The growth and survival of food-borne pathogens in sweet and fermenting brewers' wort

Garry Menz, Frank Vriesekoop *, Mehdi Zarei, Bofei Zhu, Peter Aldred

Institute of Food & Crop Science, School of Science & Engineering, University of Ballarat, Ballarat, Australia

A R T I C L E   I N F O
Article history:
Received 9 December 2009
Received in revised form 11 February 2010
Accepted 13 February 2010

Keywords:
Beer
Escherichia coli O157:H7
Salmonella Typhimurium
Staphylococcus aureus
Listeria monocytogenes
Hop iso-α-acids

A B S T R A C T
The aim of this study was to investigate the factors affecting the survival and growth of four food-borne pathogens (Escherichia coli O157:H7, Salmonella Typhimurium, Listeria monocytogenes, and Staphylococcus aureus) in sweet and fermenting brewery wort. The Gram-negative pathogens (E. coli and Salm. Typhimurium) were capable of growth during the initial stages of fermentation in hopped wort, although they were quickly inactivated when added during the later stages of fermentation. When the wort was left unpitched, the two Gram-negative pathogens grew unabated. Pathogen growth and survival was enhanced as the pH was increased, and as both the ethanol and original gravity were decreased. Although having no effect on the Gram-negative pathogens, low levels of hop iso-α-acids were sufficient to inhibit L. monocytogenes, and a synergistic antimicrobial effect between iso-α-acids and pH was observed. S. aureus failed to initiate growth in all of the test worts. There appears to be no reason for concern of the safety of a “typical” wort during fermentation, however due attention should be paid when wort is stored or antimicrobial hurdles are lowered, for example in the production of reduced and alcohol-free beer, and in unpasteurised products.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Wort is the sweet extract that is fermented by yeast (Saccharomyces cerevisiae) for the production of beer. As the fermentation proceeds, several antimicrobial hurdles arise, as ethanol and carbon dioxide are produced, and the pH and nutrient levels decrease. Along with the added hops, these intrinsic hurdles, and processing hurdles such as wort boiling and often pasteurisation (or sterile filtration), add to the antimicrobial properties of beer. However, it is not until the wort is fermented that some of these hurdles arise, therefore wort may succumb to undesirable microbial growth, especially if pitching is delayed as is common in beer production by continuous fermentation. Whilst several genera of beer spoilage bacteria have been recognised, previous studies have shown that the survival of pathogens in beer is generally poor (Bendová and Kurzová, 1968; Bunker, 1955; Felsenfeld, 1965; Lentz, 1903; Menz et al., 2009; Sachs-Müke, 1908; Sheth et al., 1988; Zikes, 1903), although Hompesch (1949) showed that Salm. Paratyphi could survive for up to 63 days, albeit from a high inoculation level. Pathogen survival and/or growth is enhanced when antimicrobial hurdles are reduced, for example, L’Anthoën and Ingleedew (1996) reported that several pathogens could grow in alcohol-free beer. Further, pathogens have been reported or inferred in some traditional African beers (Pattison et al., 1998; Shayo et al., 2000), and E. coli has been associated with beer dispense (Schindler and Metz, 1990; Taschan, 1996). However, we are aware of only one study in which the survival of pathogens in wort has been investigated, where Bendová and Kurzová (1968) reported on the survival (but not growth) of Shigella flexneri and Salmonella enteritidis in wort.

The four species chosen for this study (Escherichia coli O157:H7, Salmonella Typhimurium, Listeria monocytogenes, and Staphylococcus aureus) are common causes of food-borne illness, and they have previously been isolated from other beverages. For example, Salmonella Typhimurium and E. coli have been isolated from carbonated soft-drinks (Akond et al., 2009), and E. coli has been transmitted through unpasteurised apple cider (Besser et al., 1993), where it has been shown to survive for extended periods despite a pH below 4.0 (Miller and Kaspar, 1994; Zhao et al., 1993). Such high acid resistance may confer low infective doses, for example E. coli O157:H7 has an infective dose as low as 10–100 cells.

With the advent of such previously unreported acid-resistant strains, coupled with brewing practices trending towards a reduction in antimicrobial hurdles (such as reduced or late hopping, reduced alcohol beers, and unpasteurised beer), it seems prudent to investigate the potential for the growth and survival of pathogenic bacteria in stored and fermenting wort, and to elucidate the effects of the typical antimicrobial hurdles in wort and beer.

* Corresponding author. PO Box 663, Ballarat, 3353, Victoria, Australia. Tel.: +61 3 53279247; fax: +61 3 53279240.
E-mail address: f.vriesekoop@ballarat.edu.au (F. Vriesekoop).

0168-1605/© 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.02.018
2. Materials and methods

2.1. Strain information and inoculum preparation

In this work we evaluated the survival of the following bacteria; E. coli O157:H7-VT (N) – NCTC 12900, L. monocytogenes ATCC 7644, Salm. Typhimurium (kindly provided by the University of Melbourne, Melbourne, Australia), and S. aureus (kindly provided by Victoria University, Melbourne, Australia). A brewing strain of S. cerevisiae was used for wort fermentation. Microorganisms were subcultured weekly and maintained at 4 °C on nutrient agar for bacteria, or YPD for the yeast. Overnight cultures (stationary phase) of the pathogens were prepared in wort with an original gravity of 1.020 (prepared as described later) at 37 °C from a fresh culture, and all experiments were inoculated at approximately 10³ Colony Forming Units per mL (CFU/mL) using optical density standard curves at 600 nm in a UV–Visible spectrophotometer (Varian Cary 50, Mulgrave, Australia). Inoculums of S. cerevisiae were prepared overnight in wort (original gravity of 1.020) at 25 °C from a fresh subculture, and pitched into the experimental worts at 10⁵ CFU/mL. Yeast cell numbers (particles/mL) were estimated using a ZB Coulter Counter (Coulter Electronics, Dunstable, UK) (Barber et al., 2002).

2.2. Microbial analysis

Samples for microbiological analysis were serially diluted (0.85% NaCl) prior to enumeration on solid media. Bacteria were quantified after 24–48 h at 37 °C on nutrient agar, supplemented with cycloheximide (100 mg/L) to inhibit yeast where appropriate. Yeast populations were estimated on YPD (yeast extract peptone dextrose agar), containing (in g/L) glucose (20.0), peptone (5.0), yeast extract (5.0), and bacteriological agar (15.0), supplemented with ampicillin (100 mg/L) and chloramphenicol (100 mg/L) to inhibit bacterial growth, and incubated at 25 °C for 48–72 h. Antibiotics were obtained from Sigma-Aldrich (Castle Hill, Australia) and stock solutions were filter-sterilised (0.45 μm). All media components (with the exception of glucose) were purchased from Oxoid (Thebarton, Australia). Where described, enrichment was performed by transferring 1 mL samples into 9 mL nutrient broths (supplemented with cycloheximide) which were then incubated at 37 °C for 48 h.

2.3. Wort preparation

Wort was prepared by diluting unhopped malt extract (Coopers Brewery, Regency Park, Australia) with distilled water to the desired specific gravity, as determined using a density meter (DMA 35n, Anton Paar, Graz, Austria). The pH was adjusted to 6.0 with 10 M NaOH using a TPS-LC 80A pH meter (Springwood, Australia), resulting in a pH of 5.5 after autoclaving, and a final beer pH of approximately 4.3 when fermented, which is within the typical beer pH range (van Leeuwen, 2006). To assist with wort clarification, Whirlfloc T finings (Deltagen, Boronia, Australia) were added to the wort at 100 ppm, and the wort was then autoclaved at 121 °C for 15 min. This sterile wort was held at room temperature for 48 h, allowing the cold break to precipitate, and was subsequently decanted into sterile bottles before being aseptically aliquoted into the experimental flasks. Total nitrogen levels determined by the Kjeldahl method were similar to that of a typical beer. Wort used for inoculum preparation was further

Fig. 1. The growth and survival of E. coli O157:H7, Salm. Typhimurium, L. monocytogenes, and S. aureus in fermenting, hopped (20 IBUs) wort (original gravity of 1.040). All flasks were inoculated with S. cerevisiae at time zero. In addition, sets of three flasks were inoculated with the pathogen under investigation at time zero and every subsequent 24 h. The time of inoculation is given by the different symbols; 0 h (●), 24 h (○), 48 h (▼), 72 h (Δ), 96 h (■). Ethanol production is shown by the dotted curve. Data points are means of triplicate flasks, and error bars show positive standard deviations.
clarified by centrifugation (RC5C, Sorvall, DuPont, Newtown, USA) for 10 min at 27,500 x g to allow for consistent baseline optical density readings. When fermented with *S. cerevisiae*, the specific gravity, ethanol, pH, and viable yeast counts were typical of a beer fermentation. For the various experiments, several post autoclave modifications were made to the wort. ISO hop extract (Ellerslie Hop Estate, Mitcham, Australia) was added to reach the desired levels expressed as International Bittering Units (IBUs), determined spectrophotometrically (Varian Cary 50, Mulgrave, Australia) after iso-octane extraction, as per the Institute of Brewing method 9.16 (Institute of Brewing, 1997), with 15 min shaking with a magnetic stirrer. Ethanol was sterile filtered (0.45 μm) prior to addition, and wort pH levels were adjusted with sterile filtered (0.45 μm) 2 M HCl or 2 M NaOH using a TPS-LC 80A pH meter (Springwood, Australia).

2.4. Experimental conditions

All experiments were conducted in triplicate in 250 mL Erlenmeyer flasks, each containing 100 mL of wort. Each flask was fitted with a rubber stopper closure with a small (5 mm diameter) hole drilled through the centre, through which a cotton wool plug was fitted. This closure did not allow contaminants into the flask, but allowed the evolution of excess gas out of the flask (during fermentation). All worts were incubated statically at 25 °C.

2.5. Hop adaptation

The effect of progressive subculturing at non-lethal hop iso-α-acid levels was investigated for *L. monocytogenes*. An overnight culture was inoculated into 10 mL nutrient broth (Oxoid, Thebarton, Australia) at 37 °C until visually turbid, and this was then used to inoculate a 10 mL nutrient broth containing 2 IBUs. Once turbid, this culture was inoculated into a 10 mL nutrient broth with 4 IBUs. This adaptation procedure was continued in 2 IBU increments until the apparent minimum inhibitory concentration (MIC) was reached. This value was compared to the MIC of overnight cultures in nutrient broth with various levels of hop iso-α-acids at 2 IBU increments. The experiment was repeated using wort (original gravity of 1.040).

2.6. Ethanol quantification

Samples for ethanol analysis were centrifuged at 8500 × g for 3 min (Universal 30F, Hettich, Tuttingen, Germany) to remove yeast and carbon dioxide, and stored at −18 °C prior to analysis by static headspace gas chromatography (HS-GC). A CombiPAL AutoSampler heated and agitated samples to 55 °C at 500 rpm (2 s on, 4 s off) for 30 min and injected into a 3800 GC (Varian, Mulgrave, Australia) with nitrogen as a carrier gas, fitted with an Econo-Cap Carbowax (Alltech, Baulkham Hills, Australia) 30 m capillary column (0.32 mm ID, 0.25 μm film thickness), which was held at 65 °C for 8 min. The split ratio was 5, the injector was maintained at 200 °C, and a Flame Ionisation Detector (FID) was used at 150 °C. Results were calculated using n-propanol as an internal standard.

2.7. Statistical analysis

One-way ANOVA on the effect of hop iso-α-acids on the growth of *E. coli* and *Salm. Typhimurium* in unpitched wort was performed using SPSS 16 (SPSS Inc., Chicago, USA).

3. Results

3.1. Survival in fermenting wort

The growth and survival of four pathogens in fermenting, hopped (20 IBUs) wort, with an original gravity of 1.040, is shown as Fig. 1. All worts were pitched with *S. cerevisiae* (time zero), and at time zero and every subsequent 24 h, a set of flasks were inoculated with the pathogen under investigation, to divulge the effect of wort contamination at various points during the fermentation. The production of 5.1% (w/v) ethanol by the brewing yeast is also shown in Fig. 1.

The Gram-negative pathogens showed growth for the first 20 h of fermentation, after which their populations declined. *E. coli* O157:H7 reached 10^4 CFU/mL (from an inoculation of 10^2 CFU/mL) and *Salmonella Typhimurium* peaked at 5 × 10^5 CFU/mL, whilst no growth was observed when these bacteria were inoculated after 24 h of fermentation. To theoretically increase the detection limit from ten to one cell per mL, samples from the 96 h inoculation flasks were enriched. The two Gram-negative pathogens could be detected after 168 h, but not after 216 h.
L. monocytogenes and Staph. aureus showed minimal survival in the fermenting wort (Fig. 1), with those inoculated at the beginning of the fermentation surviving for approximately 20 h. Survival was reduced when the pathogens were inoculated into the latter stages of fermentation. It was hypothesised that the stark difference between the response of the Gram-positive and Gram-negative pathogens was due to the presence of hop iso-α-acids (20 IBUs), thus the effects of the hops (and other antimicrobial hurdles) was tested in subsequent experiments.

3.2. Effect of original gravity

Fig. 2 shows the effect of original gravity on the survival of pathogens in fermenting, hopped (20 IBUs) wort. As original gravity is an indication of the available fermentable sugars (in this work original gravity was varied by dilution), a higher original gravity leads to an increased production of ethanol and carbon dioxide by the yeast, both of which have antimicrobial properties. The Gram-negative E. coli O157:H7 and Salmonella Typhimurium grew in all fermenting worts, and in all instances reached their maximum population after approximately 20–30 h, when approximately 1% (w/v) ethanol (along with carbon dioxide) had been produced by the yeast. Both growth and survival of the pathogens was lowest in the wort with the highest original gravity (1.040). No growth could be observed and survival was poor for the Gram-positive pathogens in all of the worts, presumably due to the presence of hop iso-α-acids.

3.3. Effect of hop iso-α-acids

Hop iso-α-acids were inhibitory towards the Gram-positive pathogens in both pitched (Fig. 3) and unpitched wort (Fig. 4). However, hop iso-α-acids showed no significant effect \((p>0.05)\) on the growth of E. coli O157:H7 or Salmonella Typhimurium in wort (original gravity of 1.040) at all levels tested (up to 80 IBUs). In unpitched wort, L. monocytogenes reached a maximum population density of \(3 \times 10^7\) CFU/mL after approximately 50 h. At 5 IBUs the population was steady, whilst at levels of 10 and above the cells were reduced to the detection limit (10 CFU/mL) within 44 h. In pitched wort, L. monocytogenes reached a maximum population of \(2 \times 10^4\) CFU/mL in 24 h, after which time cell numbers decreased as the fermentation progressed, reaching the detection limit of 10 CFU/mL after 120 h. This decline can be attributed to the antimicrobial activity of ethanol and carbon dioxide produced by the pitching yeast. Again, hop iso-α-acids showed strong inhibition, with a two log cycle reduction occurring within 24 h, and enhanced death rates were observed as hop levels increased. Staph. aureus was unable to initiate growth in either the unpitched or pitched wort, and the populations declined to 10 CFU/mL within 80 h. As per L. monocytogenes, cell death was faster with increased hop iso-α-acid levels. To investigate the inability of Staph. aureus to grow in the wort (original gravity of 1.040), two further strains were evaluated (Staph. aureus ATCC 25923, and a Staph. aureus laboratory strain). Although these strains grew well in 1.020 wort, they too failed to grow in the 1.040 wort under the tested conditions. Further investigations showed this phenomenon...
was due to the inoculum size, as growth could be observed in unpitched, unhopped 1.040 wort using inoculation levels above $10^6$ CFU/mL. A similar phenomenon for inoculum size dependent growth has been reported by Robinson et al. (2001) under salt (NaCl) stress conditions for L. monocytogenes.

The synergistic antimicrobial effect between hops and pH is presented as Fig. 5. This graph shows the reduction in specific growth rate (a measure of growth retardation) for Listeria monocytogenes in 1.040 wort. Observed values are plotted beside expected values, which were calculated based on the simple addition of both the individual pH and iso-α-acid effects. The difference in these two values estimates the synergistic effect, which was greater as the pH decreased and IBUs increased. Although it would be expected that the synergy would be further enhanced at higher IBU values, these were not included as growth of L. monocytogenes could only be observed at low IBU levels (Fig. 4).

Further, the ability of Listeria monocytogenes to adapt to iso-α-acids was investigated via progressive culturing in increasing hop iso-α-acid levels. No significant difference in the minimum inhibitory concentration (MIC) in nutrient broth or 1.040 wort was observed between the hop exposed and non-hop exposed cultures.

### 3.4. Effect of ethanol in sweet wort

The effect of ethanol on the growth and survival in unpitched sweet wort is presented as Fig. 6. E. coli and Salm. Typhimurium grew rapidly at 0–2% ethanol (v/v), however the growth rate decreased as the concentration approached 4% (v/v). L. monocytogenes was more strongly influenced by increasing ethanol concentrations, and no

---

**Fig. 5.** Synergistic antimicrobial effect of hop iso-α-acids on Listeria monocytogenes in sweet wort (original gravity of 1.040). The antimicrobial effect was quantitated as the reduction in specific growth rate (SGR) (log/day). SGRs were calculated using DMFit v.2.1 with the model of (Baranyi and Roberts, 1994). The calculated/additive reduction in SGR was determined by adding the reduction in SGR due to a change in pH, and the reduction in SGR due to a change in hop level, which were determined experimentally. The change in the SGR with regard to a change in pH was calculated from the individual effect of pH reduction down from pH 6 to the respective pH, and the expected change in the SGR with regard to a change in hops loading was calculated by the same method, although from 0 IBUs to the respective value. The difference between the calculated/additive reduction and observed reduction in SGR can be taken as the synergistic action between hops and pH.

**Fig. 6.** The effect of ethanol (% v/v) on the growth of E. coli O157:H7, Salm. Typhimurium, L. monocytogenes, and S. aureus in sweet, unhopped wort (original gravity of 1.040) at 25 °C. Data points are means of triplicate flasks, error bars are ± standard deviations.
growth could be observed above 3% (v/v). As previously observed, Staph. aureus was unable to grow in the base wort with an inoculum size of 10^3 CFU/mL. None of the strains could initiate growth at 5% ethanol (v/v) in the wort.

3.5. Effect of pH in sweet wort

The pH is a major antimicrobial hurdle of beer, and its effect on the growth of pathogens in sweet wort is shown as Fig. 7. Both the E. coli and the Salm. Typhimurium strains were unable to initiate growth in wort at pH values of 4.0 and below. Growth was noticeably slowed at pH 4.5, whereas rapid growth was observed at 5.0 and above, with maximum population densities occurring at pH 6.0. L. monocytogenes was not as acid tolerant as the two Gram-negative organisms, as it was unable to grow in the sweet wort at pH 4.5 and below. Growth rates were similar to E. coli and Salm. Typhimurium for pH 6.0 and 5.5, whereas the growth rate was reduced when the pH was adjusted to 5.0. Staph. aureus failed to grow at all the experimental pH values.

4. Discussion

In this work we have investigated the growth and survival of four food-borne pathogens in wort, given various levels of several important antimicrobial hurdles. We began by assessing survival in fermenting wort by introducing the pathogenic bacteria at various stages during the fermentation, and from this “base wort”, we further elucidated the effects of several key hurdles on the growth and survival of the four pathogens in wort.

The Gram-negative pathogens (E. coli and Salm. Typhimurium) were capable of growth during the initial stages of fermentation in the hopped wort (Fig. 1), although they were quickly inactivated when added during the later stages of fermentation. When the wort was left unpitched, the two Gram-negative pathogens grew unabated (Figs. 6 and 7), highlighting the importance of prompt pitching. It is the combination of hurdles that emerge during the yeast fermentation, rather than a single factor, that confer a hostile environment for the pathogens. For example, E. coli and Salm. Typhimurium were not inactivated at 4% (v/v) ethanol (Fig. 6), and could grow at pH 4.5 (Fig. 7), but were inactivated during wort fermentation (Fig. 1). The presence of hops up to 80 IBUs (near the maximum hop level of any beer) had no effect on the growth of the Gram-negative pathogens.

Low to moderate hop levels protected the wort from the growth of the two Gram-positive pathogens, L. monocytogenes and S. aureus. These results are consistent with previous reports on the antimicrobial effects of hop iso-α-acids against these two species (Haas and Barsoumian, 1994; Larson et al., 1996; Sağdic et al., 2003; Schmalreck et al., 1975). In Fig. 3 we show that in unhopped, pitched wort, L. monocytogenes exhibits enhanced survival, although the maximum population only increased by one log cycle. We further investigated the effect of various hop levels on the growth of L. monocytogenes (Fig. 4), where growth could not be observed above 5 IBUs. As the overwhelming majority of beer contains more than 5 IBUs, it would appear that the survival of L. monocytogenes is of no concern in hopped wort or beer. However, brewing and hopping practices respond to market pressures, and there appears to be a trend towards a reduction in hop levels within some areas of the beer market.

It has been suggested (Larson et al., 1996; Shinwell, 1937; Simpson and Smith, 1992) that a synergistic antimicrobial relationship exists between the hop iso-α-acids and the pH, as the iso-α-acids act as organic acids (where such a synergistic relationship is well defined). The data presented in Fig. 5 (as L. monocytogenes only grew over a narrow hop range) shows evidence for this synergy, which
would be expected to be greater as hop levels increased. A greater understanding of this relationship may be useful for the protection of beer from hop sensitive spoilage bacteria.

Beer may be produced with varying original gravities, and as the original gravity is reduced, so too are the fermentable sugars, and thus ethanol and carbon dioxide production are lowered. In this work various original gravities were prepared by dilution, thus an original gravity of 1.020 will contain half the fermentable sugars as the 1.040 wort. As the original gravity was reduced below 1.040, survival of the Gram-positive pathogens (unaffected by the hops) was enhanced, although the yeast activity ensured that they could only survive for a limited period of time after the fermentation was complete (Fig. 2).

Throughout this work a relatively low inoculation level of 10^3 CFU/ml was used. We did not investigate the effect of inoculum size on survival, although it is anticipated that survival would be enhanced at higher (although somewhat more unrealistic) inoculum levels.

We have shown that the growth and survival of four food-borne pathogens in pitched wort is limited. However when the levels of antimicrobial hurdles are reduced, such as the hops, ethanol, and pH, survival is enhanced and growth can occur. There appears to be no reason for concern of the safety of a "typical" wort during fermentation, however due attention should be paid when wort is stored or antimicrobial hurdles are lowered, for example in the production of reduced and alcohol-free beer, and in unpasteurised products. While beer can generally be considered to be a hostile environment to pathogenic microorganisms (Menz et al., 2009), brewers should keep abreast of new and emerging pathogens with enhanced resistance to ensure the microbiologically safe reputation of their products is maintained.

References


