errors were eliminated (Pflug 2003). In particular, the temperature of the medium was measured accurately, as most heat inactivation trials (except those performed at higher temperatures) use heating times that allow the test matrix to rapidly reach the target temperature. Here, in both methods, lags in heat-transfer, first in the heating and later in the cooling of the test units were negligible.

Conclusions

This study showed that the bacterium, *Enterococcus faecium* NRRL B-2354 to be more heat resistant than *Lactobacillus brevis* or *Pediococcus acidilactici*. As *E. faecium* is used as a pathogenic surrogate in the food industry for the validation of thermal and non-thermal processes it could also be used in studies with beers or other fermented beverages. The results also suggest that at higher temperatures (>65°C), this microorganism is more heat resistant than yeast ascospores and would be of value when validating the flash pasteurisation of alcoholic and non-alcoholic beverages.

Author Contributions

Grzegorz Rachon: Conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing (original draft), visualisation, supervision, project administration.
Christopher Raleigh: Methodology, validation, investigation, formal analysis.
Harry Rothera: Methodology, validation, investigation, formal analysis, data curation.

Conflict of interest

The authors declare no conflict of interest.

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