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Survival of probiotic strain *Lactobacillus paracasei* L26 during co-fermentation with *S. cerevisiae* for the development of a novel beer beverage

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ABSTRACT

Amidst the rising popularity of craft beers, it would be opportune to develop a novel, unfiltered and unpasteurized sour beer with high probiotic live counts. However, as beer typically contains hop iso-α-acids that prevent the growth and survival of probiotic lactic acid bacteria, the use of suitable fermentation strategies is crucial. The growth, and survival of the probiotic bacterium, *Lactobacillus paracasei* L26, were assessed during a 10-day co-fermentation period with a brewer's yeast, *Saccharomyces cerevisiae* S-04, in unhopped wort. Isomerized hop extract was added prior to storage of the beers at 25 °C and 5 °C. During co-fermentation in unhopped wort, *L. paracasei* L26 maintained high viable cell counts above 8 Log CFU/mL, indicating species compatibility with the yeast. The majority of fermentable sugars were attenuated by *S. cerevisiae* S-04, with a concomitant production of alcohols and esters. Significant amounts of lactic acid were produced by *L. paracasei* L26 ($P < 0.05$). During storage with added isomerized hop extract, maximal probiotic viability enhancing effects were observed in the presence of live *S. cerevisiae* S-04, in combination with refrigeration. The results suggest that beers could be a vehicle for probiotic delivery under appropriate conditions. This was the first study demonstrating the feasibility of utilizing probiotic lactobacilli as starter cultures in beer brewing.

1. Introduction

Craft beers (also known as specialty beers) have experienced exponential growth over the last two decades, primarily driven by premiumization, and consumers' willingness to seek new, intimate, and unique drinking experiences (Donadini et al., 2016; Euromonitor International, 2018). Although there is a lack of consensus on the definition of a "craft beer" (Euromonitor International, 2018), they are typically regular beers, brewed according to classic styles but with novel flavors or ingredients, or are end-products of unconventional fermentation techniques (Yeo and Liu, 2014). As consumers eschew mass produced lager brands, reviving traditional recipes may be of particular interest, since the competitive advantage of craft beers lies in the rediscovery of ancient beer styles (Donadini et al., 2016; Euromonitor International, 2018).

Early beers were soured to some degree due to acidification by wild yeast and bacteria during spontaneous fermentation. Therefore, traditional sour beers, which are intentionally acidified through wild lactic acid bacteria (LAB) and/or acetic acid bacteria (AAB), can be considered a type of craft beer. Belgian lambics and Flanders red ales represent some of the oldest commercial sour beers, which have recently seen strong revival (Verachtert and Derdelinckx, 2014). As wild yeasts and LAB often results in long fermentation periods and inconsistencies in flavor and quality, pure or mixed commercial LAB cultures (*Lactobacillus delbrueckii*, *L. amylovorus*, and *L. amylolyticus*) are preferred by brewers for fast and reproducible biological acidification of wort (Peyer et al., 2017). More recently, lactic acid producing non-*Saccharomyces* yeasts were shown to be viable alternatives to LAB, for the production of sour beers (Osburn et al., 2017).

Nonetheless, the use of probiotics derived from LAB as starter cultures for sour beer production is a novel concept. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Commercial probiotic cultures commonly used in food applications belong to the genera *Lactobacillus* and *Bifidobacterium*. Their metabolic products may enhance nutrient bioavailability, possess antioxidant and antimicrobial activities, and improve sensory profiles of foods. Therapeutic applications include anti-carcinogenic properties, immune system modulation, and prevention of symptoms of rotavirus and antibiotic associated diarrhea, depending on species and strains within species of probiotics

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(Tamang et al., 2016; Vasiljevic and Shah, 2008). In this regard, probiotics derived from LAB will serve the same purpose of wort acidification, similar to commercial or wild LAB. More notably, the production of unfiltered, and unpasteurized beers rich in live probiotics, will possibly provide additional health benefits that regular sour beers do not provide.

According to the definition provided by Hill et al. (2014), undefined microbial consortia (wild LAB and yeast) involved in spontaneous fermentations cannot be considered as probiotics, unless they are isolated and defined, and there is evidence from well-designed clinical trials that suggests a possible health benefit (Sanders et al., 2018). Similarly, commercial LAB cultures used for the production of sour beers are often selected for their technical abilities (e.g. wort souring), and to the best of the authors' knowledge, have not been proven in human trials to substantiate their strain specific health benefits. Although probiotic strains of *L. amylovorus*, and *L. amylolyticus* (both of non-brewing origins) have been proposed to be potential probiotics (Fei et al., 2018; Finamore et al., 2014), the same health benefits cannot be extended to the strains used for brewing sour beers. Strain specificity of probiotic benefits is still the presumption, unless mechanistic and clinical evidences suggest otherwise (Sanders et al., 2018).

For application of probiotics in functional foods, it is imperative that probiotics are active, viable, and present at sufficient levels to exert optimum therapeutic effects (the recommended minimum intake is 9 Log CFU per serving of product; Hill et al., 2014). However, maintaining probiotic viability in a novel and difficult delivery carrier such as beer is a major technological challenge. Iso-α-acids, which are extracted from hops during wort boiling, are one of the more potent antimicrobial constituents that inhibit growth of Gram-positive probiotic LAB in beer. Undissociated hop compounds are able to diffuse into the bacterial cell and subsequently release protons due to the higher intracellular pH. Dissociated hop compounds then bind to divalent cations such as Mn^{2+}, and diffuse out of the cell. The electroneutral exchange of ions leads to depletion of Mn^{2+}, which is involved in energy generation and redox homeostasis. Concurrently, intracellular acidification occurs and transmembrane ion gradient is dissipated, which inhibits proton motive force uptake of nutrients, ultimately resulting in cell death (Suzuki, 2011).

Despite many hurdles, other intrinsic chemical constituents in beer may enhance probiotic viability and stability. Yeasts, which are required to produce ethanol and carbon dioxide in beer (Narvhus and Gadaga, 2003), have been shown to enhance probiotic survival in fermented milk and acidic environments, possibly due to the formation of mixed-species biofilms which protect LAB from external stress (Liu and Tsao, 2009; Suharja et al., 2014; Yeo et al., 2016). However, it remains to be seen if similar viability enhancing effects between yeast and probiotics can be observed in beer.

To the best of our knowledge, only one other study has attempted to utilize probiotic yeast as starter cultures in beer fermentation, but none thus far have investigated beer fermentation involving probiotic bacteria. Capece et al. (2018) carried out mixed fermentations using several different *Saccharomyces cerevisiae* strains, with the probiotic yeast *S. cerevisiae* var. *boulardii*. The resultant unfiltered and unpasteurized beer contained high levels of the probiotic yeast (6.9–7.8 Log CFU/mL), with a concomitant rise in antioxidant and polyphenol contents. However, probiotic viability and stability during storage, which is essential for practical considerations, was not assessed. In addition, the impacts of co-culturing probiotic LAB together with beer yeasts have yet to be evaluated. Compared to probiotic yeasts which are resistant to hop iso alpha acids (Hazelwood et al., 2010), incorporating probiotic LAB may prove to be a much more challenging task. Nonetheless, if accomplished, opportunities abound to employ other commercially important probiotic LAB (besides yeast) that possess strain specific therapeutic benefits.

Therefore, this paper's aim was to investigate the growth, viability, and stability of the probiotic *Lactobacillus paracasei* L26 in unhopped wort, when co-cultured with *Saccharomyces cerevisiae* S-04. This was followed by the addition of isomerized hop extract, and subsequent storage at refrigerated and ambient temperatures. Ultimately, the goal was to develop a novel sour beer with high probiotic bacteria live counts (9 Log CFU per serving of product, 100 g or mL).

2. Materials and methods

2.1. Wort preparation

Sweet wort was prepared by reconstituting 12.2% (w/v) dry light malt extract (Thomas Coopers Breweries, Adelaide, Australia) into pure drinking water (Fraser and Neave, Limited, Malaysia), followed by boiling for 20 min to achieve hot break. After which, 2.0% (w/v) dextrose (Thomas Coopers Breweries, Adelaide, Australia) and 0.2% (w/v) Cascade hop pellets (Yakima Chief- Hopunion, Yakima, USA) were added and boiled for another 60 min. This translated to 27 International Bitterness Units (IBUs) using the Rager, Tinseth, and Daniel methods (Daniels, 1996; Palmer, 2001; Rager, 1990). Pure drinking water was then used to top up to the original wort batch weight, and cooled using an ice bath for 60 min to achieve cold break. Subsequently, the cooled wort was filtered through double layer cheese cloths into 250-mL or 500-mL capped glass bottles, and pasteurized at 95 °C for 15 min. The effectiveness of the pasteurization process was verified by streaking the wort onto potato dextrose agar (PDA; Oxoid Ltd., Hampshire, UK). Hops were omitted in the preparation of unhopped wort, which was for use as pre-culture and for co-fermentation with yeast.

2.2. Microorganisms, cultivation conditions and enumeration

The six *Lactobacillus* probiotic strains used for preliminary screening were *L. acidophilus* NCFM, *L. paracasei* Lpc-37, *L. rhamnosus* HN001, and *L. bulgaricus* Lb-64 (all strains from Danisco A/S, Copenhagen, Denmark), as well as *L. acidophilus* L10 and *L. paracasei* L26 (both from Lallemand, Montreal, Canada). Probiotic pure cultures were propagated in MRS (de Man, Rogosa and Sharpe) broth, at 37 °C for 24 h. Freeze dried *Saccharomyces cerevisiae* Safale S-04 dry ale yeast (Lesaffre, Lille, France) was propagated at 20 °C for 48 h, in sterile yeast nutrient broth (2.5 g/L yeast extract, 2.5 g/L bacteriological peptone, 2.5 g/L malt extract; all from Oxoid Ltd., Hampshire, UK), and 20 g/L dextrose (Thomas Coopers Breweries, South Australia, Australia), adjusted to pH 5.0 with 1 M malic acid. All pure cultures were stored at −80 °C before use.

Probiotic pre-cultures were prepared by inoculating 5% (v/v) pure culture in unhopped wort, which were incubated statically at 37 °C for 24 h to achieve minimum cell counts of 8.0 Log CFU/mL. *S. cerevisiae* S-04 pure cultures at 5% (v/v) were inoculated into unhopped wort at 20 °C for pre-culture preparation, followed by 48 h incubation to achieve cell counts of at least 7.0 Log CFU/mL.

Serial dilutions were carried out in 0.1% (w/v) peptone solution, and the appropriate dilutions were spread-plated on agar selective for each microorganism. Enumeration of *Lactobacillus* strains was carried out using MRS agar (Oxoid Ltd., Hampshire, UK) supplemented with 135 ppm of natamycin (Natamax®, Danisco A/S, Copenhagen, Denmark) to inhibit yeasts. The plates were incubated at 37 °C for 48 h. For mixed cultures, *S. cerevisiae* S-04 was enumerated on oxytetracycline glucose-yeast extract agar (Oxoid Ltd., Hampshire, UK) containing 100 ppm of oxytetracycline (Oxoid Ltd., Hampshire, UK) to inhibit LAB growth. The plates were incubated at 20 °C for 72 h. Enumeration of *S. cerevisiae* S-04 mono-culture was carried out on PDA (Oxoid Ltd., Hampshire, UK), before incubation at 20 °C for 72 h.

2.3. Fermentation conditions and design

For the preliminary screening, incubations of each probiotic lactobacilli strain were first carried out in 250-mL sterile Erlenmeyer flasks,

containing 100 mL of hopped wort. Each flask was inoculated with 1% (v/v) of *L. acidophilus* NCFM, *L. acidophilus* L10, *L. paracasei* Lpc-37, *L. rhamnosus* HN001, *L. bulgaricus* Lb-64, or *L. paracasei* L26 pre-culture, and incubated statically at 37 °C for 7 days. Enumeration of the probiotics was carried out on day 0, 1, 2, and 4, and 7.

Based on the results of the screening experiment, *L. paracasei* L26 was selected for subsequent co-fermentation of unhopped wort with *S. cerevisiae* S-04. Co-fermentation was carried out in 500-mL screw capped glass bottles, containing 400 mL of unhopped wort. *L. paracasei* L26 and *S. cerevisiae* S-04 were inoculated at approximately 6.7 Log CFU/mL (1% (v/v)) and 5.0 Log CFU/mL (0.5% (v/v)), respectively, to favor the growth of *L. paracasei* L26. Mono-cultures of *L. paracasei* L26 and *S. cerevisiae* S-04 served as controls. Fermentations were first carried out statically at 30 °C from day 0 to day 2 to provide favorable conditions for probiotic growth. After maximal probiotic cell counts had been reached, the temperature was changed to 20 °C from day 2 to day 10, to cater to yeast fermentation. Samples were taken periodically for analyses of various parameters.

After 10 days of fermentation, isomerized hop extract (Brouwland, Beverlo, Belgium) was diluted in deionized water to 5% (v/v), and added to the same fermentation vessels, to achieve approximately the same hop concentration as the preliminary screening stage (27 IBUs). To assess the effect of storage temperatures on probiotic survivability, the samples were divided into 15-mL centrifuge tubes, each containing 8 mL of samples, and stored at 5 °C and 25 °C to simulate refrigerated and ambient storage, respectively. The end of shelf life for each fermentation set was determined when plate counts for *L. paracasei* L26 fell below 7 Log CFU/mL. Sampling was similarly carried out periodically for analysis.

2.4. Non-volatile and volatile analytical determinations

Non-volatile and volatile measurements were carried out according to the method described by Toh et al., 2018. Measurements for pH and °Brix were carried out using a pH meter (Metrohm, Herisau, Switzerland), and a refractometer (ATAGO, Tokyo, Japan) respectively. Sugars and organic acids were identified and quantified using high performance liquid chromatography (HPLC; Shimadzu HPLC, class-VP software version 6.1, Kyoto, Japan). Volatiles were analyzed using headspace (HS)-solid phase micro extraction (SPME) combined with gas chromatography (GC; Agilent 7890A, Santa Clara, CA, USA), triple axis mass spectrometer (MS; Agilent 5975C, Santa Clara, CA, USA), and flame ionization detector (FID; Agilent 5975C, Santa Clara, CA, USA).

2.5. Statistical analysis

Data from triplicate fermentations (n = 3) were analyzed using one-way analysis of variance (ANOVA) and Tukey's post-hoc test (SPSS Corporation, version 17.0, Chicago, IL, USA). Results were statistically significant when $P < 0.05$. Principal component analysis (PCA) was conducted using Matlab R2008a (MathWorks, Natick, MA, USA).

3. Results and discussion

3.1. Preliminary screening for the survival of different probiotic lactobacilli strains in hopped wort

Depending on the style, the levels of iso-α-acids in sour beers range from 0 to 25 IBUs (Beer Judge Certification Program, 2015). Although omitting hops is a plausibility in the production of a novel probiotic sour beer, identifying a hop resistant probiotic strain would expand the possibilities of creating other probiotic beer styles with a higher iso-α-acids content (e.g. lagers, stouts, etc.). Therefore, preliminary screening was carried out to select a suitable probiotic bacterial strain which exhibits the best survival in hopped wort.

Fig. 1 shows the survival of *L. acidophilus* NCFM, *L. acidophilus* L10, *L. bulgaricus* Lb-64, *L. paracasei* L26, *L. paracasei* Lpc-37, and *L. rhamnosus* HN001 in hopped wort at 37 °C. The cell counts of all six probiotic strains declined continuously, and no viable cells were detected after the 7-day incubation period. This indicated that the selected probiotic strains were unable to grow and survive in hopped wort. *L. paracasei* L26 exhibited the greatest survivability, which declined by 1.9 Log cycle after 4 days of incubation. This was followed by *L. paracasei* Lpc-37 and *L. rhamnosus* HN001, which were not detected in the wort on day 4, and *L. acidophilus* L10 and *L. bulgaricus* Lb-64 on day 2. Viable cell counts of *L. acidophilus* NCFM were not detectable within a day of incubation. Cell death can be attributed to the presence of hop iso-α-acids, as the probiotics could reach high viable cell counts of at least around 8.5 Log CFU/mL when propagated in unhopped wort during the pre-culturing step (data not shown).

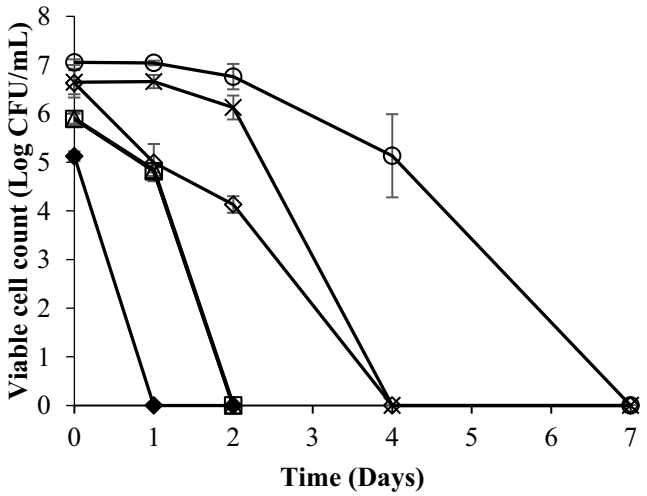

Fig. 1. Survival of single probiotic strains in hopped wort at 37 °C. (♦) *L. acidophilus* NCFM; (Δ) *L. acidophilus* L10; (□) *L. bulgaricus* Lb-64; (○) *L. paracasei* L26; (×) *L. paracasei* Lpc-37 and; (◇) *L. rhamnosus* HN001. Values are the mean of triplicate experiments ($n = 3$), with error bars representing the standard deviation of the mean values.

Since all probiotic strains were unable to grow and survive in the presence of hops, it was evident that the probiotics would first need to be co-cultured with yeast in unhopped wort. *L. paracasei* L26, which displayed the greatest survivability in hopped wort, was subsequently chosen for co-fermentation with *S. cerevisiae* S-04 ale yeast.

3.2. Co-culturing L. paracasei L26 with S. cerevisiae S-04 in unhopped wort

3.2.1. Growth of individual and mixed cultures of L. paracasei L26 and S. cerevisiae S-04

Growth kinetics of single and mixed cultures of *L. paracasei* L26 and *S. cerevisiae* S-04 during the 10-day fermentation period (30 °C for 2 days, 20 °C for subsequent 8 days), are shown in Fig. 2. In the absence of hops, *L. paracasei* L26 was not only able to grow, but maintained high and viable stationary phase counts throughout the fermentation period, with cell counts of 9.1 and 8.8 Log CFU/mL on day 10 for mono- and co-cultures respectively. The findings are in good agreement with various authors, who have reported malt-based media to be favorable to various lactobacilli, due to the abundance of energy and nutrient sources (Charalampopoulos et al., 2002; Rathore et al., 2012).

Interactions between LAB and yeast are complex. The growth and metabolic rate of LAB may either be diminished, augmented, or unaffected in the presence of yeast, depending on various factors such as food matrix or microbial strains (Sieuwerts et al., 2018). In this study, the presence of *S. cerevisiae* S-04 was not detrimental towards the viability of *L. paracasei* L26, as probiotic cell counts were not significantly different ($P > 0.05$) between single and mixed cultures on day 10. The

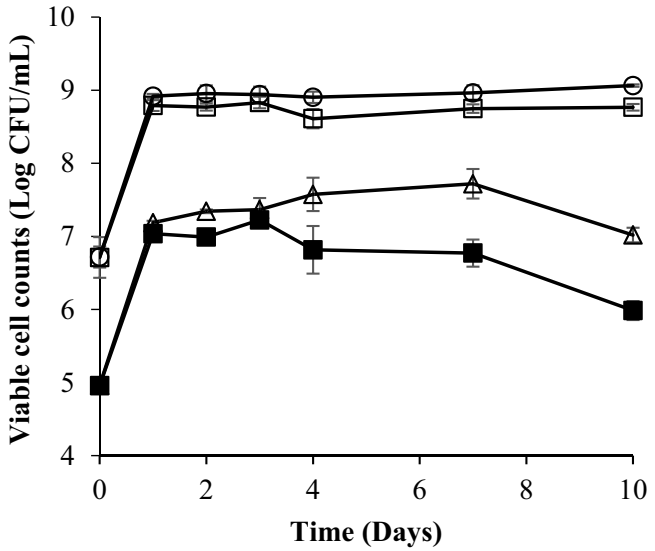

Fig. 2. Growth of *L. paracasei* L26 and *S. cerevisiae* S-04 in the co-fermentation stage. (○) *L. paracasei* L26 in mono-culture; (□) *L. paracasei* L26 in co-culture; (■) *S. cerevisiae* S-04 in co-culture and; (Δ) *S. cerevisiae* S-04 in mono-culture. Values are the mean of triplicate experiments ($n = 3$), with error bars representing the standard deviation of the mean values.

high stationary phase cell numbers also meet the minimal requirement of 9 Log CFU per serving of product (100 mL or g) in order to confer health benefits (Hill et al., 2014).

3.2.2. Changes in total soluble solids and sugars

Table 1 and Fig. 3 show the changes in °Brix and sugars in single and mixed cultures of *L. paracasei* L26 and *S. cerevisiae* S-04 during the 10-day fermentation period. Fermentation kinetics of °Brix and sugars were largely driven by *S. cerevisiae* S-04, where the significant decline in °Brix values ($P < 0.05$) coincided with the utilization of sugars by the yeast. Complete sugar utilization, as well as similar growth trends between single and mixed cultures of *S. cerevisiae* S-04 (Fig. 2) indicated that the acidic environment imposed by the probiotic bacteria was insufficient to affect yeast growth. It is imperative that adequate numbers of live viable yeast are present to produce desired quantities of ethanol and carbon dioxide in beers (Narvhus and Gadaga, 2003). More importantly, viable yeasts are required to prolong the survival of probiotics in acidic environments (Suharja et al., 2014; Yeo et al., 2016).

Table 1
Non-volatile changes in unhopped wort (day 10) by co-fermentation of *L. paracasei* L26 with *S. cerevisiae* S-04.

Parameter	Unhopped wort	Unhopped fermented wort/beer (day 10)		
		L26	L26 + S-04	S-04
pH	5.34 ± 0.04^{d}	3.46 ± 0.00^{a}	3.62 ± 0.01^{b}	4.72 ± 0.00^{c}
°Brix (%)	14.00 ± 0.00^{d}	13.83 ± 0.03^{c}	7.55 ± 0.01^{b}	6.99 ± 0.08^{a}
Sugars (g/L)				
Fructose	3.91 ± 0.42	Trace	ND	ND
Glucose	24.96 ± 1.32^{b}	22.13 ± 0.67^{a}	ND	ND
Sucrose	Trace	Trace	ND	ND
Maltose	48.46 ± 2.49^{a}	49.31 ± 1.85^{a}	Trace	ND
Organic Acids (g/L)				
Acetic acid	1.44 ± 0.06^{b}	1.41 ± 0.02^{b}	1.42 ± 0.02^{b}	1.28 ± 0.05^{a}
Citric acid	1.88 ± 0.02^{b}	1.80 ± 0.02^{b}	1.64 ± 0.02^{a}	2.04 ± 0.06^{c}
Lactic acid	ND	6.63 ± 0.06^{b}	5.25 ± 0.17^{a}	ND
Malic acid	0.68 ± 0.02	Trace	ND	Trace

ND: Not detected. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the same row with different lowercase letters are significantly different ($P < 0.05$).

It was observed that *S. cerevisiae* S-04 preferentially utilized sucrose first, followed by glucose, fructose, and finally maltose (Fig. 3). In addition, for both single and mixed cultures of *S. cerevisiae* S-04, a rise in fructose concentration from day 0 to day 1 coincided a decline in sucrose levels in the same time period. Such fermentation patterns by brewing yeasts are consistent with sugar uptake patterns in wort due to glucose catabolite repression. When first pitched into new wort containing an abundance of glucose, invertase located in the yeast periplasmic space hydrolyzes sucrose into glucose and fructose. Glucose is then depleted more rapidly than fructose, as transport of fructose is less kinetically favored than glucose. Once glucose is exhausted, catabolite repression of maltose transporter genes is relieved and yeast begins utilizing maltose (D'Amore et al., 1989).

3.2.3. Changes in pH and organic acids

Fig. 4 shows the kinetic changes in pH for single and mixed cultures during the 10-day fermentation period. The significant decline in pH ($P < 0.05$) observed in mixed cultures coincided with the production of lactic acid by *L. paracasei* L26. The probiotic bacteria in the co-cultured beer could thus contribute a sour character to beer reminiscent of commercial sour beers, as the final pH of 3.62 falls within the range of sour beers (pH 3.1 to 3.9; Tonsmeire, 2014).

It was also observed that compared to the *L. paracasei* L26 mono-culture, significantly higher pH ($P < 0.05$), and significantly lower levels of lactic acid ($P < 0.05$) were produced in the mixed culture on day 10 (Table 1). Competition for sugars by *S. cerevisiae* S-04, in particular fructose and glucose, could have reduced the monosaccharides available for *L. paracasei* L26 for lactic acid production. Significantly higher pH values in the mixed culture may have implications during storage, as the potency of hop acids is diminished due to a greater proportion of hop acids existing in the dissociated form (Blanco et al., 2007). This will be discussed further in Section 3.3.1.

A significantly lower citric acid concentration ($P < 0.05$) was detected in the mixed culture than the probiotic mono-culture on day 10 (Fig. 4 and Table 1). *L. paracasei* L26 has been shown to utilize citrate, producing lactic acid and acetic acid via citrate lyase (Lee et al., 2013). However, a rise in acetic acid concentrations was not reflected, possibly due to acetic acid catabolism by *S. cerevisiae* under low nutrient conditions for synthesis of amino acids and organic acids (Coote and Kirsop, 1974; Trček et al., 2015). Indeed, since significant amounts of acetic acid were consumed by *S. cerevisiae* S-04 in the mono-culture ($P < 0.05$), any production of acetic acid by *L. paracasei* L26 was therefore not reflected.

Malic acid was progressively depleted in both single and mixed cultures (Fig. 4 and Table 1). *L. paracasei* L26 is able to carry out malolactic fermentation (MLF) to produce lactic acid and carbon dioxide from malic acid (Lee et al., 2013; Liu, 2003), while *S. cerevisiae* S-04 inefficiently converts malic acid to pyruvate, which is further metabolized to ethanol and carbon dioxide via malo-ethanolic fermentation (MEF; Volschenk et al., 2003). Similar to wines, MLF and MEF could contribute to the development of more complex organoleptic profiles in the sour beers by reducing total acidity due to the conversion of dicarboxylic malic acid to monocarboxylic lactic acid (Varakumar et al., 2013).

Normally, the maintenance of the intracellular pH by LAB is energy consuming, due to the H^+ translocating ATP synthase required to generate the proton motive force across the membrane. The consumption of organic acids (e.g. citric and malic acids) by *L. paracasei* L26 may thus be energetically favorable, since intermediates produced (e.g. pyruvate) can be diverted to produce lactic acid, with its subsequent cellular efflux maintaining the proton motive force without consuming extra ATP (Liu, 2003; Michels et al., 1979). This may be beneficial for *L. paracasei* L26 to survive in the nutrient scarce beer.

3.2.4. Changes in volatile components before storage

A wide range of volatiles were detected (Table 2; Supplementary

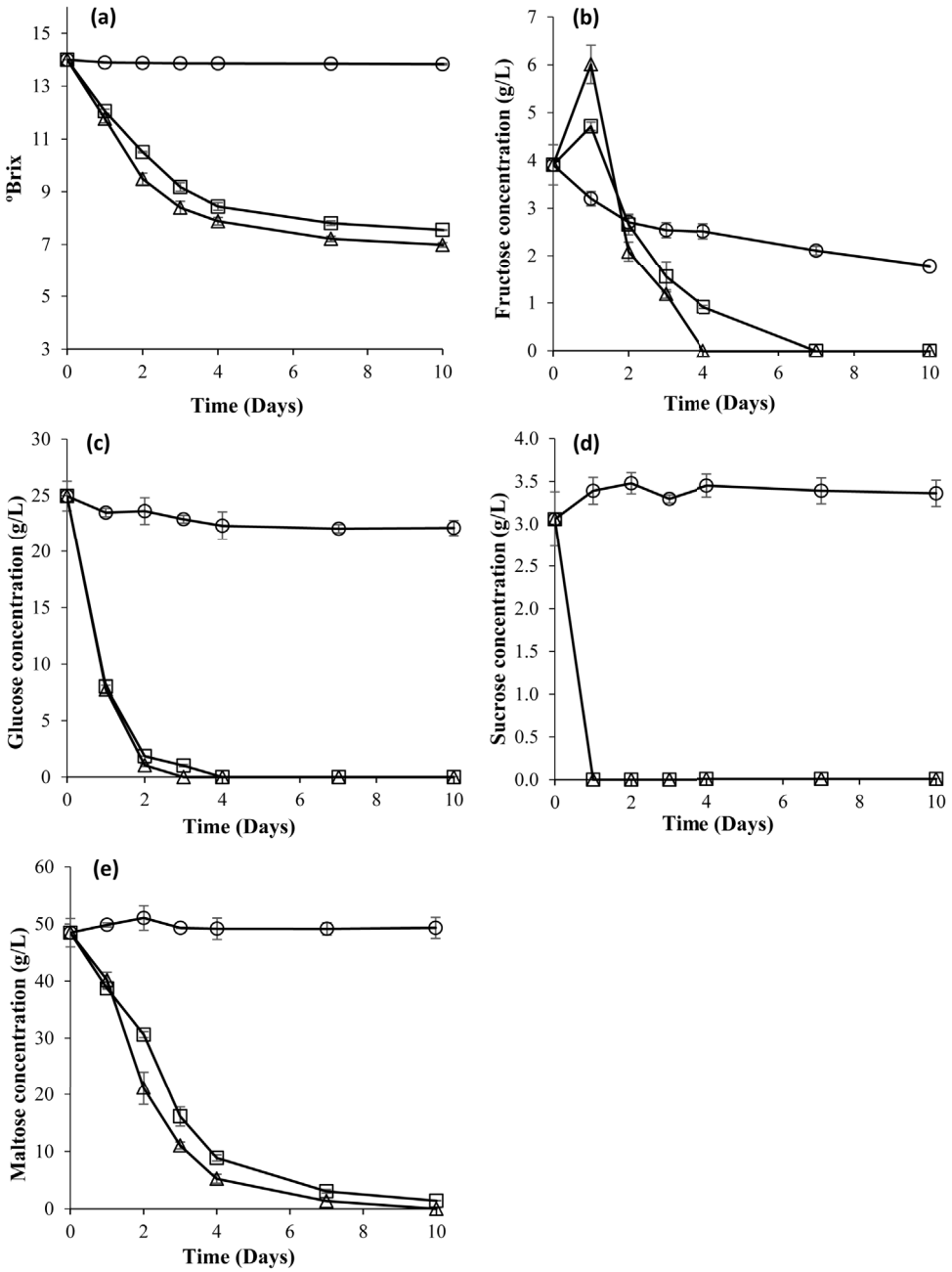

Fig. 3. Changes in (a) °Brix, and the concentration of different sugars during single and mixed fermentation with yeast and probiotics. (b) Fructose, (c) glucose, (d) sucrose and (e) maltose. (○) *L. paracasei* L26 mono-culture; (□) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 and; (Δ) *S. cerevisiae* S-04 mono-culture. Values are the mean of triplicate experiments ($n = 3$), with error bars representing the standard deviation of the mean values.

Table A), including acids, alcohols, aldehydes, esters, ketones, Maillard reaction products, terpenes and terpenoids in both the wort and fermented wort/beers prior to storage. *S. cerevisiae* S-04 was the primary producer of alcohols, mainly contributed by ethanol. Although lower amounts of ethanol were detected in the mixed culture compared to the pure yeast culture, the difference was insignificant ($P > 0.05$), highlighting that the probiotic did not compromise on ethanol production by the yeast.

Higher alcohols such as 2-phenylethyl alcohol, isobutanol, isoamyl alcohol, and 1-propanol, were detected in samples containing *S. cerevisiae* S-04, and are synthesized by the yeast from amino acids via the Ehrlich pathway (Hazelwood et al., 2008). Levels of 2-phenylethyl alcohol and isoamyl alcohol were found in significantly greater amounts in the co-culture than in the yeast mono-culture ($P < 0.05$), which may be attributed to the release of phenylalanine and leucine via proteolysis by *L. paracasei* L26 (Damiani et al., 1996; Ong and Shah, 2009), or acid stress imposed by *L. paracasei* L26 (Damiani et al., 1996; Guerzoni et al., 2007). It has been proposed that Ehrlich pathway products (e.g. higher alcohols) act as quorum-sensing compounds to induce differentiation, enabling yeast cells to adapt to environmental stresses (Hazelwood et al., 2008). Greater concentrations of 2-phenylethyl alcohol and isoamyl alcohol may favorably impact the flavor profile of mixed culture as they impart honey rose like flavor, and whiskey malt flavor respectively (Dong et al., 2013).

Esters comprised the second largest group of volatiles for beers containing *S. cerevisiae* S-04, with the predominant being ethyl esters. Overall, the co-cultured beer yielded significantly lower levels of esters compared to the single yeast culture ($P < 0.05$), suggesting that ester

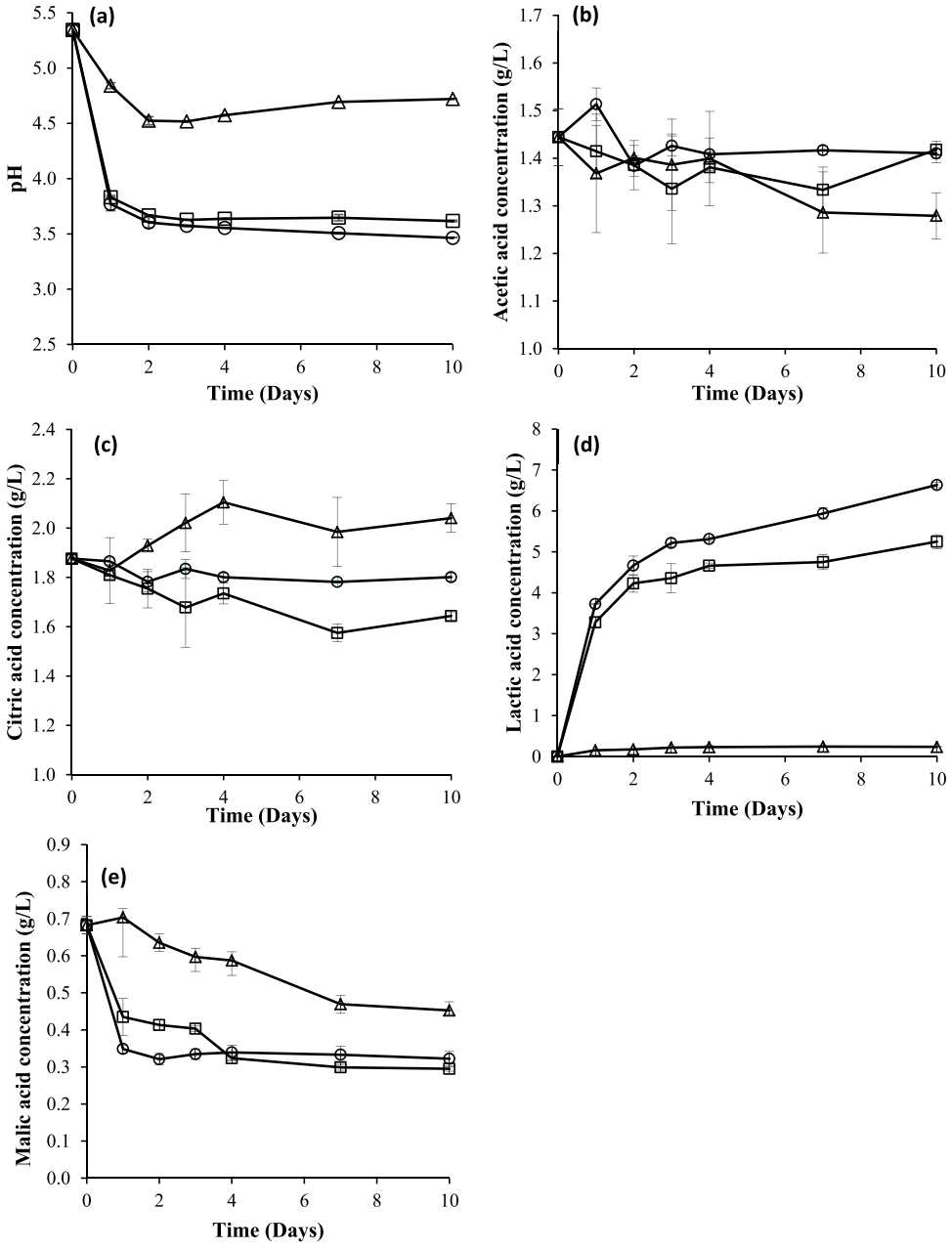

Fig. 4. Changes in (a) pH, and the concentration of different organic acids during the co-fermentation with yeast and probiotics. (b) Acetic acid, (c) citric acid, (d) lactic acid, (e) malic acid. (○) *L. paracasei* L26 mono-culture; (□) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 and; (Δ) *S. cerevisiae* S-04 mono-culture. Values are the mean of triplicate experiments (n = 3), with error bars representing the standard deviation of the mean values.

production was affected by the probiotic. Although esters produced by *S. cerevisiae* usually stayed around their respective odor detection threshold values, they have synergistic/additive effects with other volatiles and minute changes in their concentrations can have considerable consequences on the flavor profile (Saerens et al., 2010). Further sensorial studies are thus required to assess the impact of lower concentrations of esters on the overall flavor profile of esters in the mixed culture compared to the single culture.

Ketones, which were the main volatiles produced by *L. paracasei* L26, were either not detected or found in significantly lower amounts in the mixed culture compared to the single culture ($P < 0.05$), possibly due to the reduction of methyl ketones to secondary alcohols by the yeast (Damiani et al., 1996; Moore et al., 2007). This may be desirable, as the absence of potent odorants such as diacetyl and acetoin (buttery notes) is favored in commercial sour beers (Tonsmeire, 2014).

Strecker aldehydes (e.g. 2-phenylacetaldehyde, 3-methylbutanal), and Maillard reaction products (e.g. 2-acetylfuran, 2-pentylfuran, 5-methylfurfural, and furfural) were formed during wort boiling (Baert et al., 2012). As fermentation progressed, their levels largely declined, possibly arising from the reducing action by yeasts (Baert et al., 2012; Hazelwood et al., 2008; Saison et al., 2009).

3.2.5. Principal component analysis

To further understand and visualize the differences in aroma compound profiles of each fermentation trial, principal component analysis (PCA; Fig. 5) was conducted using volatile data from Table 2, and lactic and acetic acid data from Table 1. Principal components (PC) 1 and PC2 accounted for 65.02% and 22.19% of the total variance respectively.

Table 2
Selected volatiles (mean GC-FID peak area x 10^6) and their relative peak area (RPA, %) in unhopped beer and worts/beers fermented with single and mixed cultures of *L. paracasei* L26 and *S. cerevisiae* S-04.

Compound Identified	Identification Methods	LRI	Unhopped wort		Unhopped fermented wort/beer (day 10)					
					L26		L26 + S-04		S-04	
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)
Acids										
Acetic acid	MS, LRI	1447	5.58 ± 0.28^b	8.33	14.60 ± 0.65^c	21.72	1.14 ± 0.20^a	0.05	0.82 ± 0.10^a	0.02
Subtotal			6.95	10.38	16.32	24.27	59.27	2.35	105.47	3.20
Alcohols										
Ethanol	MS		ND	0.00	ND	0.00	1854.25 ± 268.55^a	73.37	1978.22 ± 167.56^a	59.93
1-Propanol	MS, LRI	1052	ND	0.00	ND	0.00	2.43 ± 0.16^a	0.10	4.37 ± 0.57^b	0.13
Isobutanol	MS, LRI	1100	ND	0.00	ND	0.00	7.24 ± 0.14^b	0.29	6.69 ± 0.85^a	0.20
Isoamyl alcohol	MS, LRI	1227	ND	0.00	11.23 ± 0.40^a	16.70	44.80 ± 3.56^c	1.77	30.52 ± 3.73^b	0.92
2-Phenylethyl alcohol	MS, LRI	2133	ND	0.00	ND	0.00	69.83 ± 3.82^b	2.76	33.34 ± 1.46^a	1.01
Subtotal			0.00	0.00	14.24	21.18	1978.55	78.29	2053.14	62.20
Aldehydes										
3-Methylbutanal	MS		26.31 ± 1.14^b	39.29	2.41 ± 0.08^a	3.58	ND	0.00	ND	0.00
Hexanal	MS, LRI	1077	3.03 ± 0.11	4.53	ND	0.00	ND	0.00	ND	0.00
(E)-2-Nonenal	MS, LRI	1530	0.77 ± 0.10^a	1.15	1.04 ± 0.09^a	1.55	1.47 ± 0.09^b	0.06	1.58 ± 0.12^b	0.05
2-Phenylacetaldehyde	MS, LRI	1648	3.34 ± 0.42^b	4.99	0.89 ± 0.09^a	1.32	ND	0.00	ND	0.00
Subtotal			37.44	55.91	5.70	8.48	3.10	0.12	7.04	0.21
Esters										
Ethyl acetate	MS		ND	0.00	ND	0.00	17.74 ± 1.96^a	0.70	21.04 ± 1.04^b	0.64
Isoamyl acetate	MS, LRI	1111	ND	0.00	ND	0.00	30.94 ± 3.63^a	1.22	26.90 ± 4.02^a	0.81
2-Phenylethyl acetate	MS, LRI	1813	ND	0.00	ND	0.00	11.59 ± 1.79^a	0.46	26.41 ± 3.86^b	0.80
Ethyl butanoate	MS, LRI	1029	ND	0.00	ND	0.00	1.83 ± 0.07^b	0.07	1.72 ± 0.04^a	0.05
Ethyl hexanoate	MS, LRI	1215	ND	0.00	ND	0.00	22.31 ± 5.41^a	0.88	21.90 ± 1.94^a	0.66
Ethyl lactate	MS, LRI	1346	ND	0.00	ND	0.00	2.75 ± 0.14	0.11	ND	0.00
Ethyl octanoate	MS, LRI	1421	0.22 ± 0.01^a	0.33	0.18 ± 0.00^a	0.27	154.80 ± 7.95^b	6.13	176.18 ± 19.57^b	5.34
Ethyl decanoate	MS, LRI	1628	0.31 ± 0.03^a	0.46	0.53 ± 0.03^a	0.79	157.64 ± 32.35^b	6.24	524.11 ± 25.08^c	15.88
Ethyl dodecanoate	MS, LRI	1832	ND	0.00	ND	0.00	25.42 ± 1.94^a	1.01	80.56 ± 2.61^b	2.44
Subtotal			0.53	0.79	0.71	1.06	481.28	19.04	1130.97	34.26
Ketones										
Diacetyl	MS		ND	0.00	6.91 ± 0.37	10.28	ND	0.00	ND	0.00
Acetoin	MS, LRI	1298	ND	0.00	8.17 ± 0.50	12.15	ND	0.00	ND	0.00
Subtotal			2.52	3.76	22.22	33.05	0.67	0.03	0.24	0.01
Maillard reaction products										
2-Pentylfuran	MS, LRI	1207	0.61 ± 0.06^b	0.91	0.54 ± 0.04^b	0.80	0.10 ± 0.00^a	0.00	0.18 ± 0.02^a	0.01
Furfural	MS, LRI	1470	13.11 ± 1.13^b	19.58	1.90 ± 0.15^a	2.83	0.50 ± 0.06^a	0.02	0.61 ± 0.07^a	0.02
2-Acetylfuran	MS, LRI	1510	0.54 ± 0.03^c	0.81	0.48 ± 0.02^b	0.71	0.22 ± 0.02^a	0.01	0.18 ± 0.00^a	0.01
5-Methylfurfural	MS, LRI	1582	0.18 ± 0.01^b	0.27	0.21 ± 0.00^c	0.31	0.15 ± 0.01^a	0.01	0.14 ± 0.00^a	0.00
Subtotal			17.26	25.78	5.21	7.75	0.97	0.04	1.11	0.03
Total			66.96	100.00	67.23	100.00	2527.27	100.00	3300.97	100.00

LRI, linear retention index, which was determined on a DB-FFAP column relative to C_5−C_{40} hydrocarbons.
RPA (%) = ((peak area/total) x 100%).
ND: Not detected. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the same row with different lowercase letters are significantly different ($P < 0.05$).

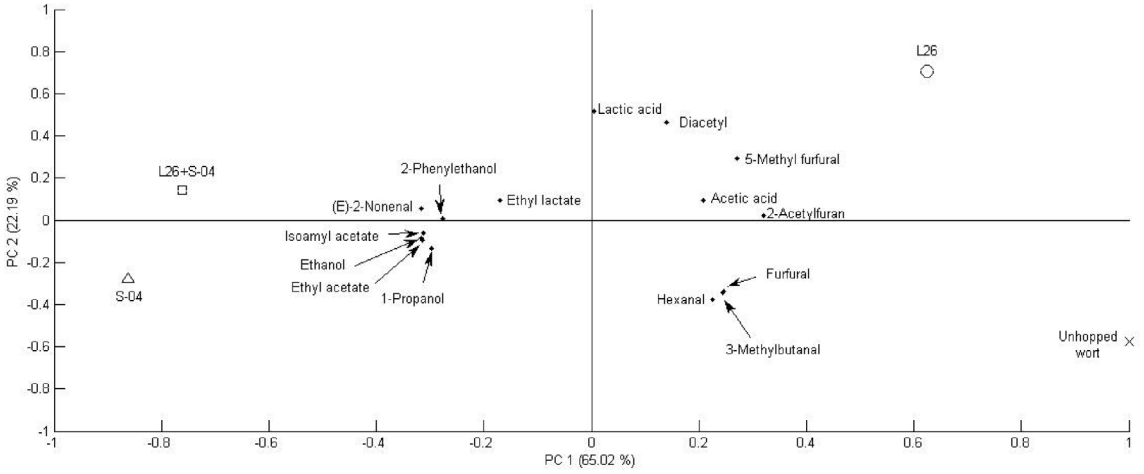

Fig. 5. Principal component analysis bi-plot for selected volatile compounds, and organic acids of unhopped wort, and unhopped wort fermented with yeast and probiotics. (×) Unhopped wort; (○) *L. paracasei* L26 mono-culture; (□) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 and; (Δ) *S. cerevisiae* S-04 mono-culture.

From the bi-plot, unhopped wort is positioned on the positive semi-axis of PC1, characterized by endogenous aldehydes (hexanal), Maillard reaction products (furfural), and Strecker aldehydes (3-methylbutanal). After fermentation, PCA clearly discriminated each fermentation trial, each occupying separate quadrants, indicating dissimilar profiles. The single culture of *L. paracasei* L26 occupied the positive semi-axes of PC1 and PC2, distinguished by metabolites from the probiotic (lactic acid, acetic acid, diacetyl), as well as endogenous wort volatiles (5-methyl furfural, 2-acetylfuran). Single and mixed cultures of *S. cerevisiae* S-04 occupied the negative semi-axis of PC1, but differed from each other by being situated at opposite positions of PC2. The mixed culture contained greater amounts of 2-phenylethanol, ethyl lactate, and *(E)*-2-nonenal, while the yeast mono-culture possessed greater amounts of isoamyl acetate, ethyl acetate, and 1-propanol. Therefore, the mixed culture of *L. paracasei* L26 and *S. cerevisiae* S-04 possessed different volatile profiles compared to fermentation using single cultures of either probiotic or yeast.

3.3. Impact of iso-α-acids on the survival of L. paracasei L26 during storage

To determine if *S. cerevisiae* S-04 was able to confer protective effects towards *L. paracasei* L26 against hop iso-α-acids at different temperatures, isomerized hop extract was added on day 10 of fermentation to each of the unhopped beer/wort samples, followed by storage at 5 °C and 25 °C.

3.3.1. L. paracasei L26 and S. cerevisiae S-04 populations during storage

Survival kinetics of *L. paracasei* L26 and *S. cerevisiae* S-04 during the co-fermentation storage period, and after addition of isomerized hop extract, are summarized in Fig. 6a and b respectively. With the addition of isomerized hop extract, *L. paracasei* L26 cell numbers in both single and mixed cultures declined during storage, with the rate of death being dependent on the storage temperature and the presence of *S. cerevisiae* S-04. At 25 °C, *L. paracasei* L26 in the mono-culture was not detectable within 1 day of storage, while *L. paracasei* L26 cell counts in the co-culture dropped below 7 Log CFU/mL by day 3 of storage. On the other hand, the viability-enhancing effect of the yeast was more pronounced under refrigeration. At 5 °C, *L. paracasei* L26 cell counts in the mono-culture and co-culture fell below 7 Log CFU/mL by day 6 and 22 of storage respectively. Such findings suggest that the viability enhancing effects can be attributed to both refrigerated storage temperatures as well as the presence of live yeast.

To the best of our knowledge, this is the first study that has provided compelling evidence that live yeasts, coupled with refrigerated storage, are able to confer probiotic viability enhancing effects against hop iso-α-acids. The temperature dependence of hop iso-α-acids has been investigated by Simpson (1993), who explained that at lower temperatures, the ionophoric effects of *trans*-isohumulone is diminished due to its restricted mobility across the membrane. However, the underlying mechanism as to how live yeasts are able to enhance probiotic survival against hop iso-α-acids remains to be elucidated.

It is possible that innate yeast resistance mechanisms to prevent intracellular accumulation of iso-α-acids, such as alteration of cell wall structures, and vacuolar sequestration to trap iso-α-acids (Hazelwood et al., 2010), may have indirectly lowered the concentration of hop acids in the mixed culture, therefore conferring additional viability enhancing effects to *L. paracasei* L26. Indeed, brewer's yeasts possess high affinity for hop iso-α-acids, and significant amounts are removed together with spent yeasts after filtration (Bryant and Cohen, 2015). A reduction in hop acid levels (above the minimum inhibitory concentration; MIC) has also been shown to reduce the death rate of *Lactobacillus brevis* (Simpson, 1993). To justify that yeasts are able to lower the levels of hop iso-α-acids in the external medium, differential vacuolar and cytosolic pool fractionation could be carried out in the future, to determine the affinity for hop iso-α-acids by *S. cerevisiae* S-04.

Alternatively, a greater degree of acidification observed in the *L. paracasei* L26 mono-culture (pH 3.46; 6.63 g/L lactic acid) compared to the co-culture (pH 3.62, 5.25 g/L lactic acid; Table 1) may have led to accelerated probiotic death rates in the mono-culture. The MIC of iso-α-acids is lower under acidic conditions due to a greater proportion of hop acids existing in the undissociated form, which is able to diffuse across the probiotic cell membrane (Blanco et al., 2007).

It is also hypothesized that additional stresses induced by *S. cerevisiae* S-04 (e.g. ethanol, sugar depletion etc. Table 1) may have elicited *L. paracasei* L26 to undergo physiological changes (e.g. cellular membrane composition), which would confer protection against new and unrelated stresses, such as the addition of hop extract prior to storage. Research on other LAB have demonstrated that exposure to stressful conditions aided with increased tolerance to other unrelated stresses (Beales, 2004; Chu-ky et al., 2013; Zhang et al., 2013). Nevertheless, further work is required to verify these postulations.

3.3.2. Changes in non-volatile and volatile components during storage

As the final product must be unfiltered and unpasteurized, non-volatile and volatile analyses were conducted to assess if the presence of live probiotic and yeast would generate any off-flavors during storage. Supplementary Table B summarizes the changes in non-volatile compositions in the co-fermented beer during storage at 25 and 5 °C. Changes in volatile components are reflected in Table 3 and Supplementary Table C.

Generally, there were no substantial changes in non-volatile components. On the other hand, there were substantial losses in esters

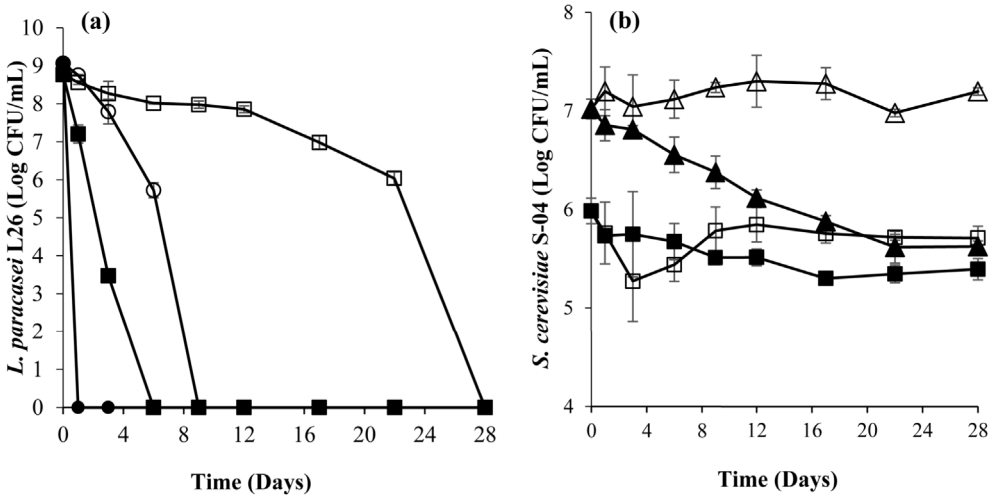

Fig. 6. Survival kinetics of (a) *L. paracasei* L26 and (b) *S. cerevisiae* S-04 during storage of beer at 5 °C and 25 °C. (○) *L. paracasei* L26 at 5 °C; (●) *L. paracasei* L26 at 25 °C; (□) co-culture of *L. paracasei* L26 with *S. cerevisiae* S-04 at 5 °C; (■) co-culture of *L. paracasei* L26 with *S. cerevisiae* S-04 at 25 °C; (Δ) *S. cerevisiae* S-04 at 5 °C and; (▲) *S. cerevisiae* S-04 at 25 °C. Values are the mean of triplicate experiments (n = 3), with error bars representing the standard deviation of the mean values. * 27 IBUs of isomerized hop extract was added prior to storage.

Table 3
Changes in selected volatiles (mean GC-FID peak area x 10^6) and their relative peak area (RPA, %) in hopped beers fermented with co-cultures of *L. paracasei* L26 and *S. cerevisiae* S-04 stored at 25 °C and 5 °C.

Compound Identified	Identification Methods	LRI	L26 + S-04 fermented hopped beer (day 0 storage)		End shelf life			
					L26 + S-04 (25 °C; day 3 storage)		L26 + S-04 (5 °C; day 22 storage)	
			Peak Area	RPA (%)	Peak Area	RPA (%)	Peak Area	RPA (%)
Acids								
Acetic acid	MS, LRI	1447	4.72 ± 0.42^{c}	0.15	1.30 ± 0.11^{a}	0.04	3.43 ± 0.01^{b}	0.13
Subtotal			72.57	2.38	42.33	1.44	52.09	2.03
Alcohols								
Ethanol	MS		2192.34 ± 204.11^{a}	71.93	2287.52 ± 337.88^{a}	77.91	2045.90 ± 205.19^{a}	79.85
1-Propanol	MS, LRI	1052	2.44 ± 0.18^{a}	0.08	2.98 ± 0.09^{b}	0.10	3.00 ± 0.10^{b}	0.12
Isobutanol	MS, LRI	1100	6.65 ± 0.43^{ab}	0.22	7.52 ± 0.56^{b}	0.26	6.18 ± 0.30^{a}	0.24
Isoamyl alcohol	MS, LRI	1227	16.02 ± 0.32^{a}	0.53	44.04 ± 4.25^{b}	1.50	9.73 ± 1.57^{a}	0.38
2-Phenylethyl alcohol	MS, LRI	2133	75.61 ± 9.84^{a}	2.48	95.37 ± 8.41^{a}	3.25	78.62 ± 11.60^{a}	3.07
Subtotal			2294.07	75.26	2437.92	83.03	2144.46	83.70
Esters								
Ethyl acetate	MS		15.78 ± 0.21^{a}	0.52	15.32 ± 1.25^{a}	0.52	16.99 ± 1.25^{a}	0.66
Isoamyl acetate	MS, LRI	1111	20.45 ± 2.53^{a}	0.67	16.65 ± 0.82^{a}	0.57	18.12 ± 1.35^{a}	0.71
2-Phenylethyl acetate	MS, LRI	1813	12.20 ± 1.16^{b}	0.40	9.39 ± 0.50^{a}	0.32	11.43 ± 1.07^{ab}	0.45
Ethyl butanoate	MS, LRI	1029	1.59 ± 0.08^{a}	0.05	1.73 ± 0.10^{ab}	0.06	1.80 ± 0.05^{b}	0.07
Ethyl hexanoate	MS, LRI	1215	38.75 ± 4.21^{b}	1.27	22.39 ± 3.18^{a}	0.76	42.65 ± 2.13^{b}	1.66
Ethyl lactate	MS, LRI	1346	2.78 ± 0.03^{a}	0.09	2.93 ± 0.22^{a}	0.10	4.52 ± 0.28^{b}	0.18
Ethyl octanoate	MS, LRI	1421	202.67 ± 15.44^{c}	6.65	151.80 ± 1.00^{b}	5.17	125.40 ± 3.96^{a}	4.89
Ethyl decanoate	MS, LRI	1628	230.38 ± 44.85^{b}	7.56	129.23 ± 17.18^{a}	4.40	62.32 ± 0.40^{a}	2.43
Ethyl dodecanoate	MS, LRI	1832	23.89 ± 5.04^{b}	0.78	17.31 ± 0.85^{ab}	0.59	10.60 ± 0.67^{a}	0.41
Diethyl succinate	MS, LRI	1667	4.07 ± 0.83^{b}	0.13	1.47 ± 0.03^{a}	0.05	1.09 ± 0.15^{a}	0.04
Subtotal			662.70	21.74	439.17	14.96	353.07	13.78
Maillard reaction products								
2-Pentylfuran	MS, LRI	1207	0.19 ± 0.01^{a}	0.01	0.34 ± 0.00^{b}	0.01	0.20 ± 0.01^{a}	0.01
Furfural	MS, LRI	1470	0.33 ± 0.07^{a}	0.01	0.57 ± 0.05^{b}	0.02	0.23 ± 0.02^{a}	0.01
2-Acetylfuran	MS, LRI	1510	0.20 ± 0.02^{a}	0.01	0.25 ± 0.01^{a}	0.01	0.20 ± 0.03^{a}	0.01
5-Methylfurfural	MS, LRI	1582	ND	0.00	ND	0.00	0.12 ± 0.01	0.00
Subtotal			0.72	0.02	1.16	0.04	0.75	0.03
Total			3048.09	100.00	2936.24	100.00	2562.04	100.00

LRI, linear retention index, which was determined on a DB-FFAP column relative to C_5—C_{40} hydrocarbons.
RPA (%) = ((peak area/total) x 100%).
ND: Not detected. Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the same row with different lowercase letters are significantly different ($P < 0.05$).

during storage, with a significantly lower overall relative peak area for mixed culture beers stored at 25 °C and 5 °C ($P < 0.05$). This could be attributed to the escape of volatiles into the ambient environment, or hydrolysis of esters by yeasts (Saerens et al., 2010). The loss of fresh, floral, fruity, and estery aromas may reduce the masking effect of undesirable flavors (e.g. stale, bitter flavors) that develop during beer storage (Baert et al., 2012; Pires et al., 2014; Saison et al., 2009). However, levels of the beer staling aroma compound, (*E*)-2-nonenal, were also lost during storage, which may offset the loss of any masking effect from esters.

In contrast to the yeast mono-culture (data not shown), ethyl lactate was only detected in the co-fermented beer. The increase in relative peak areas of the co-fermented beers stored at 25 °C (5.40%) and 5 °C (62.59%) was significant ($P < 0.05$). Continuous production of the ester during storage may indicate yeast adaptation to high amounts of lactic acid produced by the probiotic (Table 1), as it has been hypothesized that the formation of medium chain fatty acid esters prevents accumulation and intracellular acidification in yeasts (Saerens et al., 2010). Ethyl lactate imparts fruity and buttery notes, and is considered an indicator of beer aging (Pires et al., 2014). On the other hand, Harayama et al. (1995) observed that its increase during storage did not significantly impact the flavor profile of aged beers, possibly due to low levels present.

4. Conclusion

This was the first study demonstrating the feasibility of utilizing probiotic lactobacilli as starter cultures in beer brewing. Unfiltered and unpasteurized hopped beers rich in live probiotics is possible, only if correct strategies are employed. Mono-cultures of probiotic LAB exhibited poor viability in hopped wort. However, when co-cultured with *S. cerevisiae* S-04 in unhopped wort, the probiotic *L. paracasei* L26 exhibited excellent growth and stability, produced significant amounts of lactic acid ($P < 0.05$), and satisfied the minimal requirement of 9 Log CFU per serving of product or 100 g or mL, in order to confer health benefits. The fermentation performance of *S. cerevisiae* S-04 was not detrimentally affected, with excellent levels of sugar attenuation, and significant production of alcohols and esters ($P < 0.05$). A combination of live *S. cerevisiae* S-04 and low temperature significantly enhanced the survival of the probiotic in beer containing isomerized hop extract, as compared to only either variable. Nevertheless, further work is required to understand the mechanisms involved, sensorial impact and consumer acceptance, and overall therapeutic benefits of the probiotic beer to substantiate any health claims.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.04.001.

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