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The impact of isomerised hop extract on the heat resistance of yeast ascospores and *Lactobacillus brevis* in premium and alcohol-free lager

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The effect of isomerised hop extract on the heat resistance of *Saccharomyces cerevisiae* BRYC 501 ascospores and *Lactobacillus brevis* BSO 566 was investigated. Heat resistance of yeast ascospores and *L. brevis* was determined in premium lager (4.5% ABV) and alcohol-free lager (0% ABV) at their initial bitterness and after adjustment with isomerised hop extract to 25 and 50 IBU. Results showed that D_{60} of yeast ascospores in alcohol-free lager was reduced by >30% at 25 IBU and >50% at 50 IBU compared to lager with no added hop extract. In premium lager, D_{60} was reduced by >10% at 25 IBU and >30% at 50 IBU. The addition of isomerised hop extract also had a significant impact on the z-values, which increased with higher concentrations of isomerised hop acids in both premium and alcohol-free lager. Slightly higher z-values were observed in premium lager (z = 4.0, 4.5 and 5.0°C for IBU = 6.3, 25 and 50 respectively) than in alcohol-free lager (z = 3.6, 4.0 and 4.2°C for IBU = 8.6, 25 and 50). The addition of isomerised hop extract also reduced the heat resistance of *Lactobacillus brevis* BSO 566. The findings in this study suggest that less bitter (low IBU) lagers should be pasteurised with higher pasteurisation units (PUs) than more bitter lagers (high IBU) which can be pasteurised at lower PUs.

Keywords: Lager; pasteurisation; hop extract; ascospores; *Lactobacillus brevis*; heat inactivation

Introduction

The impact of hop extracts on the behaviour of microorganisms has been explored by many researchers *(1–5)* but there is no published data on the impact of hop extracts on the heat resistance of yeast or *Lactobacillus*. Our earlier work *(6)* showed that heat resistance of beer spoilage microorganisms was significantly lower in more bitter, German style lagers, and higher in less bitter, American style lagers. Consequently, the calculated pasteurisation units (PU) required for German style (more bitter) were half those for the American style lagers. In this work, we sought to validate this hypothesis and show the extent of the impact of hop extracts on heat resistance.

Individual hop resin compounds are differentially effective. Although the α-acids and β-acids are generally more effective against bacteria than iso α-acids, in practice, their solubility in beer is significantly lower and, accordingly, their antimicrobial efficiency is less than for iso α-acids *(7,8)*. Beer spoilage bacteria including *Lactobacillus* and *Pediococcus* species are resistant to the antimicrobial effects of humulone, colupulone, and transisohumulone whereas strains that do not spoil beer are sensitive *(7)*. Mitchel et al. *(1)* showed that different hop compounds inhibit beer spoilage bacteria at different levels and that tetrahydro iso α-acids and α-acids inhibited the growth of spoilage bacteria the most.

The mechanism of inhibition of sensitive bacterial cells is well understood *(8, 9)*. β-acids, α-acids and iso α-acids act as mobile carrier type ionophores and attack the plasma membranes of susceptible cells leading to the dissipation of the trans-membrane proton gradient which, in turn, leads to a decrease in the proton motive force, starvation and cell death. Hop-resistant bacteria can protect themselves from hop compounds in various ways. These include a horA-gene dependent hop resistance mechanism and use a multidrug resistance pump (HorA) or a proton motive force -dependent transporter as well as by pumping out protons by overexpressed H +ATPase following exposure to hop resins.

The aim of this study was to determine if hop extract impacted on the heat resistance of yeast ascospores and *Lactobacillus brevis* when added to lager at two levels (25 and 50 IBU) which mimic medium and high bitter beers.

Materials and methods

Beers

Two American-style lagers, a premium lager (4.5% ABV) and an alcohol-free lager (0.0% ABV) of low bitterness were purchased locally. The raw materials used in the production of both lagers included barley malt, rice, hops and natural flavours. Bitterness, pH and ABV of the lagers was measured and the concentration of carbohydrates, sugars and protein was taken from the package labels (Table 1). Bitterness was measured by spectrophotometry using EBC Analytica method 9.8 and was expressed as IBU (International Bitterness Units), ABV was measured as ethanol by volume by gas chromatography (GC) using EBC Analytica method 9.3.2. The pH

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Table 1. Beer parameters

Parameters	Alcohol free lager	Premium lager
ABV (%) - label	0.0	4.5
ABV (%) - analysed	0.0	4.5
pH	4.4	4.4
Bitterness (IBU)	8.6	6.3
Carbohydrates (g/100mL)	3.3	3.1
Sugars (g/100mL)	0.1	0.1
Protein (g/100mL)	0.1	0.3

was measured with an AR15 pH meter (Accumet Research, USA). Both lagers were supplemented with the isomerised hop extract (ISOLONE®, supplied by Kalsec Europe Limited) containing 28.5-31.5% of iso-alpha acids. Lagers were supplemented with this extract to 25 and 50 IBU. In addition, unsupplemented beers (of initial low bitterness) were also evaluated. Lagers (10 mL) were supplemented with 10 μL of isomerised extract in ethanol. Concentration of the isomerised extract solution (10 μL supplement) for adjusting bitterness to 25 and 50 IBU was established in preliminary trials where 1 mg/L of isomerised alpha acids = 1 IBU. The measured bitterness of supplemented lagers was lower than calculated but successfully adjusted and verified by measurement of IBU.

Microorganisms

Saccharomyces cerevisiae BRYC 501 (yeast which produces the most heat resistant ascospores) *(6)* and *Lactobacillus brevis* BSO 566 (the most heat resistant bacterium in our previous study) *(10)* were used in this work and were stored in liquid nitrogen at -196°C (Air Products, UK).

Ascospore production and preparation of testing solution

Saccharomyces cerevisiae BRYC 501 was resuscitated from liquid nitrogen in Yeast and Mould broth (YM; Oxoid, UK) at 25°C for 2 days. The culture (0.2 mL) in the exponential phase growth was spread on plates of Ascospore Agar (AA; HiMedia, India) and incubated aerobically for 10 days. The surface of AA plates was then covered with 10 mL of sterile distilled water and the ascospores were removed using a disposable sterile L-shape spreader. The presence of ascospores was confirmed microscopically *(6)* as follows. Heat fixed slides were flooded with 1% (w/v) brilliant green (Sigma-Aldrich UK) and gently heated from underneath with a Bunsen burner. Once the stain was warm and began to evaporate, the heat source was removed and reapplied once the evaporation stopped. The heating process was maintained for 10 minutes. Any residual stain was washed off with tap water and slides were counterstained with safranin (Sigma-Aldrich UK) for 40 seconds. Slides were examined microscopically at 400 x and 1,000 x (oil immersion) magnification. In addition, wet mounts (unstained) were prepared, and the presence of spores was determined. The ascospore suspension was centrifuged in a microcentrifuge at 3,000 x *g* for 5 minutes and the pellet was re-suspended in the test liquid prior to use. The ascospore solution was stored at 2-4°C and used within one month of preparation.

Heat inactivation of yeast ascospores in premium and alcohol-free lager

The heat resistance of *Saccharomyces cerevisiae* BRYC 501 ascospores was determined at four temperatures (58, 60, 62 and 64°C) in premium lager and at 60, 62, 64 and 65°C in alcohol-free lager. Heat resistance of ascospores was studied at these temperatures as they have the most impact on heat resistance and result in a wide range of D-values. Measurement of the heat resistance of ascospores in premium lager at 65°C was not possible due to rapid inactivation of microorganisms under these conditions. Lagers were investigated at three bitterness values: initial bitterness (6.3 for premium lager and 8.6 for alcohol-free lager) and supplemented to 25 and 50 IBU. The capillary tubes method was as described previously *(6, 10)* with the test solution (50 μL) inoculated with microorganisms at 10^7-10^8 CFU/mL pipetted into soda glass capillary tubes G119/02 (Fisher Scientific, UK). The tube ends were heat sealed and then placed in a water bath at the test temperature (58, 60, 62, 64 or 65°C) and held for the required pre-established time. Although the ramp up time (heating time required to reach the target temperature) was not determined in this study, Rachon et al. *(6)* Jordan et al. *(11)* and Basaran-Akgul *(12)* showed that the ramp up time in glass capillary tubes was minimal (7-10 seconds) but is included in the holding times reported here. After each heat interval the tubes were removed from the water bath and were cooled in ice water. The test suspension from the capillary tubes was recovered in Maximum Recovery Diluent (MRD; Oxoid, UK) and the number of viable cells enumerated by spread plating. Live yeast spores were recovered on YM agar and colonies were counted after 10 days of aerobic incubation at 25°C. D- and z-value calculations and statistical analysis (one-way ANOVA) were performed using Minitab 20 software.

Each experiment was performed in triplicate. Preliminary trials were performed to select suitable holding times, to ensure an adequate $\log_{10}$ decrease in viable microorganisms. For each heat inactivation trial, the number of viable cells was enumerated at a minimum of five holding times.

Heat inactivation of *Lactobacillus brevis* in premium and alcohol-free lager

The heat resistance of *Lactobacillus brevis* BSO 566 was measured in premium lager and alcohol-free lager at three bitterness values: initial bitterness (6.3 for premium lager and 8.6 for alcohol-free lager) and at 25 and 50 IBU. *Lactobacillus brevis* BSO 566 was recovered from liquid nitrogen and was grown anaerobically in de Man, Rogosa and Sharpe Broth (MRSB; Oxoid, UK) at 25°C for 2 days. The broth was centrifuged at 3,000 x *g* for 5 minutes and the pellet was resuspended in the test lager. The bacterial heat resistance was measured at one temperature (60°C) using the capillary tube method described above. Live cells were recovered on de Man, Rogosa and Sharpe Agar (MRSA) after anaerobic incubation at 25°C for 10 days. The D_{60} values were calculated, and statistical analysis (one-way ANOVA) performed using Minitab 20 software. The heat resistance (D_{60}) of the tested *Lactobacillus* spp in different bitterness values was then compared.

Calculating the minimum pasteurisation requirement

Based on the determined D- and z-values, the minimum PU required for the elimination of 6 logs of yeast ascospores in the lager were calculated.

Results

Lagers were supplemented with isomerised hop extract to achieve a bitterness of 25 and 50 IBU. Preliminary results (not shown) showed no significant changes to pH or ABV of the lagers.

The results of the yeast ascospore and *Lactobacillus brevis* heat inactivation experiments in premium lager and alcohol-free lager showed that all inactivation curves were linear (Figures 1, 2), and first order kinetics were suitable to describe the inactivation patterns. All R^2 values were above 0.95 except for three cases; two in alcohol-free lager at 65°C where the R^2 values were 0.935 and 0.945 for 8.6 and 25 IBU respectively and in one case in premium lager at 62°C where R^2 was 0.932. The D-values for ascospores at all four temperatures and the z-values for all bitterness values in both lagers were calculated and reported in Table 2. The D_{60} values for *Lactobacillus brevis* BSO 566 are shown in Table 3.

The addition of isomerised hop acids significantly reduced the heat resistance of yeast ascospores of *Saccharomyces cerevisiae* BRYC 501 in premium lager and alcohol-free lager. The D_{60} of yeast ascospores in alcohol-free lager was reduced by over 30% at 25 IBU and by over 50% at 50 IBU. In premium lager, the impact of isomerised acids on the heat resistance of yeast ascospores was lower; D_{60} was reduced by over 10% at 25 IBU and by over 30% at 50 IBU. However, these changes were statistically significant as shown by one-way ANOVA ($p<0.05$). The addition of isomerised hop extracts also had a significant impact on z-values, which increased with higher concentrations of isomerised hop acids in both premium lager and alcohol-free lager. Slightly higher z-values were recorded in premium lager (z = 4.0, 4.5 and 5.0 for IBU = 6.3, 25 and 50 respectively) than in alcohol-free lager (z = 3.6, 4.0 and 4.2 for IBU = 8.6, 25 and 50 respectively) (Table 3).

The addition of isomerised hop extracts also reduced the heat resistance of *Lactobacillus brevis* BSO 566. D_{60} of this strain was reduced by over 20% (at 25 IBU) and by over 35% (at 50 IBU) in alcohol-free lager and by >10% (at 25 IBU) and over 35% (at 50 IBU) in premium lager (all differences are statistically significant; $p<0.05$) (Table 3).

Discussion

This study showed that the addition of isomerised hop extract at 25 and 50 IBU significantly reduces the heat resistance of the *Saccharomyces cerevisiae* BRYC 501 ascospores and *Lactobacillus brevis* BSO 566 in premium and alcohol-free lager. This suggests that less bitter lagers with low IBU, should be pasteurised with higher PUs than more bitter lagers (medium and high IBU) which can be pasteurised at lower PUs. This was highlighted in our earlier study *(6)* where an American-style lager (5.6 and 6.9 IBU), which had a significantly lower bitterness than the German lager (25-26 IBU), required higher pasteurisation units. There are no publications investigating the effect of bitterness or hop extracts on the heat resistance of yeast or beer spoilage bacteria and this study is the first report to investigate this topic. Furthermore, there are few published studies that investigated the heat resistance of beer spoilage microorganisms in beer. Kilgour and Smith *(13)* reported the D_{60} of ascospores from two *Saccharomyces* species in alcohol-free beer (D_{60} = 7.7–23 minutes; z = 3.9-4.1°C) and in 3.7% ABV beer (D_{60} = 1.7–2.9 minutes; z = 5.6–6.9°C) which are similar to those reported here. Interestingly, Kilgour and Smith also reported lower z-values for alcohol-free beers and slightly higher z-values for 3.7% ABV beers. Milani et al. *(14)* reported the highest D-values for ascospores of *Saccharomyces cerevisiae* strains. They

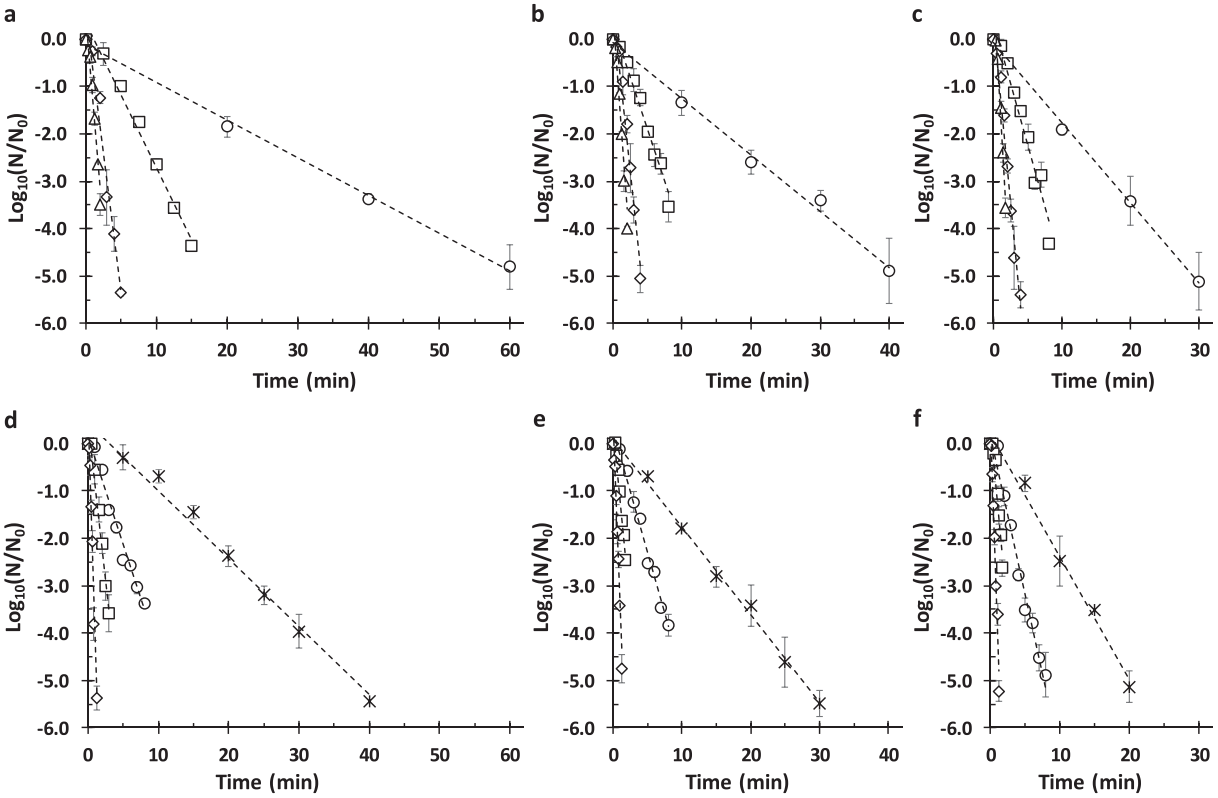

Figure 1. Inactivation curves for *Saccharomyces cerevisiae* BRYC 501 in alcohol-free lager at different bitterness values: a) 8.6 IBU, b) 25 IBU, c) 50 IBU and in premium lager d) 6.3 IBU, e) 25 IBU and f) 50 IBU at 58°C (✱), 60°C (○), 62°C (□), 64°C (◊) and 65°C (△)

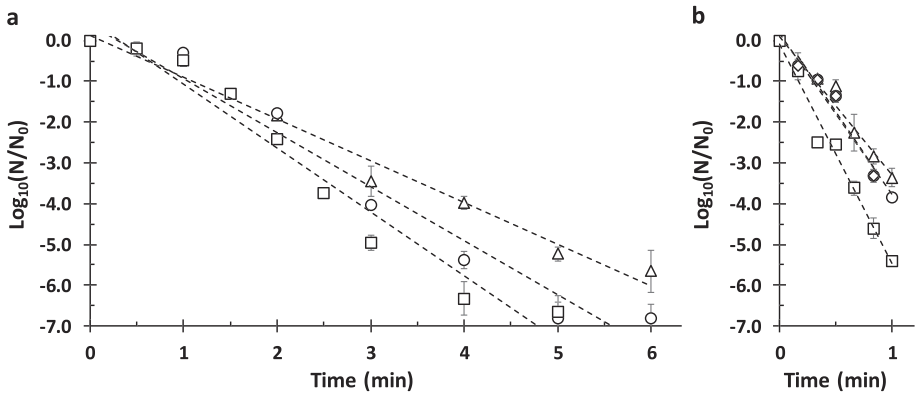

Figure 2. Inactivation curves for *Lactobacillus brevis* BSO 566 at 60°C in a) alcohol-free lager and b) premium lager at different bitterness values: initial IBU (△), 25 IBU (○) and 50 IBU (□)

Table 2. D-values and z-values of *Saccharomyces cerevisiae* BRYC 501 in beer together with 95% Confidence Interval (95 % CI), standard error (SE), R-squared and the calculated minimum pasteurisation units ('min. PU') to achieve a 6-log reduction in ascospores

Beer	Bitterness (IBU)	Temp (°C)	D (min)	95% CI	SE	R^2	z- value	SE	R^2	min. PU's
Non-alcoholic lager (0.0% ABV)	8.6	60	**12.54**	(11.4, 13.9)	0.535	0.997	**3.6**	0.16	0.996	**83**
		62	**3.29**	(3.09, 3.52)	0.103	0.986				
		64	**0.87**	(0.77, 0.98)	0.049	0.964				
		65	**0.56**	(0.50, 0.65)	0.037	0.935				
	25	60	**8.59**	(7.80, 9.56)	0.401	0.994	**4.0**	0.21	0.995	**57**
		62	**2.30**	(2.11, 2.52)	0.099	0.974				
		64	**0.74**	(0.66, 0.83)	0.040	0.955				
		65	**0.49**	(0.44, 0.55)	0.028	0.945				
	50	60	**5.94**	(5.28, 6.80)	0.339	0.998	**4.2**	0.04	0.999	**41**
		62	**1.95**	(1.77, 2.17)	0.094	0.957				
		64	**0.67**	(0.61, 0.73)	0.028	0.977				
		65	**0.37**	(0.33, 0.42)	0.019	0.977				
Premium lager (4.5% ABV)	6.3	58	**6.97**	(6.52, 7.50)	0.237	0.980	**4.0**	0.10	0.999	**14**
		60	**2.19**	(2.05, 2.34)	0.071	0.977				
		62	**0.76**	(0.68, 0.87)	0.043	0.968				
		64	**0.21**	(0.19, 0.24)	0.012	0.950				
	25	58	**5.40**	(5.06, 5.80)	0.176	0.996	**4.5**	0.09	0.999	**12**
		60	**1.94**	(1.83, 2.06)	0.056	0.986				
		62	**0.66**	(0.60, 0.74)	0.032	0.957				
		64	**0.26**	(0.24, 0.29)	0.013	0.959				
	50	58	**3.87**	(3.52, 4.29)	0.178	0.990	**5.0**	0.12	0.999	**10**
		60	**1.48**	(1.39, 1.60)	0.052	0.981				
		62	**0.64**	(0.57, 0.74)	0.041	0.932				
		64	**0.23**	(0.22, 0.26)	0.010	0.969				

Table 3. D-values of *Lactobacillus brevis* BSO 566 in beer together with 95% Confidence Interval (95% CI), standard error (SE), R-squared and the calculated difference of D_{60} in %

Beer	Bitterness (IBU)	D_{60} (min)	95% CI	SE	R^2	δ D_{60} (min)
Non-alcoholic lager (0.0% ABV)	8.6	**0.98**	(0.90, 1.07)	0.040	0.979	N/A
	25	**0.76**	(0.68, 0.84)	0.038	0.959	-23%
	50	**0.62**	(0.57, 0.69)	0.029	0.956	-36%
Premium lager (4.5% ABV)	6.3	**0.29**	(0.26, 0.34)	0.017	0.968	N/A
	25	**0.25**	(0.23, 0.28)	0.011	0.974	-14%
	50	**0.19**	(0.17, 0.20)	0.007	0.978	-37%

reported D_{60} values between 4.6 and 11.2 minutes and z-values between 11.7 and 14.3°C. These values are significantly higher than the values reported for other (non-*Saccharomyces*) species. Garg *(15)* reported D_{55} of 15.33 minutes for ascospores from *Zygosaccharomyces bailii*, Raso et al. *(16)* reported D_{50} for *Zygosaccharomyces bailii* ascospores in different fruit juices between 10.4 and 37 minutes. However, despite using the same yeast strain as Milani et al. *(14)*, the heat resistance of ascospores was significantly different in the two studies. According to Milani et al. the D_{60} of *Saccharomyces cerevisiae* ATCC 9080 in degassed and filtered 4% ABV beer was 4.6 minutes and z = 12.4°C but in our study, the values were significantly lower with D_{60}-values for premium lager between 1.48 and 2.19 minutes with z = 4.0 – 5.0°C. This may be explained by differences in the methodology used for the preparation of ascospores (growth medium, washing), differences in the enumeration of microorganisms (recovery agar, incubation temperature/time) and/or significant differences in the methods used for the heat inactivation trials (pouches, capillary tubes, minimum number of timepoints, level of inactivation, pre-treatment of beer).

The D- and z-values reported in Table 2 suggest that the heat resistance of yeast ascospores and spoilage bacteria are not only affected by the alcohol content of the lager, but also its bitterness. In this study, the bitterness of lagers was adjusted by a commercial hop extract (ISOLONE®) which may have a different impact on the heat resistance of microorganisms to other extracts or hop products. There may also be other unidentified compounds in beer that could influence the survival of ascospores when heat treated. Whilst the effect of alcohol on the heat resistance of microorganisms *(14,17,18)* is well documented, the effect of bitterness has, to our knowledge, not been reported before. However, there is a report of hop acids affecting yeast viability *(19)*.

This study also showed that the heat resistance of yeast ascospores in alcohol-free lager was 2.9-6 times more than in premium lager but also 9.5-20 times greater than the heat resistance of *Lactobacillus brevis*. This microorganism was also the most heat resistant vegetative bacterium when tested in ale and stout in our previous study *(10)* and has shown similar resistance when tested in American and German style lagers *(6)*. This indicates that when yeast ascospores are effectively inactivated during thermal processing, any vegetative spoilage bacteria should also be inactivated at significantly greater numbers. Several studies report that the heat resistance of vegetative bacteria is significantly lower than the heat resistance of yeast ascospores *(14,17, 20–23)*.

The D_{60} values for the *Lactobacillus* strains tested in this study are similar to those published by other researchers. According to Adams et al. *(24)*, the D_{60} for *Lactobacillus* E93 was 0.31 minutes in 4.4% ABV lager and 2.56 minutes in alcohol-free lagers. According to Reveron et al. *(25)*, the D_{60} of *Lactobacillus paracasei* in Pilsner beer was 0.02 minutes and L'Anthoën and Ingledew *(17)* reported D_{55} for *Lactobacillus delbrueckii* of 7.6 minutes in alcohol-free lager and 2.8 minutes in 5% ABV lager. However, in a small number of reports the heat resistance of vegetative bacteria was much higher. For example, Oliver-Daumen *(26)*, reported that D_{59} = 0.7 minutes, D_{69} = 0.5 minutes, and z = 54.8°C for *Lactobacillus lindneri* in alcohol-free lager. However, in this thesis, the z-value was calculated from only two temperature data points. Similarly, L'Anthoën and Ingledew's *(17)* reported a D_{60} for *Pediococcus acidilactici* of 7.66 minutes for alcohol-free lager and 1.33 minutes in 5% ABV lager. However, the D_{60} values were extrapolated from a thermal death curve between 47.0-53.3°C and 49.2-55.0°C and therefore the reported D- and z-values are less reliable.

Conclusion

Isomerised hop extract reduces the heat resistance of beer pasteurisation indicator organisms – *Saccharomyces cerevisiae* BRYC 501 ascospores and *Lactobacillus brevis* BSO 566 – in both premium lager and alcohol-free lager. This suggests that less bitter beers (low IBU) should be pasteurised at higher PUs than more bitter beers (high IBU) which can be pasteurised at lower PUs. Differences in PU requirements were more significant in the non-alcoholic lagers than in premium lagers. This work will enable brewers to adjust pasteurisation parameters for specific beers and guarantee product safety while maintaining environmental sustainability.

Author contributions

Grzegorz Rachon: conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing (original draft, review and editing), visualization, supervision, project administration, funding acquisition.
Christopher Raleigh: formal analysis, writing (review and editing).
Gail Betts: writing – writing (review and editing), funding acquisition.

Conflict of Interest

The authors declare no conflict of interest.

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