Received: 02/07/2024 Revised: 10/08/2024 Accepted: 29/09/2024 Published: 16/10/2024

ORIGINAL ARTICLE

DOI 10.58430/jib.v130i4.61

INSTITUTE OF BREWING & DISTILLING

Survival of *Escherichia coli* O157, *Salmonella* Enteritidis, *Bacillus cereus* and *Clostridium botulinum* in non-alcoholic beers

- Grzegorz Rachon
- Harry Rothera
- Sabina O'Reilly
- Gail Betts

Campden BRI, Station Road, Chipping Campden, GL55 6LD, UK

Grzegorz.Rachon@campdenbri.co.uk

Abstract

Why was the work done: To (i) determine whether microbial pathogens were present in packaged alcohol-free and low alcohol beers, (ii) to assess whether pathogens can survive or grow in non-alcoholic beers, and (iii) to determine the impact of pH and bitterness on their growth and survival of pathogens in alcohol-free beer.
How was the work done: : 50 alcohol-free and low alcohol beers, available in the UK, were screened for pathogens and analysed for ABV, pH and bitterness (IBU). One of the alcohol-free beers (with the lowest IBU) was adjusted to 25 and 50 IBU and pH 3.8, 4.2, 4.6 and 4.9. Challenge testing of these beers was performed with *Escherichia coli* O157, *Salmonella* Enteritidis, *Bacillus cereus* and *Clostridium botulinum*. In addition, the heat resistance (D_{60} value) of the pathogens, spoilage bacteria and *Saccharomyces cerevisiae* ascospores in these beers was determined.
What are the main findings: *Salmonella*, *E. coli*, *Enterobacteriaceae*, *Bacillus cereus* and sulphite reducing Clostridia were not found in any of the 50 beers. However, two emerging opportunistic pathogens (*Cupriavidus gilardii* and *Sphingomonas paucimobilis*) were found in the low alcohol keg beers. None of the pathogens used in this study could grow in the alcohol-free beer at low pH (pH 3.8). *E. coli* O157 was unable to grow at pH 4.2 but could grow at pH 4.6 but only with reduced levels of carbon dioxide and increased oxygen. *Salmonella* Enteritidis was able to grow at pH 4.2 and 4.6 but also with reduced levels of CO_2 and increased O_2. Although *Bacillus cereus* and *C. botulinum* were unable to grow in any of the tested conditions, both pathogens were able to survive. Survival and/or growth of the microorganisms was impacted by pH; bitterness had no effect.
Why is the work important: *Salmonella* Enteritidis and *E. coli* O157 only grew in alcohol free beer at a higher pH (4.2 and 4.6 for *Salmonella* and 4.6 for *E. coli*) together with with reduced levels of CO_2 and increased O_2. This suggests that packaged beer with appreciable levels of carbon dioxide and negligible levels of oxygen will not support the growth of pathogens. However, draught alcohol free beer may be vulnerable to pathogens.

Keywords

Non-alcoholic beer, pathogens, *Salmonella*, *Escherichia coli*, heat resistance, yeast ascospores

Introduction

Pathogens are unable to grow in alcoholic beers (Menz et al. 2011) and only some, those producing spores, are able to survive for a prolonged time in the presence of alcohol (Munford et al. 2017). Vegetative pathogens such as *Escherichia coli* or *Salmonella* survive for a short time (Gaglio et al. 2017) or are rapidly inactivated by other factors. Beers are protected from pathogens by low pH, the presence of hops, alcohol, high carbonation and low oxygen level. If one or more of these factors is removed, the risk of the growth or survival of pathogens increases. Accordingly, the absence of ethanol in low or alcohol-free beers makes them more susceptible to microbial spoilage (Quain 2021) and enhances the possible survival (or growth) of pathogenic bacteria.

The survival of pathogens in non-alcoholic beers is poorly understood as there are few reported studies (Menz et al. 2011, Kim et al. 2014, Cobo et al. 2023). The pathogens of concern are those that can survive or grow at low pH (<4.5), in the presence of carbon dioxide with negliable levels of oxygen at ambient temperature. Potentially such microorganisms include species of *Salmonella*, *E. coli* or *Listeria* (US Food and Drug Association/FDA. 2012; Jones 2012). These microorganisms do not produce spores, are not heat resistant and would be eliminated by the pasteurisation. However, they may cause problems in unpasteurised beers, or contaminate beers after sterile filtration, and in draught dispense systems with low and alcohol-free beers (Quain 2021). Spore forming pathogenic microorganisms such as *Bacillus cereus* or *Clostridium botulinum* will survive pasteurisation and could grow in beer but at a higher pH (Munford et al. 2017). The minimum pH for the growth of *Bacillus* species is 4.3 (Jones 2012) but they are sensitive to hops (Rój et al. 2015, Munford et al. 2017). However, they can potentially grow in non-alcoholic beer with low bitterness and a higher pH.

Escherichia coli

E. coli are gram-negative, facultative anaerobes, and non-spore forming. The minimum pH for the growth of *E. coli* is 4.0 but some strains such as *E. coli* O157 – verocytotoxigenic (VTEC) or Shiga like toxin-producing (STEC) - can survive at pH 3.6.

The optimum growth temperature for *E. coli* is 37°C but the bacterium can grow between 7-46°C. *E. coli* is a not heat-resistant with a D_{63} = 0.5 minutes, and is killed by mild pasteurisation. The most common foods associated with contamination by *E. coli* are ground meats, unpasteurised (‘raw’) milk, unpasteurised fruit juice, lettuce, spinach, or sprouts (FDA. 2012; Jones 2012; EU. 2024).

Shiga like toxin-producing (STEC) *E. coli* can cause serious illness, including diarrhoea, blood clotting, kidney failure, and death. Globally, STEC causes >2,800,000 acute illnesses annually and leads to almost 4,000 cases of haemolytic uraemic syndrome, 270 cases of end-stage renal disease and 230 deaths (Majowicz et al. 2014).

Salmonella

For growth of *Salmonella,* the minimum pH is 4.1 but some strains can survive at pH 3.8. The optimum growth temperature is 37°C with a range of 7-46°C. *Salmonella* is a facultative anaerobe growing in the presence or absence of oxygen and is tolerant to the presence of CO_2. *Salmonella* is not affected by hop compounds and is relatively easy to eliminate by pasteurisation with a D_{60} = 1-10 minutes (Jones 2012). *Salmonella* Typhimurium accounts for most foodborne illnesses and annually almost one in ten people fall ill and losing 33 million of ‘healthy life years’ (the number of years that a person is expected to continue to live in a healthy condition). *Salmonella* is one of four global causes of diarrhoeal diseases (WHO 2018) and is a ubiquitous and hardy bacterium that can survive for weeks in a dry environment and several months in water. *Salmonella* bacteria are often found in raw meat, undercooked poultry, eggs or unpasteurised milk with several cases linked with water and soft drinks (FDA. 2012; Jones 2012; Billah and Rahman 2024; EU. 2024)

Listeria monocytogenes

The minimum growth pH for *L. monocytogenes* is 4.4 with an optimum growth temperature between 30-37°C. Significantly, *Listeria* can grow at refrigeration temperatures (0-4°C) but growth is slow, requiring days to double in numbers. *Listeria* is not a heat-resistant organism, and is destroyed by mild pasteurisation.

Typical D-values in foods for *Listeria* range between 5-8 minutes at 60°C and 0.1 – 0.3 minutes at 70°C depending on the strain and matrix (Gandhi and Chikindas 2007; FDA 2012; Jones 2012). *L. monocytogenes* causes listeriosis with fever, muscle aches, nausea, vomiting and diarrhoea. In the USA, there about 1,600 cases each year with some 260 deaths (Center for Disease Control 2024). The foods most commonly associated with *Listeria* are meat, cheese and salad, hot dogs, fermented or dry sausages, pâté or meat spreads, cold smoked fish, sprouts, melons, raw (unpasteurised) milk and associated products (EU. 2024). However, as the minimum pH for the growth of *Listeria monocytogenes* is comparatively high (4.4) it was not considered in this study.

Bacillus cereus

Bacillus cereus is a gram-positive, facultative anaerobe, endospore forming, large rod. *B. cereus* can grow between pH 4.3-9.3 and between 4-48°C with an optimum of 28-35°C. *B. cereus* produces heat resistant spores which will survive pasteurisation with D_{85} = 33.8-106 minutes and D_{95} = 1.5-36.2 minutes (Jones 2012; Logan 2012). There are two types of illness caused by toxins of *B. cereus*: emetic and diarrhoeal. The emetic type is associated with ingestion of preformed toxin leading to nausea and vomiting. The diarrhoeal type results from ingestion of vegetative organism or spores with subsequent germination and toxin production in the intestinal tract leading to watery diarrhoea and occasional nausea. Although rare, the emetic enterotoxin of *B. cereus* foodborne illness has been implicated in liver failure and death in otherwise healthy individuals. A wide variety of foods, including meats, milk, vegetables, and fish, have been associated with diarrheal-type food poisoning. Vomiting outbreaks have been associated with rice products; with other starchy foods also implicated. Other foods include sauces, puddings, soups, casseroles and pastries. *B. cereus* cannot grow in alcoholic beers but there is insufficient evidence about growth in non-alcoholic beers. Unpublished work at Campden BRI suggests that hop compounds in beer suppress the growth of *B. cereus*, but it is not known what the limiting bitterness would be in alcohol-free or low alcohol beers (Ehling-Schulz et al. 2019).

Clostridium botulinum

C. botulinum is a strict anaerobe, gram-positive, spore-forming rod that produces a neurotoxin. The minimum pH for growth is 4.6 (proteolytic strains, group I) and pH 5 (non-proteolytic strains, group II) (Jones 2012). All *C. botulinum* strains grow in the temperature range 20-45°C, with the minimum growth temperature of 10°C (proteolytic) and 3°C (non-proteolytic strains). Spores of *C. botulinum* are heat resistant, surviving pasteurisation with a decimal reduction time of D_{121} = 0.13 minutes and z = 11°C. These values were adopted by the food industry with a safety margin ('botulinum cook') to achieve a 12-log reduction after 3.6 minutes at 121°C (FDA. 2012; Jones 2012). Ingestion of the *C. botulinum* toxin causes 'foodborne botulism' which is a serious, sometimes fatal, disease caused by the neurotoxin. Symptoms include malaise, dizziness, diarrhoea, vomiting with, during later stages, speech, vision and swallowing affected. Foods associated with botulism are canned meats, fish and vegetables, home cured sausage (Rawson et al. 2023; Tiwari and Nagalli 2024). There were no recorded cases linking *C. botulinum* with beverages. Although *Clostridium* are sensitive to hops (Johnson and Haas 2001) it is not known if these microorganisms can grow in non-alcoholic, low bitterness beers. Certainly, the increasingly stringent oxygen specifications for packaged beer (standard and alcohol free) may be a suitable environment for *Clostridium* as a strict anaerobe.

Aims of this work

There were several objectives in this study; (i) to evaluate whether there were any pathogens in packaged non-alcoholic beers from the UK market, (ii) to determine the ability of pathogens - *E. coli*, *Salmonella*, *B. cereus* and *C. botulinum* - to grow and survive in alcohol-free beer, (iii) to determine the limits of bitterness and pH which allow pahtogenic microorganisms to grow in beer and (iv) to measure the heat resistance (D_{60}) of *Salmonella* Senftenberg 775W (Ng et al. 1969) and *E. coli* O157 (Kim and Song 2023) in beers at different bitterness and pH compared to that of *Saccharomyces cerevisiae* BRYC 501 (pasteurisation bioindicator) (Rachon et al. 2021) and *Lactobacillus brevis* BSO 566 (heat resistant beer spoilage bacteria) (Rachon et al. 2018).

Materials and methods

Samples

Beers were purchased in July 2023 from three UK online stores with three keg beers donated by brewers. In all 25 lagers, 15 ales (nine Pale (P), one Blonde (B), two Golden/Amber (A) and three Dark (D)), four IPAs, three stouts and three wheat beers were used in this study (Table 1). The alcohol content, bitterness units and production location was sourced from the packaging.

Table 1.

Analysis and microbiology of 50 alcohol-free and low alcohol beers.

Beer style		Package	% ABV		Bitterness	pH	Aerobes	Anaerobes	Spores
			on label	tested	(IBU)		WLN (CFU/100mL)	RR (CFU/100mL)	PCA (CFU/mL)
Ale	A1 - A	500mL bottle	0.0	0.00	19	4.0	98	<1	<1
	A2 - A	500mL bottle	0.0	0.00	32	4.3	<1	<1	<1
	A3 - B	330mL can	0.5	0.01	20	3.8	moulds	<1	<1
	A4 - D	500mL bottle	0.5	0.48	22	4.0	<1	<1	<1
	A5 - D	330mL bottle	0.0	0.00	45	4.5	<10	<10	<1
	A6 - D	500mL bottle	0.0	0.00	36	4.4	<1	<1	<1
	A7 - P	330mL can	0.5	0.49	32	4.2	<10	<10	<1
	A8 - P	330mL bottle	0.0	0.01	24	4.1	<1	<1	1
	A9 - P	330mL can	0.4	0.57	28	4.6	<10	<10	<1
	A10 - P	330mL can	0.5	0.44	18	4.5	3	<1	<1
	A11 - P	330mL can	0.5	0.39	39	4.1	<10	<10	<1
	A12 - P	330mL bottle	0.0	0.42	25	4.2	TNTC	1	<1
	A13 - P	330mL can	0.5	0.53	34	4.5	1	<1	<1
	A14 - P	330mL bottle	0.5	0.53	32	4.4	<1	<1	<1
	A15 - P	330mL can	0.3	0.36	21	4.2	<10	<10	<1
Lager	L1	330mL bottle	0.5	0.47	17	4.1	<2	<2	3
	L2	330mL bottle	0.0	0.00	14	4.2	<1	<1	<1
	L3	500mL can	0.0	0.01	26	4.5	<1	<1	<1
	L4	330mL bottle	0.4	0.55	34	4.0	<1	<1	<1
	L5	330mL bottle	0.5	0.47	19	4.8	<1	<1	<1
	L6	330mL bottle	0.0	0.00	11	4.2	<1	<1	<1
	L7	330mL bottle	0.0	0.00	14	4.3	<1	<1	<1
	L8	330mL bottle	0.0	0.04	16	4.0	<1	<1	<1
	L9	330mL bottle	0.0	0.01	14	4.1	<1	<1	<1
	L10	330mL bottle	0.0	0.00	14	4.2	1	<1	4
	L11	330mL bottle	0.0	0.00	11	4.3	<1	<1	1
	L12	330mL bottle	0.5	0.33	29	4.2	<1	<1	<1
	L13	250mL bottle	0.0	0.01	18	4.0	<1	<1	<1
	L14	500mL bottle	0.5	0.03	20	4.1	<1	<1	<1
	L15	330mL bottle	0.5	0.37	25	4.4	<10	<10	<1
	L16	30L keg	0.5	0.79	15	4.3	22,000,000	950,000	5
	L17	30L keg	0.5	0.94	16	4.3	430,000	700	110
	L18	30L keg	0.5	0.47	18	4.3	1,000	<100	18
	L19	330mL bottle	0.0	0.04	19	4.2	<1	<1	<1
	L20	330mL bottle	0.3	0.40	15	4.5	<1	<1	<1
	L21	330mL can	0.0	0.07	21	4.3	<1	<1	<1
	L22	330mL bottle	0.3	0.01	14	4.1	<1	<1	<1
	L23	330mL can	0.0	0.01	16	4.0	<1	<1	<1
	L24	330mL bottle	0.1	0.03	17	4.1	1	<1	101
	L25	330mL can	0.5	0.63	8	4.4	1	<1	<1
IPA	I1	330mL can	0.3	0.35	18	4.4	<1	<1	1
	I2	330mL can	0.5	0.47	14	4.3	10	<10	1
	I3	330mL can	0.5	0.56	37	4.3	<1	<1	<1
	I4	330mL can	0.5	0.29	12	4.0	<1	<1	<1
Stout	S1	330mL can	0.5	0.49	29	4.5	<1	<1	<1
	S2	440mL can	0.0	0.03	24	3.8	10	<1	<1
	S3	330mL bottle	0.5	0.39	22	4.2	<2	<2	<1
Wheat	W1	500mL bottle	0.5	0.38	16	4.3	<10	<10	<1
	W2	500mL can	0.5	0.46	9	4.4	<10	<1	<1
	W3	500mL bottle	0.5	0.31	12	4.3	<10	<10	<1

Pathogens were not detected in any of the above samples. *Salmonella* was not detected in 25g, *E. coli* <1 CFU/mL, *Enterobacteriaceae* <1 CFU/mL, *B. cereus* < 1CFU/mL, Sulphite Reducing Clostridia < 1CFU/mL

Analytical and microbiological analysis

All beers were analysed for physicochemical parameters (IBU, pH and ABV) and microbiology (aerobic count, anaerobic count, enumeration of aerobic spores, enumeration of *Enterobacteriaceae*, detection of *Salmonella*, detection of *E. coli*, enumeration of *B. cereus* and detection of sulphite-reducing Clostridia. Any microorganisms found were purified and identified via 16S rRNA (bacteria) or by 26S rDNA D1/D2 sequence analysis (yeast). Bitterness was measured by spectrophotometry using the EBC Analytica method 9.8 and expressed in IBU (International Bitterness Units), the ABV was measured as ethanol by volume by gas chromatography following the EBC Analytica method 9.3.2. The pH was measured with an AR15 pH meter (Accumet Research, USA).

Aerobic counts were performed using the EBC Analytica method 4.3.2.1 with Wallerstein Laboratory Nutrient agar (WLN, Oxoid) and results were expressed as CFU/100 mL. Anaerobic counts were performed using the EBC Analytica method 4.2.3 with Raka-Ray agar (RR, Oxoid) and results were expressed as CFU/100 mL. Aerobic spores were enumerated following the Campden BRI TES-MB-050 method with PCA agar and results were expressed as CFU/mL. *Enterobacteriaceae* count was performed using the ISO 21528-2:2004 method with Violet Red Bile Glucose Agar (VRBGA, Oxoid) and the results were expressed as CFU/mL. *Salmonella* detection was performed following the ISO 6579-1:2017 method, results were expressed as '*Salmonella* detected/not detected in 25g'. Analysis for *E. coli* was performed using the ISO 16649-1:2018 method with Mineral Modified Glutamate Agar (MMG, Oxoid) followed by the Tryptone Bile x-glucuronide Agar (TBX, Oxoid), and results were expressed as CFU/mL. *B. cereus* count was performed using the ISO 7932:2004 method with Mannitol Egg Yolk Polymixin Agar (MYP, Oxoid), and results were expressed as CFU/mL. The sulphite reducing bacteria count was performed following the ISO 15213:2003 method with Egg Yolk-Free Tryptose Sulfite Cycloserine Agar (TSC, Oxoid), and results were expressed as CFU/mL.

Challenge testing

Sample preparation

Lager L6 (11 IBU, pH 4.2), was selected for use in challenge tests. The beer contained residual sugars - 3.9 g/100 mL of carbohydrates (sugars 0.7 g/100 mL), 0.4 g/100 mL of protein and traces (0.006 g/100 mL) of salts. The beer was adjusted to 25 and 50 IBU using isomerised iso-alpha acids (Isofresh 30%; Hopsteiner, UK). The base beer was adjusted to pH 3.8, 4.2, 4.6 and 4.9 using 2M HCl and 5M NaOH. The physicochemical stability of all variants was verified by measuring IBU and pH immediately after supplementation and after one day and one month with no significant changes noted.

Preparation of microorganisms

Four different inocula, each containing three different strains of each species, were prepared for challenge testing. The *E. coli* O157 strains were; *E. coli* O157:H7 - NCTC 12900 (stx1 (Shiga toxin 1) and stx2 (Shiga toxin 2) negative) - quality control strain, *E. coli* O157:H7 Sakai associated with consumption of white radish sprouts (Michino et al. 1999) and *E. coli* O157:H7 strain EDL932 (ATCC 43894) a ground beef isolate from a 1983 haemorrhagic colitis outbreak (Wells et al. 1983). The *Salmonella* enterica strains were; *Salmonella* Enteritidis phage type 13A – chicken isolate (Bucher et al. 2007), *Salmonella* Enteritidis CRA 1947 - mayonnaise isolate and *Salmonella* Enteritidis CRA 3736 – cereal isolate. The *Bacillus cereus* strains were: *B. cereus* NCTC 11143 - vomit isolate (vomit food poisoning), Serotype 1 (emetic) (Taylor and Gilbert 1975), *B. cereus* NCTC 11145 - Meat loaf isolate (diarrhoeal food poisoning) Serotype 2 (diarrhoeal) (Midura et al. 1970) and *B. cereus* CRA 1761 – dairy product isolate. Two proteolytic *C. botulinum* strains; strain 213B and strain 62A were used in this study. *C. botulinum* 213B was obtained from Dr. D. Zink, Campbell Institute for Research and Technology, United States, and was checked for production of type B toxin (Brown and Martinez 1992). *C. botulinum* strain 62A produces an A1 serotype/subtype botulinum neurotoxin and is frequently used in food challenge and detection studies (Johnson and Bradshaw 2001, Wentz et al. 2018).

The *E. coli* O157 and *Salmonella* Enteritidis strains used in this study were recovered from long term storage (TS/80-BL Cryobeads at -70°C) and were grown in Nutrient broth (NB; Oxoid). These cultures were grown aerobically for 24 h at 37 ± 1°C. Cultures were adapted to products by cultivation in broth/product mix at 1:1 ratio. The levels present in the adopted broths were enumerated prior to inoculation and were stored chilled (<3°C) prior to use. Enumeration was carried out on Plate Count Agar (PCA; Oxoid) and colonies were counted after 24 h aerobic incubation at 37°C.

Bacillus cereus strains were inoculated into products as a spores using prepared spore solutions. *B. cereus* was recovered from storage (TS/80-BL Cryobeads at -70°C) and grown on Nutrient agar (Oxoid) at 30°C for 48 h and subsequently in Nutrient broth at 30°C for 48 h. Culture (0.5 mL) was spread onto the surface of approximately 350-400 pre-poured Nutrient agar plates. Plates were incubated for 5 days at 30°C and the presence of high number of spores (>90%) was verified by microscopy. Agar plates were flooded with 10 mL of sterile distilled water and spores were harvested using L-shape spreader. Spore solution was centrifuged at 2,160 x g for 20 minutes and washed twice with SDW. The spore suspension was stored at 2-8°C and used within one month.

C. botulinum strains were recovered from storage (TS/80-BL Cryobeads at -70°C) and transferred to 20 mL Cooked Meat Media (CMM, Oxoid) and incubated anaerobically at 37°C for 48 h. CMM culture (20 mL) was transferred to 90 mL of steamed (≥ 20 minutes) and cooled CMM and incubated anaerobically at 37°C for 48 h. CMM (90 mL) was transferred into 900 mL of CMM (prepared as above0 and incubated at 37°C for 120 h. The CMM culture was filtered through a sterile glass funnel containing glass wool. The liquid was centrifuged at 2,160 x g for 20 minutes, the pellet washed in SDW twice, resuspended in SDW, stored at 2-8°C and used within one year. To reduce the hazard posed by pre-formed neurotoxin in spore suspensions, the presence of toxin was analysed by ELISA and if toxin was found additional centrifugation and washing was performed until the absence of toxin was established.

Inoculation and testing

Inoculum were enumerated, diluted in sterile diluent and inoculated. Bottles were recapped with sterilised crowns using a manual crown capper (Vigo, UK), shaken for 10 s and stored in an incubator at 25 ± 1°C. The level of inoculated microorganisms was determined immediately by plating out 'as is' and decimally diluted (as required) in triplicate. Bottles were opened and poured into 500 mL bottles, mixed and degassed by vigorous shaking (20 min).

E. coli O157 was enumerated using Sorbitol MacConkey Agar (Oxoid) supplemented with potassium tellurite and cefixime (Oxoid). Colonies were counted after 24 h of aerobic incubation at 37 ± 1°C. Degassed beer in bottles were stored with lids lightly closed (to allow gas exchange) and tested alongside closed bottles at each timepoint (two weeks, one, two, three and six months) to determine the effect of low carbonation on microbial behaviour. At each timepoint, the content of the closed bottles was poured into stomacher bags and mixed before testing. Results were expressed as CFU/mL and Log_{10} values, Mean Log10, SD (standard deviation) and log reductions were calculated.

Salmonella Enteritidis was enumerated after inoculation on Xylose Lysine Desoxycholate Agar (XLD, CM0469 - Oxoid) and colonies were counted after 24 h of aerobic incubation at 37 ± 1°C. Samples inoculated with *Salmonella* were tested at the same timepoints as *E. coli* O157 with the results presented similarly.

Samples inoculated with *B. cereus* and *C. botulinum* were tested immediately and at the same timepoints as the *Salmonella* and *E. coli* samples. However, only closed 'anaerobic' bottles were tested (degassed 'aerobic' samples were not tested). *B. cereus* was enumerated on Mannitol Egg Yolk Polymyxin Agar (Oxoid) supplemented with Polymyxin B supplement (Oxoid) and colonies were counted after 24 h of aerobic incubation at 30 ± 1°C. *C. botulinum* was enumerated on Tryptose Sulphite Cycloserine agar (Oxoid) supplemented with D-cycloserine (Oxoid) and colonies were counted after two and five days of anaerobic incubation at 30 ± 1°C.

Heat resistance

The heat resistance (D_{60}) of *E. coli* O157:H7 - NCTC 12900, *Salmonella* Senftenberg 775W (heat resistant strain), *Lactobacillus brevis* BSO 566 (heat resistant strain) and ascospores of *Saccharomyces cerevisiae* BRYC 501 (pasteurisation bio-indicator) was determined. Trials were performed in alcohol free beer at two bitterness levels (11 IBU, adjusted to 25 IBU at three pH values - 4.2, adjusted to 4.6 and 4.9). Microorganisms were grown under optimum conditions and ascospores were prepared on acetate agar (Rachon et al. 2021). *E. coli* O157:H7 and *Salmonella* Senftenberg 775W were grown overnight in Tryptone Soya Broth (Oxoid) at 37 ± 1°C. Bacteria were centrifuged and washed twice in the sterile distilled water with the cells mixed with the beer variant and tested within 20 minutes. *Lactobacillus brevis* BSO 566 was growth in MRS broth anaerobically at 27 ± 1°C for two days. The broth was centrifuged at 3,000 x g for five minutes and the pellet was resuspended in the beer and tested within 20 minutes. Yeast ascospores were prepared on the Ascospore agar (HiMedia, India) inoculated with 0.2 mL of exponential phase culture grown in YM broth. After 10 days incubation, the Ascospore Agar plates were flooded with 10 mL of sterile distilled water and the ascospores were harvested with an L-shape spreader. This suspension was centrifuged at 3,000 x g for five minutes and the pellet suspended in the test beer. The ascospore solution was stored at 2-8°C and used after two weeks of maturation storage.

The heat resistance of the four microorganisms was measured at 60°C using the capillary tube method. The capillary tubes method was as described previously (Rachon et al. 2018, 2021). The test solution (50 µL) was inoculated with microorganisms at 10^7-10^8 CFU/mL pipetted into soda glass capillary tubes G119/02 (Fisher Scientific). The tube ends were heat sealed and then placed in a water bath (60°C) and held for the required time. After each heat interval the tubes were removed from the water bath and cooled in ice water. The test suspension from the capillary tubes was recovered in Maximum Recovery Diluent (Oxoid) and the number of viable cells determined by spread plating. *E. coli* O157:H7 and *Salmonella* Senftenberg 775W were recovered on Tryptone Soya Agar (Oxoid) incubated aerobically for 24 h at 37 ± 1°C. *Lactobacillus brevis* BSO 566 was recovered on MRS agar after 7 days of anaerobic incubation at 27 ± 1°C. Yeast spores were recovered on YM agar after 10 days of aerobic incubation at 27 ± 1°C. D_{60} values were calculated from the regression curves 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.

Results

Analysis of the 50 beers

Eight of the beers contained a higher ABV than declared (Table 1). The bitterness of beers was between 8 and 45 IBU without correlation to beer style. The bitterness of the majority (70%) of the beers were in range of 8-24 IBU with the remaining beers in the range 25-45 IBU. The pH of beers was also variable, the highest pH was 4.8 (L5 - lager at 19 IBU) and the lowest was 3.8 (S2 – stout at 24 IBU and A3B – blond ale at 20 IBU). 50% of the beers had a pH ≥ 4.3 (Table 1).

No pathogens were found in any of the beers but over 40 different microorganisms were isolated from 13 different beers. The microorganisms included - *Dekkera anomala*, *Hannaella surugaensis*, *Naumovozyma castellii* (x 2), *Saccharomyces cerevisiae*, *Acetobacter*, *Leuconostoc mesenteroides*, *Levilactobacillus brevis* (x 2), *Loigolactobacillus backii*, *Pediococcus inopinatus*. Non beer spoilage microorganisms (spore forming bacteria) included *Bacillus* species (x 17), *Paenibacillus humicus* (x 2) with poor hygiene indicators - *Staphylococcus capitis*, *Staphylococcus warneri*, *Klebsiella/Kosakonia/Enterobacter* species and *Pseudomonas* species. Emerging opportunistic pathogens - *Cupriavidus gilardii* (x 3) and *Sphingomonas paucimobilis* – were isolated from keg beers.

Cupriavidus gilardii is emerging opportunistic pathogen (Hazard Group 2) which is a risk to human health especially in immunocompromised or elderly patients (Ruiz et al. 2019). *C. gilardii* is a gram-negative, rod-shaped, motile, glucose non-fermenting bacteria belonging to the *Betaproteobacteria* class and the *Burkholderiaceae*

family. *C. gilardii* has been isolated from untreated drinking water, urban pond water, agricultural soil, soil contaminated with heavy metals, soil containing natural asphalt, plants and human clinical samples. The microorganism is resistant to multiple antibiotics (Kobayashi et al. 2016).

Sphingomonas paucimobilis is also an opportunistic pathogen which can take advantage of people with underlying conditions and diseases (Ryan and Adley 2010). *Sphingomonas* species are aerobic gram-negative, oxidase positive, non-fermentative rods that are found in water (sea, river, waste, mineral), water systems in hospital and drinking water distribution systems (Koskinen et al. 2000). *Sphingomonas* species are associated with problems in clinical settings, and is a widespread cause of hospital acquired infections (Ryan and Adley 2010).

Quantitatively, the most microorganisms were isolated from three keg lager beers which had high aerobic, anaerobic and spore counts. High aerobic counts were also recorded in three ales (A1-A, A3-B, A12-P).

Beers with a lower IBU were considered for challenge testing. The lowest bitterness was found with L25 (8 IBU), W2 (9 IBU), L6 and L11 (11 IBU). With a target pH (4.2), lager L6 was chosen over L25 (0.63% ABV), W2 (Wheat beer) and L11 (pH 4.3) (Table 1).

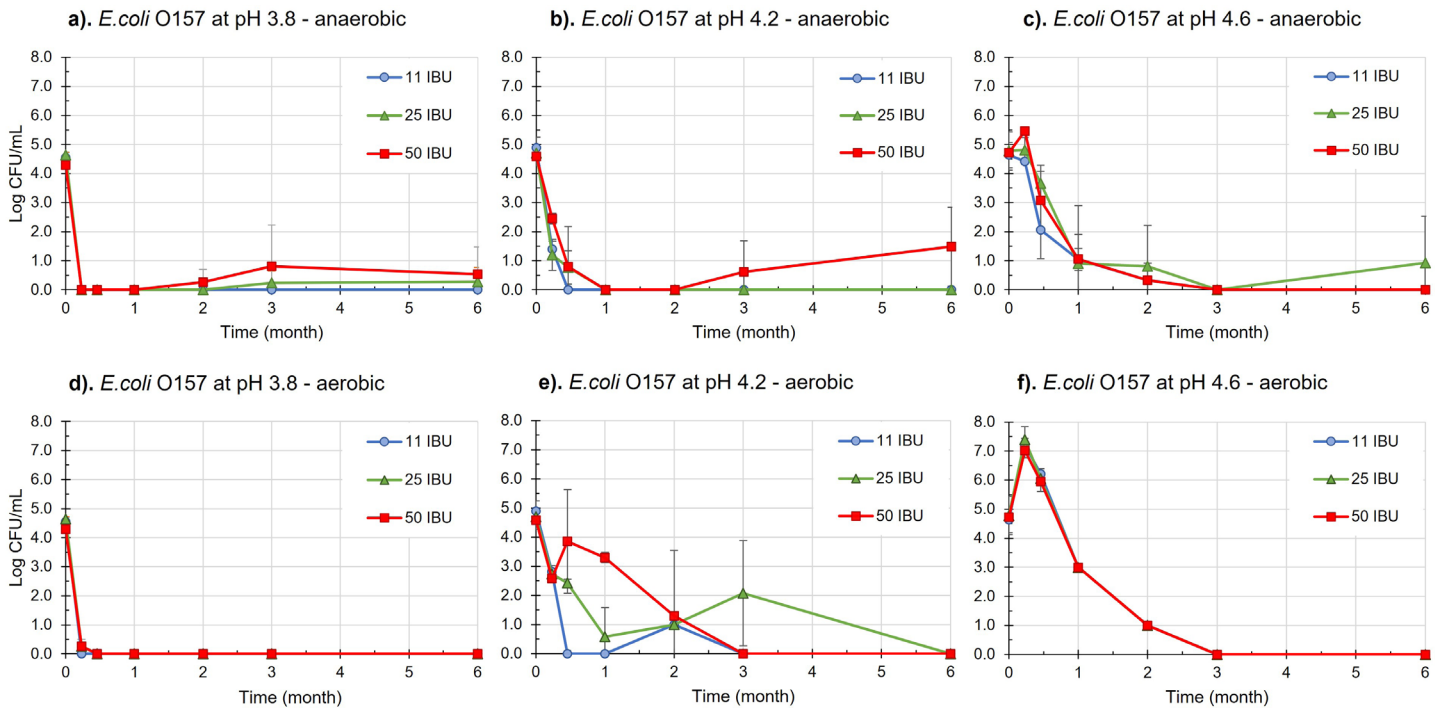
Challenge testing

Growth and survival of *E. coli* O157 and *Salmonella* Enteritidis was evaluated in nine pH/IBU variants of lager L6. Beers were adjusted to pH 3.8, 4.2 and 4.6 with each variant with a different bitterness (11, 25 and 50 IBU). Growth and survival of *B. cereus* and *C. botulinum* was evaluated at pH 4.6 and 4.9 with three bitterness values (11, 25 and 50 IBU).

E. coli O157 was unable to grow in any of the beer variants where a high CO_2 level and low O_2 was maintained ('anaerobic') (Figure 1). At pH 3.8 (Figure 1a), the inoculum of *E. coli* O157 was killed within a week but a low number of viable microorganisms were recovered at months two and three. At pH 4.2 (Figure 1b), *E. coli* O157 was killed within the first month, was not recovered at month two but low levels were recovered at month three. At pH 4.6 (Figure 1c) *E. coli* O157 was slowly inactivated and still present at month two with low levels at 25 IBU at month three.

Fugure 1.

***E. coli* O157 in non-alcoholic beer at pH 3.8, 4.2 and 4.6 and 11, 25 and 50 IBUs in anaerobic (closed bottles a, b and c) and aerobic conditions (bottles with loose closures - d, e and f).**



In degassed ('aerobic') samples, at pH 3.8 (Figure 1d) *E. coli* O157 was completely inactivated within a week but at a higher pH 4.2 (Figure 1e) no growth of *E. coli* O157 was observed but viable cells were recovered over three months storage. At pH 4.6 (Figure 1f) growth of *E. coli* O157 was initially observed in all bitterness variants. Cell viability then declined between week two and month three without any further recovery.

Salmonella Enteritidis was unable to grow in any of the beer variants if the conditions were 'anaerobic' (high CO_2 level and low O_2 - closed bottle) (Figure 2). At 3.8 (Figure 2a) all microorganisms were inactivated within a week without any recovery thereafter. At pH 4.2 (Figure 2b) *Salmonella* Enteritidis was killed within a week of inoculation. At pH 4.6 (Figure 2c) the viable count of *S.* Enteritidis dropped slowly within first month with no viable cells recovered after first two months after which low numbers of viable cells (20 and 12 CFU/mL) were recovered. In degassed ('aerobic') samples at pH 3.8 (Figure 2d), *Salmonella* Enteritidis was killed within a week.

At pH 4.2 (Figure 2e) *Salmonella* Enteritidis grew (2 log increase) between inoculation and week two but was undetectable after two months. At pH 4.6 (Figure 2f), growth of *S.* Enteritidis was observed in the first week of incubation for the lowest bitterness variant (> 2 log increase at 11 IBU). At 25 and 50 IBU no growth was observed with no viable cells recovered after two weeks incubation.

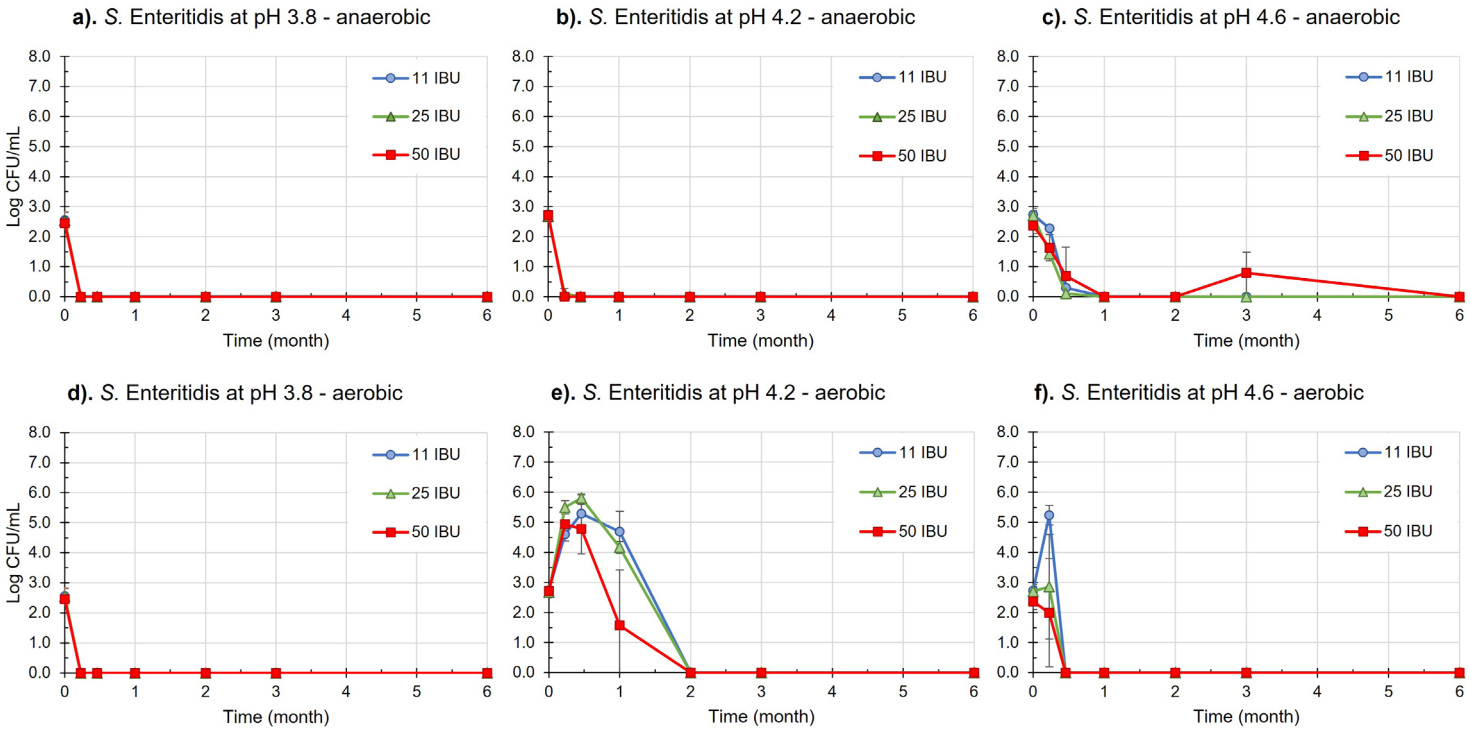
Neither *B. cereus* and *C. botulinum* grew in any the pH and bitterness variants. However, the number of these microorganisms did not change throughout storage indicating, importantly, that the initial inoculum remained viable (Figures 3 and 4).

Heat resistance

The heat resistance (D_{60}) of yeast ascospores in 11 and 25 IBU beers at pH 3.8, 4.2 and 4.6 was significantly greater than the heat resistance of *Lactobacillus brevis* BSO 566, *Salmonella* Senftenberg 775W or *E. coli* O157 - NCTC 12900. In 11 IBU beers, D_{60} values for yeast ascospores were >10 times greater than D_{60} of other tested microorganisms.

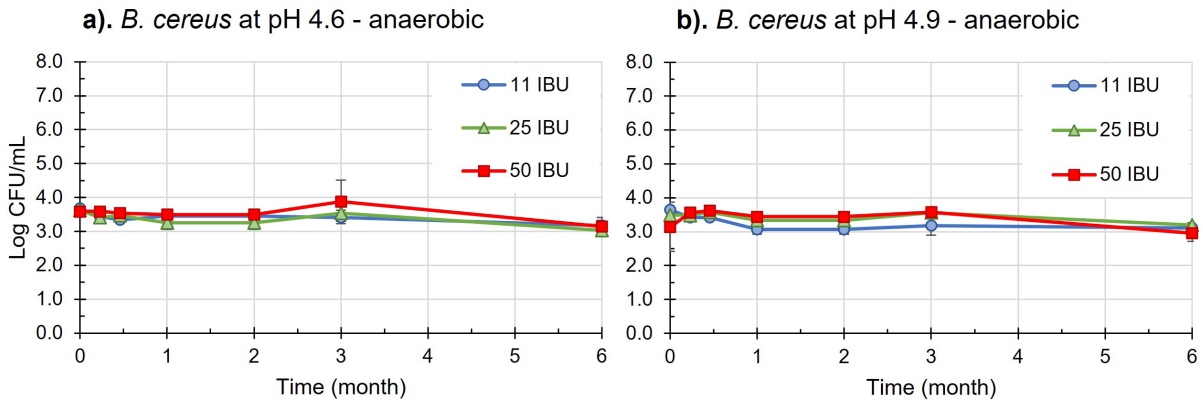
Fugure 2.

***E. coli* O157 in alcohol-free beer at pH 3.8, 4.2 and 4.6 and 11, 25 and 50 IBUs in anaerobic (closed bottles a, b and c) and aerobic conditions (bottles with loose closures - d, e and f).**



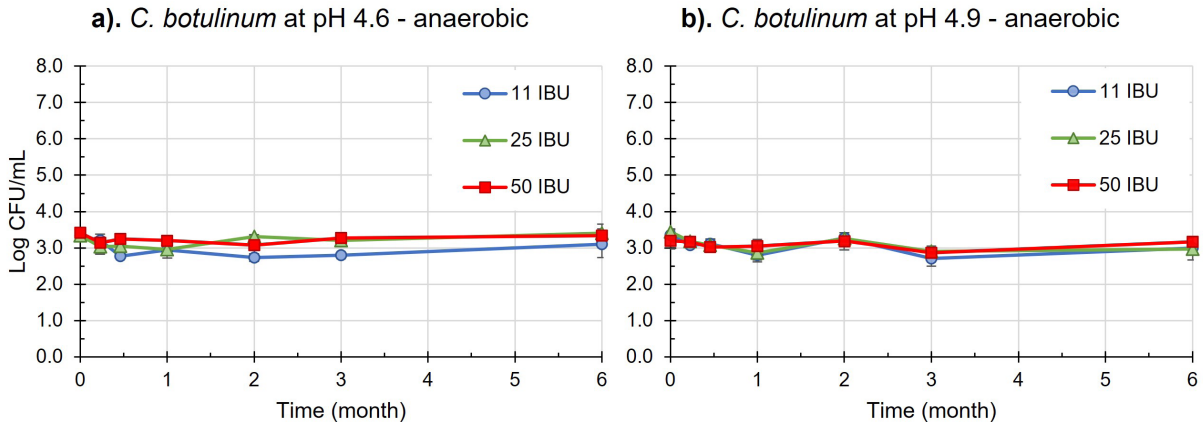
Fugure 3.

Bacillus cereus in alcohol-free beer at (a) pH 4.6 and (b) 4.9 and 11, 25 and 50 IBU.



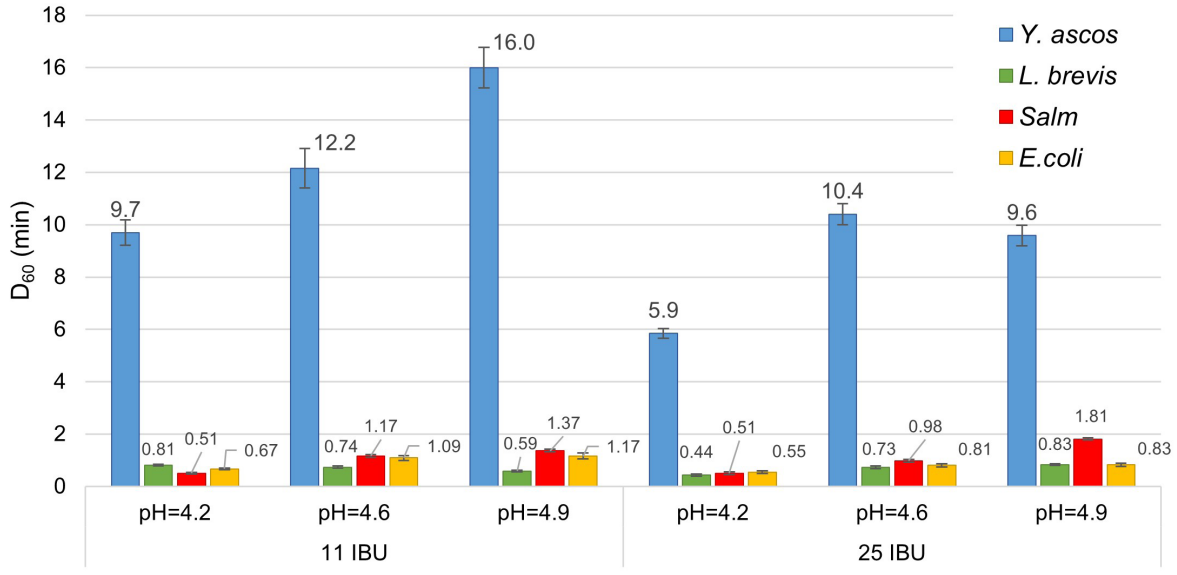
Fugure 4.

Clostridium botulinum in alcohol-free beer at (a) pH 4.6 and (b) 4.9 and 11, 25 and 50 IBU.



Fugure 5.

D_{60} values of _Saccharomyces cerevisiae_ BRYC 501 ascospores ('_Y. ascos_'), _Lactobacillus brevis_ BSO 566, _Salmonella_ Senftenberg 775W ('_Salm_') and _E. coli_ O157.



In the 25 IBU beers at pH 4.9, the D_{60} of yeast ascospores was five times greater than D_{60} of *Lactobacillus brevis* BSO 566, *E. coli* O157 and *Salmonella* Senftenberg 775W. At lower pH values the D_{60} was 10 times greater as in the low IBU beers. The heat resistance of all microorganisms was significantly greater at lower IBU (11 IBU) then higher IBU (25 IBU) beers. Furthermore, at higher pH, the D_{60} was higher except for *Lactobacillus brevis* BSO 566 in beer at 11 IBU where the D_{60} at pH 4.9 was lower than at pH 4.6 or 4.2 (Figure 5).

Discussion

The published studies on the survival of pathogenic microorganisms in non-alcoholic beers is limited. Of the three reports (Menz et al. 2010, Menz et al. 2011, Cobo et al. 2023) none investigated the survival/growth of pathogens in fully carbonated beers.

Pathogens were shown to grow in wort (Menz et al. 2010) and in non-alcoholic beers (Menz et al. 2011, Cobo et al. 2023). However, in the experiments the liquid was dispensed into flasks or vials which would impact on the concentration of CO_2 and O_2 (which were not measured). Menz et al (2011) tested the growth of pathogens in stoppered 250 mL Erlenmeyer flasks, containing 100 mL of beer. Cobo et al (2023) tested the survival of pathogens in beer (27 mL) sealed in 50 mL sterile cylindroconical vials wrapped with parafilm. Menz (2011) concluded that depleting the level of CO_2 had no significant impact on the survival of the pathogens *E. coli* in alcoholic beer.

In the study reported here, the growth of *E. coli* O157 and *Salmonella* Enteritidis differed between forcibly degassed beer (in loosely capped bottles) and beer retaining full levels of CO_2 (closed bottles). While the impact of CO_2 on microorganisms has been investigated in the past, the impact of CO_2 level on the growth of pathogenic microorganisms in beer has not been investigated. It is well established that CO_2 at high pressure can be bactericidal while at lower pressure reduces growth rate. King et al (2009) suggested that elevated levels of dissolved CO_2 extend the lag phase of microorganisms. However, Martin et al (2003) showed that dissolved CO_2 significantly inhibited the growth of bacteria in raw milk, influencing lag, exponential, and stationary growth phases. Loss et al (2002) showed that addition of CO_2 during pasteurisation significantly reduces heat resistance (D-values) of the tested microorganisms.

As the most pathogenic microorganisms are facultative anaerobes - which can grow at both aerobic and anaerobic conditions - it is expected that the inhibitory impact of CO_2 on these microorganisms would be lower than on aerobic microorganisms. Lacoursiere et al (1986) reported that the optimum growth rate for *E. coli* was 1.3 mmol/L (0.57 g/L) of dissolved CO_2, close to that in the mammalian gut, where this microorganism naturally resides. Above this level of CO_2 growth was inhibited. As beer is carbonated with 1-4 volumes of CO_2 (1.96-7.84 g/L) and with oxygen actively minimised (<100 µg/L), the growth of pathogens is unlikely in packaged beer. However, pathogens are a potential threat to draught alcohol-free beer (Quain 2021) epecially as oxygen ingress and CO_2 egress occurs during dispense (Jevons and Quain 2022).

The survival and/or growth of pathogenic spore forming bacteria was investigated. Spores of *B. cereus* and *C. botulinum* were unable to germinate in any of the beer variants with the number of inoculated spores unchanged over six months. Spores of both these microorganisms were unaffected by pH or hop bitterness. This is surprising, as reports suggest hop extract negatively affect both species (Johnson and Haas 2001, Rój et al. 2015). Nevertheless, spore forming bacteria were found in this work (Table 1) and have been reported in standard and alcohol-free beers (Munford et al. 2017).

Conclusions

This study showed that carbonation together with the pH of alcohol-free beer are factors determining the ability of enteric pathogens - *E. coli* and *Salmonella* - to grow. It is suggested that draught alcohol free-beers with reduced carbonation and comparatively elevated levels of oxygen are at greatest risk of contamination by pathogens. It is surprising that there are so few studies on the survival of pathogens in non-alcoholic beers and, accordingly, it is suggested that more research is required in this important area.

Author contributions

Grzegorz Rachon: conceptualisation, methodology, validation, investigation, resources, data curation, writing (original draft, review and editing), visualisation, supervision, project administration, funding acquisition.
Harry Rothera: validation, formal analysis.
Sabina O'Reilly: formal analysis.
Gail Betts: writing (review and editing).

Acknowledgements

The technical support of Tina Shilham and technical advice from Linda Everis and Rob Limburn was greatly appreciated.

Conflict of interest

The authors declare no conflict of interest.

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