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Growth and Survival of Foodborne Pathogens in Beer

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ABSTRACT

This work aimed to assess the growth and survival of four foodborne pathogens (*Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes,* and *Staphylococcus aureus*) in beer. The effects of ethanol, pH, and storage temperature were investigated for the gram-negative pathogens (*E. coli* O157:H7 and *Salmonella* Typhimurium), whereas the presence of hops ensured that the gram-positive pathogens (*L. monocytogenes* and *S. aureus*) were rapidly inactivated in alcohol-free beer. The pathogens *E. coli* O157:H7 and *Salmonella* Typhimurium could not grow in the mid-strength or full-strength beers, although they could survive for more than 30 days in the mid-strength beer when held at 4°C. These pathogens grew rapidly in the alcohol-free beer; however, growth was prevented when the pH of the alcohol-free beer was lowered from the ''as received'' value of 4.3 to 4.0. Pathogen survival in all beers was prolonged at lowered storage temperatures.

Beer is considered to be a microbiologically safe beverage due to a number of intrinsic antimicrobial hurdles. These hurdles include ethanol (typically 3.5 to 5.0% [vol/vol]), hop bittering compounds (approximately 17 to 55 ppm of iso-α-acids), low pH (3.9 to 4.4), elevated carbon dioxide (approximately 0.5% [wt/wt]), low oxygen (<0.1 ppm), and a lack of nutritive substances *(24).* In addition, extrinsic processing hurdles such as mashing, wort boiling, pasteurization, sterile filtration, and cold storage provide further protection against pathogenic microorganisms *(24).*

Due to the antimicrobial hurdles, it is widely assumed that pathogens cannot survive in beer, and several studies have shown that the survival of pathogens in beer is generally poor *(4, 8, 13, 21, 30, 33, 41).* However, it has been suggested that beer may not be as hostile to pathogens as some have presumed *(14, 18, 26, 29, 30, 32).* For example, Hompesch *(14)* showed that *Salmonella* Paratyphi could survive in beer for up to 63 days, while L'Anthoën and Ingledew *(20)* reported that a range of pathogens can grow in alcohol-free beer. Foodborne pathogens have also been reported or inferred to exist in some traditional African beers *(28, 32), Escherichia coli* and coliforms have been detected in draught beer *(36),* and coliforms have also been isolated from beer mugs and tankards *(31).*

In this study we tested the assumption that pathogens cannot survive in beer. This was achieved by evaluating the survival of the foodborne pathogens *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes,* and *Staphylococcus aureus* in beers with various levels of key antimicrobial hurdles. The selected *E. coli* O157:H7 strain was of particular interest, as it is known for its acid resistance and has been transmitted through unpasteurized apple cider *(6),* where it has been shown to survive for extended periods despite a pH below 4.0 *(26, 40).* Due to its acid resistance, this strain has an infective dose as low as 10 to 100 cells *(37).* In addition to the emergence of acid-resistant strains, further impetus for the study was provided by the wider availability of beers with reduced levels of protective antimicrobial hurdles, such as alcohol-free beer, unpasteurized beer, and beer with reduced hop levels.

MATERIALS AND METHODS

Microorganisms and inoculum preparation. In this work we evaluated the survival of the bacteria *E. coli* O157:H7-VT (N) – NCTC 12900, *L. monocytogenes* ATCC 7644, *Salmonella* Typhimurium (kindly provided by the University of Melbourne, Melbourne, Australia), and *S. aureus* (kindly provided by Victoria University, Melbourne, Australia). Bacterial cultures were maintained by weekly subculturing on nutrient agar (Oxoid, Thebarton, Australia). Prior to inoculation, fresh cultures were grown overnight at 37°C in wort with a specific original gravity of 1.020. Wort was prepared by diluting unhopped malt extract (Coopers Brewery, Regency Park, Australia) with distilled water to the desired specific gravity, as determined by a density meter (DMA 35n, Anton Paar, Graz, Austria). The pH was adjusted to 6.0 with 10 M NaOH, and the wort was then autoclaved at 121°C for 15 min and clarified as described elsewhere *(25).* Following sterilization, the pH of the wort was 5.5, resulting in a final beer pH of approximately 4.3, which is within the typical pH range of beer *(39).* Cultures were diluted in wort (specific gravity of 1.020) to the target inoculum (typically 10^3 CFU/ml) with the assistance of optical density standard curves at 600 nm in a UV-visible spectrophotometer (Varian Cary 50, Mulgrave, Australia).

Beers used for microbial challenge tests. A series of commercially available, pasteurized beers were used in the

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TABLE 1. *Alcohol, pH, and hop levels of beers used for microbial challenge tests*

Beer	ABV (%)	pH	Hop level (IBU/ppm iso-α-acids)
Alcohol-free	0.5	4.3	15
Mid-strength (1)	2.6	3.79	10
Mid-strength (2)	2.9	4.05	24
Mid-strength (3)	2.6	4.11	26
Mid-strength (4)	2.3	3.9	16
Full strength	5	4.3	12

microbial challenge tests. Beers may be classified according to their alcohol by volume (ABV) content into alcohol-free (<0.5% ABV), mid-strength (2.5 to 3.6% ABV), and full-strength (4.0 to 5.0% ABV) beers *(1)*. Using this classification, we selected six commercially available bottled Australian lagers (Table 1).

The effects of ethanol, pH, and storage temperature on pathogen growth and survival were investigated. Ethanol levels were tested at 0.5, 2.7, and 5.0% ABV by modifying the alcohol-free beer (Table 1) with sterile-filtered (0.45-μm-pore-size filter) ethanol. Survival at beer pH levels of 4.0, 4.3, 4.5, and 5.0 was evaluated by adjusting alcohol-free beer from pH 4.3 with sterile-filtered 2 M HCl or 2 M NaOH. The effect of storage temperature on survival was evaluated by incubating inoculated beers at 4, 14, 25, and 37°C.

Experimental conditions. As elevated levels of dissolved CO_2 have been recognized as being inhibitory toward the growth of various foodborne pathogens *(19, 22, 23),* and because dissolved CO_2 was not part of our main investigation, an exploratory trial was conducted to elucidate the potential loss of antimicrobial activity in the beer due to the effects of escaping CO_2 when held in stoppered flasks (compared with a sealed beer bottle). This trial was performed as follows. A bottle-conditioned beer was produced (4.5% ABV, pH 4.30, 20 international bittering units [IBU]) in bottles sealed with a rubber septum (the septum allowed inoculation and sampling without significant CO_2 loss). These bottles represented full CO_2 levels. A subset of the bottle-conditioned beer was dispensed in 100-ml volumes into stoppered 250-ml Erlenmeyer flasks, which represented depleting CO_2 levels. The bacterium *E. coli* O157:H7 was inoculated at approximately 10^3 CFU/ml into both sets of beer, and the beers were incubated statically at 4°C. Periodic enumeration revealed no significant differences ($P > 0.05$) in the survival of *E. coli* O157:H7 between the unopened bottles (full CO_2) and the Erlenmeyer flasks (depleting CO_2). Furthermore, no significant differences ($P > 0.05$) in *E. coli* O157:H7 survival at 4°C were observed in the alcohol-free beer when fully degassed compared with when it was poured into the Erlenmeyer flasks. Therefore, all further experiments were conducted in triplicate in stoppered 250-ml Erlenmeyer flasks, each containing 100 ml of beer. All experimental flasks were incubated statically.

Acid adaptation. The effect of acid adaptation on the survival of *E. coli* O157:H7 in alcohol-free beer was assessed by using two previously reported methods *(7, 27)*. In one approach, an acid shock was used *(27)*. The culture was grown to stationary phase in 1.020 wort at 37°C, and cells were harvested by centrifugation at 7,500 RCF (relative centrifugal force) for 5 min (Mikro 20, Hettich, Tuttlingen, Germany), washed (0.85% NaCl), and resuspended in 1.020 wort with a pH of 4.5 (adjusted using 2 M HCl). The acidified wort was then incubated for 1 h at 37°C, diluted with 1.020 wort, and inoculated into the test beer (alcohol-free beer, pH 3.5). A control culture (using 1.020 wort, pH 5.75) was run in parallel. The second acid adaptation approach cultured the inoculum in tryptic soy broth (TSB) supplemented with 1% glucose *(7)*. Cultures were grown to stationary phase in TSB (Oxoid) with glucose and TSB without glucose at 37°C. Cells were again harvested by centrifugation, washed, diluted in 1.020 wort (pH 5.75), and inoculated into the test beer (alcohol-free beer, pH 3.5). The various cultures were inoculated at 10^5 CFU/ml into 10-ml volumes of the alcohol-free beer (0.5% ABV, pH modified to 3.5 with 2 M HCl) and incubated at 25°C. Bacterial survival was monitored over time.

Microbial analysis. Samples for microbiological analysis were serially diluted in saline water (0.85% NaCl) prior to enumeration on solid media. Bacteria were quantified after 24 to 48 h at 37°C on nutrient agar (Oxoid), which was supplemented with filter-sterilized (0.45-μm pore size) cycloheximide (100 mg/liter; Sigma-Aldrich, Castle Hill, Australia) to inhibit yeast when appropriate. Specific growth rates (μ, per hour), specific death rates (*k,* per hour), and lag phases were calculated by using DMFit v2.1 (a macro for Microsoft Excel) following the model of Baranyi and Roberts *(3)*. Coefficients of variation for growth rates, death rates, and lag phases were always less than 4%. Where *P* values are provided, differences between means were investigated using two-tailed *t* tests in SPSS 17 (SPSS Inc., Chicago, IL).

RESULTS

Survival in commercial mid- and full-strength beer. To investigate the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium in beer, the pathogens were inoculated into a lager beer (5.0% ABV) and incubated at various temperatures. *E. coli* O157:H7 survived longest at lower temperatures (Fig. 1), where at 4°C (near typical

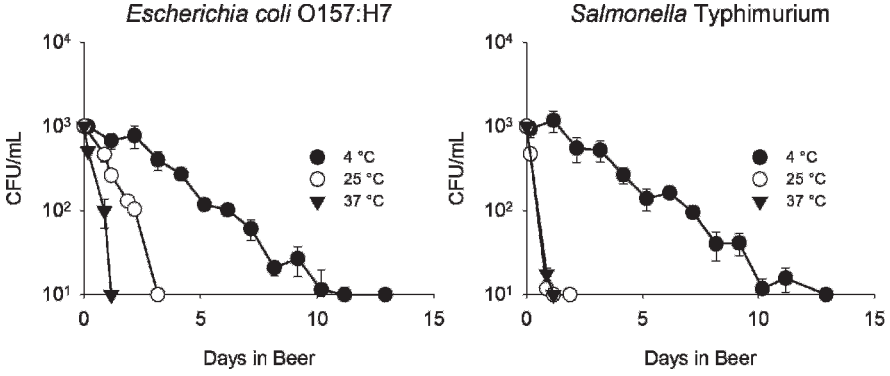

FIGURE 1. *Effects of storage temperature on the survival of* E. coli *O157:H7 and* Salmonella *Typhimurium in lager beer (5.0% ABV). Data points are the means of triplicate flasks; error bars are ±standard deviations.*

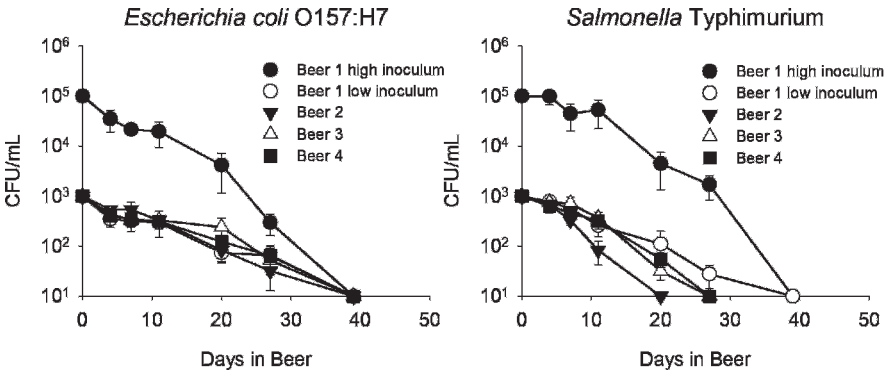

FIGURE 2. *Survival of* E. coli *O157:H7 and* Salmonella *Typhimurium in four commercial mid-strength beers at 14°C. Beer 1 was inoculated at two levels (10^5 and 10^3 CFU/ml). Descriptions of the beers are given in ''Materials and Methods.'' Data points are the means of triplicate flasks; error bars are ±standard deviations.*

serving and refrigeration temperatures) more than 10 days was required to reduce the bacterial population by 2 log cycles. The specific death rate (k, per hour) was −0.0099. Inactivation was faster at 25°C ($k = -0.0254$) and faster again at 37°C ($k = -0.0641$). Similar trends were observed for *Salmonella* Typhimurium; however, inactivation was faster at the higher incubation temperatures than for *E. coli* O157:H7 (Fig. 1). Specific death rates (k) for *Salmonella* Typhimurium were −0.0072 at 4°C, −0.0926 at 25°C, and −0.0838 at 37°C. Growth was not observed in this full-strength lager beer.

Based upon trial data for the growth and survival in full-strength and alcohol-free beer, 14°C was chosen as the preferred incubation temperature for the challenge tests. Survival was too poor at temperatures of 25°C and above, while incubation at 4°C was deemed impractical for further experimentation as it excluded the possibility of growth. Hence, 14°C was chosen as the incubation temperature, as this allowed the assessment of both survival and growth.

After observing the inability of the two gram-negative pathogens to grow in a full-strength beer, survival in commercial mid-strength beers (those with a reduced ethanol content) was investigated at 14°C. Four different beers were used for the challenge test, and one of these (Beer 1) was inoculated at two levels (10^3 and 10^5 CFU/ml) to investigate inoculum size effects. The pH and ABV were as follows: beer 1, pH 3.79, 2.6% ABV; beer 2, pH 4.05, 2.9% ABV; beer 3, pH 4.11, 2.6% ABV; and beer 4, pH 3.90, 2.3% ABV. *E. coli* O157:H7 (Fig. 2) was somewhat more resistant to the antimicrobial hurdles of the beers than *Salmonella* Typhimurium (Fig. 2). No growth was observed in any of the beers, and the bacteria were inactivated to the detection limit (10 CFU/ml) by approximately 30 to 40 days. Specific death rates (k) for *E. coli* O157:H7 were as follows: beer 1 with high inoculum, −0.0041; beer 1 with low inoculum, −0.0019; beer 2, −0.0022; beer 3, −0.0019; and beer 4, −0.0019. Similar specific death rates were observed for *Salmonella* Typhimurium: beer 1 with high inoculum, −0.0063; beer 1 with low inoculum, −0.0023; beer 2, −0.0044; beer 3, −0.0042; beer 4, −0.0031.

Effects of temperature, pH, and ethanol on growth and survival in alcohol-free beer. The effects of incubation temperatures on the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium in alcohol-free beer are shown in Figure 3. Populations of both bacteria reached maximum density within a few days at 25°C, with specific growth rates (μ, per hour) of 0.5794 (for *E. coli* O157:H7) and 0.1100 (for *Salmonella* Typhimurium). Considerable lag phases (19.4 days for *E. coli* O157:H7 and 18.2 days for *Salmonella* Typhimurium) and slower growth rates ($\mu = 0.0137$ for *E. coli* O157:H7 and 0.0104 for *Salmonella* Typhimurium) were observed at 14°C. Neither of the pathogens grew at 4°C, however, nor were they completely inactivated after 46 days ($k = -0.0012$ and −0.0007 for *E. coli* O157:H7 and *Salmonella* Typhimurium, respectively).

Attention was next turned to investigating the effects of two of the major antimicrobial hurdles of beer on pathogen survival, the ethanol content and the pH, by using the alcohol-free beer as a basal medium. Similar responses to ethanol were observed for *E. coli* O157:H7 and *Salmonella* Typhimurium (Fig. 4). As shown in Figure 3, *E. coli* O157:H7 and *Salmonella* Typhimurium grew in the alcohol-free beer at 14°C ($\mu = 0.0137$ and 0.0104, respectively) after an extended lag phase (19.4 and

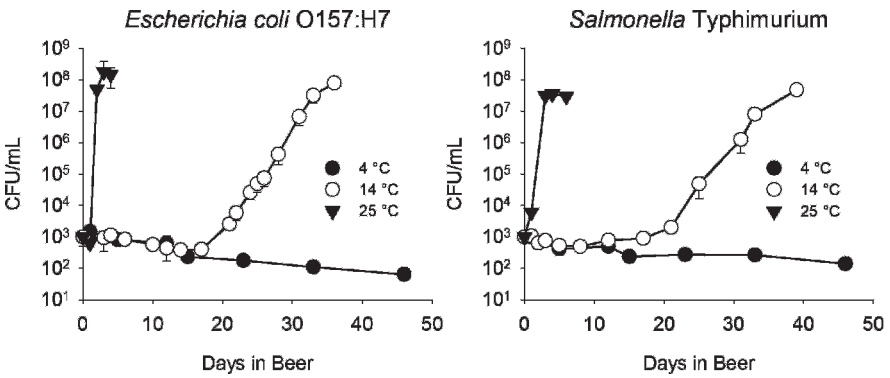

FIGURE 3. *Effects of storage temperature on the survival of* E. coli *O157:H7 and* Salmonella *Typhimurium in alcohol-free beer. Data points are the means of triplicate flasks; error bars are ±standard deviations.*

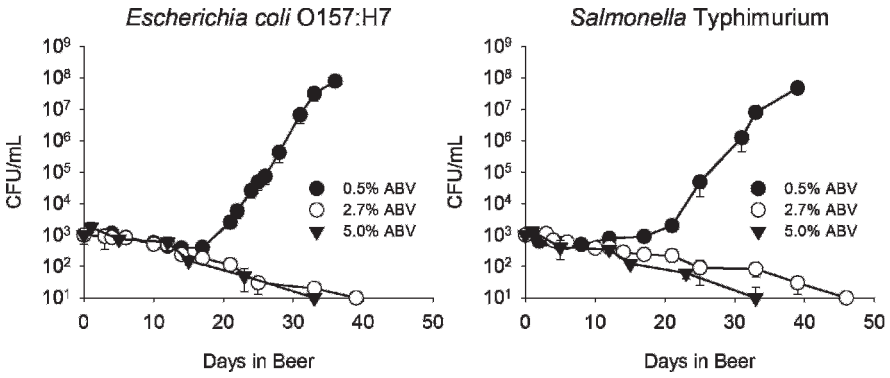

FIGURE 4. *Effects of ethanol on the survival of* E. coli *O157:H7 and* Salmonella *Typhimurium at 14°C, using alcohol-free beer (0.5% ABV) as a basal medium. Data points are the means of triplicate flasks; error bars are ±standard deviations.*

18.2 days, respectively). When the ethanol content was increased to 2.7 and 5.0% ABV, the cells were slowly inactivated; however, the detection limit (10 CFU/ml) was not reached until after 30 days. Similar responses were obtained for both *E. coli* O157:H7 and *Salmonella* Typhimurium. Specific death rates for *E. coli* O157:H7 and *Salmonella* Typhimurium were −0.0024 and −0.0017, respectively, at 2.7% ABV and −0.0027 and −0.0025 at 5.0% ABV.

As the pH of the alcohol-free beer (pH 4.3 ''as received'') was increased, the growth of the two pathogens was enhanced. Figure 5 shows that both *E. coli* O157:H7 and *Salmonella* Typhimurium grew at faster rates and with shorter lag phases as the pH was increased from the as received value of 4.3 to 4.5 and 5.0. Specific growth rates for *E. coli* O157:H7 were 0.0137 at pH 4.3, 0.0162 at pH 4.5, and 0.0517 at pH 5.0, with corresponding lag phases of 19.4, 10.5, and 2.2 days, respectively. For *Salmonella* Typhimurium, specific growth rates were 0.0104 at pH 4.3, 0.505 at pH 4.5, and 0.0609 at pH 5.0, with lag phases of 18.2, 17.1, and 2.3 days, respectively. At pH 4.0, both pathogens were inactivated, although more than 20 days were required to cause a 2-log reduction in the population. In an attempt to more fully assess pathogen survival at low beer pH values, the effect of acid adaptation via preexposure to sublethal pH values was assessed by two independent methods. Irrespective of the approach taken, acid adaptation did not significantly ($P > 0.05$) influence the survival of *E. coli* O157:H7 compared with nonadapted cultures when inoculated into alcohol-free beer at pH 3.5 (data not shown).

We have previously reported *(25)* that low levels of hop iso-α-acids in wort provide protection against the growth of the gram-positive pathogens *S. aureus* and *L. monocytogenes*. In this study we have shown this to hold true for beer, as these two pathogens were inactivated within 3 days in the alcohol-free beer when held at 14°C (Fig. 6). Specific death rates were −0.0027 for *S. aureus* and −0.0028 for *L. monocytogenes*.

DISCUSSION

E. coli O157:H7 and *Salmonella* Typhimurium were unable to initiate growth in full-strength and mid-strength beer. However, these foodborne pathogens could survive in beer for extended periods when held at lower temperatures. When an inoculum of 10^3 CFU/ml was used, the bacteria survived for more than 10 days in full-strength beer at 4°C and more than 30 days in mid-strength beer. At higher temperatures (25 and 37°C) survival was reduced to a few days. Various studies *(2, 9–12, 15, 38)* involving other acidic foods have reported that the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium is enhanced at lower temperatures. This enhanced survival is assisted by modifications in the bacterial fatty acid profile and by the production of cold shock proteins at low temperatures *(5, 18)*. When other environmental factors precluded growth, Ross et al. *(29)* reported that the temperature had a strong positive effect on the inactivation rate of *E. coli* and other bacteria, whereas other factors such as pH and water activity did not. Such an effect was observed in the present study. Growth was not detected in the full- and mid-strength beers even when stored at a temperature usually conducive to growth (37°C), presumably due to the action of antimicrobial hurdles such as pH and ethanol *(24)*. In such instances, lower temperatures greatly enhanced survival. As the pathogens tested were not rapidly inactivated upon contact

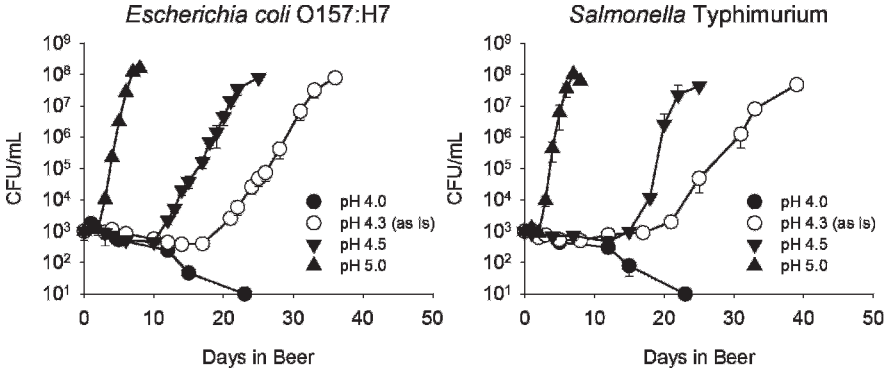

FIGURE 5. *Effects of pH on the survival of* E. coli *O157:H7 and* Salmonella *Typhimurium at 14°C, using alcohol-free beer as a basal medium. Data points are the means of triplicate flasks; error bars are ±standard deviations.*

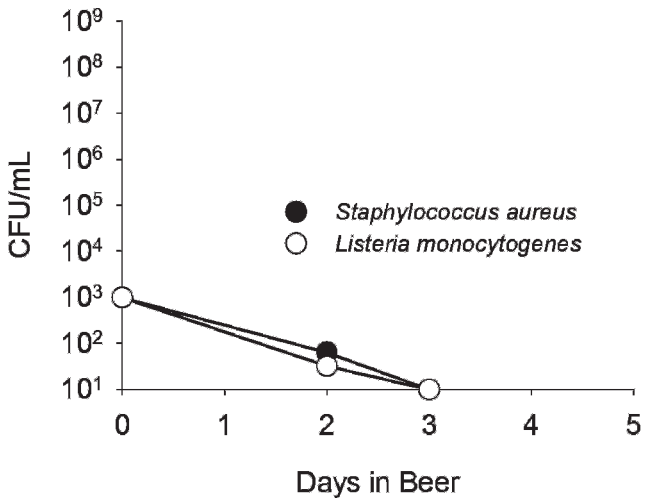

FIGURE 6. *Survival of* S. aureus *and* L. monocytogenes *in alcohol-free beer at 14°C. Data points are the means of triplicate flasks; error bars are ±standard deviation.*

with beer, there remains potential for the transmission of pathogens in beer dispense, especially for acid-resistant microorganisms such as *E. coli* O157:H7, which has an infective dose as low as 10 to 100 cells *(37).* The infective dose for *Salmonella* spp. is estimated to be approximately 10^5 cells; however, outbreaks have been reported to occur from much lower infective doses (10 to 100 cells), and the infective dose is lower in liquids, as these pass through the stomach faster than solid foods *(16).*

After establishing inactivation in full- and mid-strength beers, the potential for growth and survival of the pathogens in alcohol-free beer (0.5% ABV) was investigated. We observed that both *E. coli* O157:H7 and *Salmonella* Typhimurium were capable of vigorous growth in alcohol-free beer (Fig. 3), which is in agreement with an earlier report by L'Anthoën and Ingledew *(20).* Growth in alcohol-free beer was significantly faster (reduced lag phase and increased growth rate) at 25°C than at 14°C, while growth did not occur at 4°C.

In addition to storage temperature, the ethanol content and pH affected the growth and survival of the pathogens. The alcohol-free beer was modified by the addition of ethanol to typical levels of mid-strength (2.7% ABV) and full-strength (5.0% ABV) beer. At these ethanol contents growth did not occur, although the pathogens survived for at least 30 days in the alcohol-free beer adjusted to 5% ABV. Increasing the pH values of the alcohol-free beer to 4.5 and 5.0 from the as received value of 4.3 caused an increase in growth rates and a reduction in lag phases, while lowering the pH to 4.0 inactivated the pathogens. Beer pH values typically fall between 3.95 and 4.29 *(39).*

Acid adaptation did not significantly ($P > 0.05$) increase survival in alcohol-free beer at pH 3.5, which may be explained by the fact that the inocula used throughout this work were in the stationary phase, which has previously been shown to induce a level of acid resistance in cultures *(17, 27).*

This study focused on the survival of gram-negative pathogens only, as we have previously shown *(25)* that the presence of even low levels of hop iso-α-acids prohibit the growth and limit survival of gram-positive pathogens (*L. monocytogenes* and *S. aureus*). Further evidence of the protective effect of the hop iso-α-acids is provided in Figure 6, showing that *L. monocytogenes* and *S. aureus* were inactivated within 3 days in alcohol-free beer, despite only a moderately low level of hops (15 IBU). This can be compared with the effect of the other hurdles on the gram-negative pathogens in alcohol-free beer, where they survived for more than 20 days at pH 4.0 and more than 30 days at 5% ABV. Previous studies in our laboratory have shown that even at 80 IBU, hop iso-α-acids had no effect on the growth of *E. coli* O157:H7 and *Salmonella* Typhimurium *(25).* As large numbers of *S. aureus* cells are required to produce sufficient enterotoxin to cause illness *(34),* there appears to be no risk associated to this organism in hopped beer. Although *L. monocytogenes* has a much lower infective dose (potentially as low as 10^2 to 10^3 cells, depending upon many factors including the susceptibility of the victim) *(35),* risk remains very low due to the organism's intolerance to hop iso-α-acids.

In this work we have investigated the growth and survival of foodborne pathogens in beer. The gram-negative *E. coli* O157:H7 and *Salmonella* Typhimurium were unable to grow in mid- and full-strength beer, although these bacteria could survive for periods exceeding 30 days in mid-strength beer. These pathogens grew rapidly in alcohol-free beer; however, lowering the pH of the beer prevented growth. The gram-positive pathogens *L. monocytogenes* and *S. aureus* were inactivated in alcohol-free beer, presumably due to the presence of hop iso-α-acids. There appears to be no danger of the transmission of pathogens in bottled full-strength beer; however, as the inactivation rate is slowed at low temperatures (4°C), a small risk of pathogen transmission remains with draught beer dispense via cross-contamination. Furthermore, due attention must be paid in the production of alcohol-free beer, as it can support the growth of pathogens, especially at slightly elevated pH levels. Therefore, pasteurization and pH values should be closely monitored, and the production of unpasteurized alcohol-free beer is not risk free.

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