

An alcoholic beverageTechnical Field

The present invention relates to an alcoholic beverage. In particular, the alcoholic
5 beverage may comprise probiotic bacteria.

Background

There has been an increase in interest in the consumption of probiotics in view of the health benefits one can derive from them. At present, many food and beverages comprise probiotics. However, most of the food and beverages are dairy-based
10 products and therefore, these may not be ideal for a lactose intolerant person. There has therefore been ongoing research on novel delivery methods of probiotics in new food environments outside the dairy sector.

Malt-based beverages have been shown to exhibit protective effects on the viability of several *Lactobacillus* probiotic strains. Alcoholic beverages, particularly specialty beers
15 are seeing rising popularity amongst consumers. Beer is a malt-based beverage. However, maintaining probiotic viability in beer is still a major technological challenge as beer has many intrinsic antimicrobial hurdles that prevent the growth and impair survival of probiotic bacteria. The main antimicrobial compound in beer is iso- α -acid, which acts as an ionophore and limits the growth of Gram-positive bacteria through
20 various mechanisms such as intracellular acidification and inhibition of energy generation and redox homeostasis. Probiotic bacteria, which are not isolated from beers, do not have any mechanism to overcome the inhibitory effects of the acid to grow and survive in beer.

Summary of the invention

25 The present invention seeks to address these problems, and/or to provide an improved beverage comprising probiotic bacteria.

In general terms, the invention relates to an alcoholic beverage comprising probiotic bacteria. The alcoholic beverage of the present invention may provide health benefits as compared to other alcoholic beverages in view of the beverage comprising probiotic
30 bacteria. In particular, probiotic bacteria have been shown to provide health benefits such as enhanced gut health and immune system function.

According to a first aspect, the present invention provides an alcoholic beverage comprising probiotic bacteria.

The alcoholic beverage may have a suitable alcohol content. According to a particular aspect, the alcohol content of the alcoholic beverage may be $\geq 0.5\%$ by volume. In particular, the alcohol content may be 0.5-10%, 1.0-9.0%, 1.5-8.0%, 2.0-7.5%, 2.5-7.0%, 3.0-6.5%, 3.5-6.0%, 4.0-5.5%, 4.5-5.0%. Even more in particular, the alcohol content may be 2.0-5.0%.

The alcoholic beverage may be any suitable alcoholic beverage. In particular, the alcoholic beverage is beer. According to a particular aspect, the alcoholic beverage may further comprise a hop. For the purposes of the present invention, reference to hop refers to a hop and/or its derivatives. The hop may be any suitable hop. For example, the hop may be an isomerised hop extract. The hop may have a suitable bitterness. In particular, the hop may have a bitterness of ≤ 30 IBU. Even more in particular, the bitterness may be 0-30 IBU, 3-27 IBU, 5-25 IBU, 7.5-20 IBU, 9-18 IBU, 10-16 IBU, 12-15 IBU, 13-14 IBU.

The probiotic bacteria comprised in the alcoholic beverage may be any suitable probiotic bacteria. For example, the probiotic bacteria may comprise lactobacilli, bifidobacteria, or a combination thereof. In particular, the lactobacilli may comprise, but is not limited to, *Lactobacillus* (*L.*) *paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum* or a combination thereof.

A suitable amount of probiotic bacteria may be comprised in the alcoholic beverage. For example, the probiotic bacteria may have a cell count of ≥ 5.0 log CFU/mL. In particular, the probiotic bacteria comprised in the alcoholic beverage may have a cell count of 5.0-12.0 log CFU/mL, 5.5-11.5 log CFU/mL, 6.0-11.0 log CFU/mL, 6.5-10.5 log CFU/mL, 7.0-10.0 log CFU/mL, 7.5-9.5 log CFU/mL, 8.0-9.0 log CFU/mL. Even more in particular, the probiotic bacteria comprised in the alcoholic beverage may have a cell count of about 7.0 log CFU/mL.

The alcoholic beverage may have a suitable pH. For example, the pH of the alcoholic beverage may be 2-6. In particular, the pH may be 2.5-5.5, 3-5, 3.5-4.5, 3.75-4.0. Even more in particular, the pH may be about 3-5.

The alcoholic beverage may have a suitable Brix. For example, the Brix of the alcoholic beverage may be 4-20 °Bx. In particular, the Brix may be 5-18°Bx, 6-15 °Bx, 7-12 °Bx, 8-10 °Bx, 9-9.5 °Bx. Even more in particular, the Brix may be about 5-15 °Bx.

According to a second aspect of the present invention, there is provided a method of forming an alcoholic beverage described above, the method comprising:

- providing a wort or must;
- adding a probiotic bacteria to the wort or must;
- adding a yeast to the wort or must; and
- fermenting the wort or must at a pre-determined period of time and at a pre-determined temperature to form the alcoholic beverage.

The wort or must may be any suitable wort or must for the purposes of the present invention.

The probiotic bacteria added to the wort or must may be any suitable probiotic bacteria. For example, the probiotic bacteria may be as described above.

The yeast added to the wort or must may be any suitable yeast for the purposes of the present invention. For example, the yeast may be *Saccharomyces* yeast, non-*Saccharomyces* yeast, or a combination thereof. According to a particular aspect, the yeast may be, but not limited to, *Saccharomyces* (*S.*) *cerevisiae*, *S. pasteurianus*, *Torulaspora* (*T.*) *delbrueckii*, *Lachancea* (*L.*) *thermotolerans*, *Pichia* (*P.*) *kluveri*, *Metschnikowia* (*M.*) *pulcherrima*, or a combination thereof.

The method may further comprise adding a hop to the wort or must. The hop added to the wort or must may be any suitable hop as described above.

The adding a probiotic bacteria and the adding a yeast may be carried out simultaneously or sequentially.

According to a particular aspect, the adding a probiotic bacteria and the adding a yeast may be carried out simultaneously. The fermenting may then be carried out under suitable conditions. For example, the fermenting may comprise fermenting the wort or must at a first temperature for a first period of time and subsequently fermenting the

wort or must at a second temperature for a second period of time. The method may further comprise adding a hop at the end of the second period of time.

According to a particular aspect, the adding a probiotic bacteria and the adding a yeast may be carried out sequentially. In particular, the adding a yeast may be carried out
5 after a third period of time following the adding a probiotic bacteria. The fermenting may then be carried out under suitable conditions. For example, the fermenting comprises fermenting the wort or must at a third temperature for a fourth period of time following the adding a yeast and subsequently fermenting the wort or must at a fourth
10 temperature for a fifth period of time. The method may further comprise adding a hop at the end of the fifth period of time.

The first period of time, second period of time, third period of time, fourth period of time and fifth period of time may be any suitable amount of time. The periods of time may be selected depending on the probiotic bacteria and the yeast added to the wort or must.

The first temperature, second temperature, third temperature and fourth temperature
15 may be any suitable temperature for the purposes of the present invention. For example, the first temperature and the third temperature may be the same. For example, the second temperature and the fourth temperature may be the same.

In particular, the first temperature and the third temperature may be the same or different and may be 20-42°C. Even more in particular, the first temperature and the
20 third temperature may be the same or different and may be about 30°C.

In particular, the second temperature and the fourth temperature may be the same or different and may be 18-30°C. Even more in particular, the second temperature and the fourth temperature may be the same or different and may be about 20°C.

According to a particular aspect, the formed alcoholic beverage may be stored at a
25 suitable temperature following the fermentation. For example, the alcoholic beverage formed from the method of the present invention may be stored at a temperature of $\leq 20^{\circ}\text{C}$. In particular, the alcoholic beverage may be stored at a temperature of about $\leq 10^{\circ}\text{C}$. Even more in particular, the alcoholic beverage may be stored at a temperature of about 1-5°C.

Brief Description of the Drawings

In order that the invention may be fully understood and readily put into practical effect there shall now be described by way of non-limitative example only exemplary embodiments, the description being with reference to the accompanying illustrative drawings. In the drawings:

Figure 1 shows a graph of the survival of three probiotic bacterial strains during 10 days of fermentation in hopped wort: (●) *L. paracasei* L26; (X) *L. paracasei* Lpc-37 and; (◆) *L. rhamnosus* HN001. #Error bars indicate standard deviations (n=3). *Fermentation temperature was carried out at 37 °C throughout;

Figure 2 shows growth and survival of *L. paracasei* L26 during the co-fermentation and storage periods at 5°C and 25°C: (○) *L. paracasei* L26 at 5°C; (●) *L. paracasei* L26 at 25°C; (□) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 at 5°C and; (■) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 at 25°C. *Fermentation temperature was carried out at 30°C from day 0 to day 2, and 20°C from day 2 to day 10. *27 IBUs of isomerized hop extract was added on day 10;

Figure 3 shows growth and survival of *L. paracasei* L26 during the sequential fermentation and storage periods at 5°C and 25°C: (○) *L. paracasei* L26 at 5°C; (●) *L. paracasei* L26 at 25°C; (□) sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 at 5°C and; (■) sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04 at 25°C. *Fermentation temperature was carried out at 30°C from day 0 to day 2, and 20°C from day 2 to day 10. ^*S. cerevisiae* S-04 was added on day 2. #27 IBUs of isomerized hop extract was added on day 10;

Figure 4 shows growth and survival of co-inoculated *L. paracasei* L26 during storage of beer at 5°C: (□) 0 IBUs at 5°C; (x) 7.5 IBUs at 5°C; (○) 15 IBUs at 5°C; (Δ) 22.5 IBUs at 5°C. *Fermentation temperature was carried out at 30°C from day 0 to day 2, and 20°C from day 2 to day 10. #Isomerized hop extract was added on day 10;

Figure 5 shows growth and survival of co-inoculated *L. paracasei* L26 during storage of beer at 25°C: (■) 0 IBUs at 25°C; (+) 7.5 IBUs at 25°C; (●) 15 IBUs at 25°C; (▲) 22.5 IBUs at 25°C. * Fermentation temperature was carried out at 30°C from day 0 to day 2, and 20°C from day 2 to day 10. #Isomerized hop extract was added on day 10;

Figure 6(a) shows changes in pH during fermentation and storage of beer at 5°C: (□) 0 IBUs at 5°C; (x) 7.5 IBUs at 5°C; (○) 15 IBUs at 5°C; (Δ) 22.5 IBUs at 5°C, and Figure 6(b) shows the changes in pH during fermentation and storage of beer at 25°C: (■) 0 IBUs at 25°C; (+) 7.5 IBUs at 25°C; (●) 15 IBUs at 25°C; (▲) 22.5 IBUs at 25°C;

- 5 Figure 7(a) shows changes in °Brix during fermentation and storage of beer at 5°C: (□) 0 IBUs at 5°C; (x) 7.5 IBUs at 5°C; (○) 15 IBUs at 5°C; (Δ) 22.5 IBUs at 5°C, and Figure 7(b) shows changes in Brix during fermentation and storage of beer at 25°C: (■) 0 IBUs at 25°C; (+) 7.5 IBUs at 25°C; (●) 15 IBUs at 25°C; (▲) 22.5 IBUs at 25°C;

- Figure 8 shows survival of sequentially inoculated *L. paracasei* L26 (after yeast
10 fermentation prior) during storage of beer at 5°C and 25°C: (○) 5°C; (Δ) 25°C. #Hopped wort (equivalent to 27 IBUs) was fermented with *S. cerevisiae* S-04 for 10 days prior to the addition of *L. paracasei* L26;

Figure 9 shows changes in pH during fermentation and storage of the sequentially inoculated probiotic beer at 5°C and 25°C: (○) 5°C; (Δ) 25°C;

- 15 Figure 10 shows changes in °Brix during fermentation and storage of the sequentially inoculated probiotic beer at 5°C and 25°C: (○) 5°C; (Δ) 25°C;

- Figure 11 shows growth and survival of co-inoculated probiotics during fermentation and storage of beer at 5°C: (♦) *L. paracasei* L26; (■) *L. rhamnosus* HN001; (▲) *L. acidophilus* NCFM. * Fermentation temperature was carried out at 30°C from day 0 to day 2, and 20°C from day 2 to day 10. #7.5 IBUs of isomerized hop extract was added
20 on day 10;

- Figure 12 shows growth and survival of co-inoculated probiotics during fermentation and storage of beer at 25°C: (◇) *L. paracasei* L26; (□) *L. rhamnosus* HN001; (Δ) *L. acidophilus* NCFM. *Fermentation temperature was carried out at 30°C from day 0 to day 2, and 20°C from day 2 to day 10. #27 IBUs of isomerized hop extract was added
25 on day 10;

- Figure 13(a) shows changes in pH during fermentation and storage of beer at 5°C: (♦) *L. paracasei* L26; (■) *L. rhamnosus* HN001; (▲) *L. acidophilus* NCFM, and Figure 13(b) shows changes in pH during fermentation and storage of beer at 25°C: (◇) *L. paracasei*
30 L26; (□) *L. rhamnosus* HN001; (Δ) *L. acidophilus* NCFM;

Figure 14(a) shows changes in °Brix during fermentation and storage of beer at 5°C: (◆) *L. paracasei* L26; (■) *L. rhamnosus* HN001; (▲) *L. acidophilus* NCFM, and Figure 14(b) shows changes in °Brix during fermentation and storage of beer at 25°C: (◇) *L. paracasei* L26; (□) *L. rhamnosus* HN001; (Δ) *L. acidophilus* NCFM;

- 5 Figure 15 shows growth of *L. paracasei* L26 during co-fermentation with non-*Saccharomyces* yeasts and subsequent storage at 5°C: (■) *L. paracasei* L26 with *T. delbrueckii* Prelude; (●) *L. paracasei* L26 with *M. pulcherrima* Flavia. Fermentation was carried out at 30°C from day 0 to 2, and at 20°C from day 2 to 12. #7.5 IBUs of isomerized hop extract was added on day 12;
- 10 Figure 16 shows growth of *L. paracasei* L26 during co-fermentation with non-*Saccharomyces* yeasts and subsequent storage at 25°C: (□) *L. paracasei* L26 with *T. delbrueckii* Prelude; (○) *L. paracasei* L26 with *M. pulcherrima* Flavia. Fermentation was carried out at 30°C from day 0 to 2, and at 20°C from day 2 to 12. #7.5 IBUs of isomerized hop extract was added on day 12;
- 15 Figure 17(a) shows pH changes of unhopped wort during fermentation and storage at 5°C and Figure 17(b) shows pH changes of unhopped wort during fermentation and storage at 25°C: (■) *L. paracasei* L26 with *T. delbrueckii* Prelude; (●) *L. paracasei* L26 with *M. pulcherrima* Flavia. (□); *T. delbrueckii* Prelude only; (○) *M. pulcherrima* Flavia only;
- 20 Figure 18(a) shows °Brix changes of unhopped wort during fermentation and storage at 5°C and Figure 18(b) shows °Brix changes of unhopped wort during fermentation and storage at 25°C: (■) *L. paracasei* L26 with *T. delbrueckii* Prelude; (●) *L. paracasei* L26 with *M. pulcherrima* Flavia. (□); *T. delbrueckii* Prelude only; (○) *M. pulcherrima* Flavia only;
- 25 Figure 19 shows growth and survival of *L. paracasei* L26 during co-inoculation and sequential inoculation with *S. cerevisiae* W-34/70 during fermentation and storage of beer at 5°C: (●) co-inoculation of *L. paracasei* L26 and *S. cerevisiae* W-34/70 at 5°C; (■) sequential inoculation of *L. paracasei* L26 and *S. cerevisiae* W-34/70 at 5°C. *Fermentation temperature was carried out at 30°C from day 0 to day 1, and 20°C from
- 30 day 1 to day 22. #7.5 IBUs of isomerized hop extract was added on day 22. ^*S. cerevisiae* W-34/70 was added on day 1 of fermentation for sequential inoculation;

Figure 20 shows growth and survival of *L. paracasei* L26 during co-inoculation and sequential inoculation with *S. cerevisiae* W-34/70 during fermentation and storage of beer at 25°C: (○) co-inoculation of *L. paracasei* L26 and *S. cerevisiae* W-34/70 at 25°C; (□) sequential inoculation of *L. paracasei* L26 and *S. cerevisiae* W-34/70 at 25°C.

- 5 *Fermentation temperature was carried out at 30°C from day 0 to day 1, and 20°C from day 1 to day 22. #7.5 IBUs of isomerized hop extract was added on day 22. ^*S. cerevisiae* W-34/70 was added on day 1 of fermentation for sequential inoculation;

- Figure 21(a) shows changes in pH during fermentation and storage of beer at 5°C: (●) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 5°C; (■) sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 5°C; (▲) *S. cerevisiae* W-34/70 mono-culture at 5°C, and Figure 21(b) shows changes in pH during fermentation and storage of beer at 25°C: (○) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 25°C; (□) sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 25°C; (Δ) *S. cerevisiae* W-34/70 mono-culture at 25°C; and

- 15 Figure 22(a) shows changes in °Brix during fermentation and storage of beer at 5°C: (●) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 5°C; (■) sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 5°C; (▲) *S. cerevisiae* W-34/70 mono-culture at 5°C, and Figure 22(b) shows changes in °Brix during fermentation and storage of beer at 25°C: (○) co-inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 25°C; (□) sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* W-34/70 at 25°C; (Δ) *S. cerevisiae* W-34/70 mono-culture at 25°C.

Detailed Description

As explained above, there is a need for a non-dairy beverage as a means for delivering probiotic bacteria.

- 25 The present invention relates to an alcoholic beverage comprising probiotic bacteria. Generally, it is difficult to expect probiotic bacteria to be incorporated into an alcoholic beverage such as beer due to the presence of antimicrobial compounds which would prevent the growth and impair survival of the probiotic bacteria. Accordingly, the present invention provides a surprising and unexpected alcoholic beverage in which
- 30 probiotic bacteria is able to grow and survive despite the presence of the antimicrobial compounds in the alcoholic beverage.

Probiotic bacteria are known to provide health benefits. For example, it has been shown that certain strains of probiotic bacteria can bring about improvement in gut health and digestion. Accordingly, the alcoholic beverage according to the present invention may bring about health benefits such as improvement in gut health and
5 boosting immunity.

According to a first aspect, the present invention provides an alcoholic beverage comprising probiotic bacteria.

For the purposes of the present invention, an alcoholic beverage is defined as a beverage which comprises an alcohol, such as ethanol or ethyl alcohol. In particular,
10 the alcoholic beverage may have an alcohol content $\geq 0.5\%$ by volume.

The alcoholic beverage may have suitable alcohol content. According to a particular aspect, the alcohol content of the alcoholic beverage may be $\geq 0.5\%$ by volume. For example, the alcohol content may be 0.5-10%, 1.0-9.0%, 1.5-8.0%, 2.0-7.5%, 2.5-7.0%, 3.0-6.5%, 3.5-6.0%, 4.0-5.5%, 4.5-5.0%. In particular, the alcohol content may
15 be 2.0-5.0%. Even more in particular, the alcohol content may be about 3-4%.

The alcoholic beverage may be any suitable alcoholic beverage. Examples of an alcoholic beverage may include, but is not limited to, beer, wine, cider, spirits and the like. According to a particular aspect, the alcoholic beverage may be beer.

The probiotic bacteria comprised in the alcoholic beverage may be any suitable
20 probiotic bacteria. The probiotic bacteria may be any suitable live microorganism which, when provided in an adequate amount, confers a health benefit on the host. For example, the probiotic bacteria may comprise lactobacilli, bifidobacteria, or a combination thereof. In particular, the lactobacilli may comprise, but is not limited to, *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum* or a
25 combination thereof. Even more in particular, the lactobacilli may comprise *Lactobacillus paracasei* LAFTI L26, *Lactobacillus paracasei* Lpc-37, *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* NCFM, or a combination thereof.

A suitable amount of probiotic bacteria may be comprised in the alcoholic beverage. For example, the probiotic bacteria may have a cell count of ≥ 5.0 log CFU/mL. In
30 particular, the probiotic bacteria comprised in the alcoholic beverage may have a cell

count of 5.0-12.0 log CFU/mL, 5.5-11.5 log CFU/mL, 6.0-11.0 log CFU/mL, 6.5-10.5 log CFU/mL, 7.0-10.0 log CFU/mL, 7.5-9.5 log CFU/mL, 8.0-9.0 log CFU/mL. Even more in particular, the probiotic bacteria comprised in the alcoholic beverage may have a cell count of about 7.0 log CFU/mL.

- 5 According to a particular aspect, the alcoholic beverage may further comprise a hop. For the purposes of the present invention, hop comprises a hop and/or its derivatives. The hop may be any suitable hop comprising isomerised alpha acids. The hop may be in any suitable form such as, but not limited to, hop cones, hop pellets, hop resins, hop powder, isomerised hop extracts and the like.
- 10 The hop may be any suitable hop. For example, the hop may be an isomerised hop extract. The hop may have a suitable bitterness. In particular, the hop may have a bitterness of ≤ 30 IBU. Even more in particular, the bitterness may be 0-30 IBU, 3-27 IBU, 5-25 IBU, 7.5-20 IBU, 9-18 IBU, 10-16 IBU, 12-15 IBU, 13-14 IBU. IBU refers to International bittering units and is a measure of the concentration of hop compounds in
- 15 the alcoholic beverage. In particular, the IBU measures the parts per million (ppm) of isohumulone in the alcoholic beverage.

The alcoholic beverage may have a suitable pH. For example, the pH of the alcoholic beverage may be 2-6. In particular, the pH may be 2.5-5.5, 3-5, 3.5-4.5, 3.7-4.0. Even more in particular, the pH may be about 3-5.

- 20 The alcoholic beverage may have a suitable Brix or its specific gravity equivalent. Brix is a measure of the amount of sugars in the alcoholic beverage. For example, 1 °Bx refers to 1 g of sucrose in 100 g of the alcoholic beverage. Accordingly, the higher the Brix, the higher the alcohol content may be in the alcoholic beverage. The Brix of the alcoholic beverage may be 4-20 °Bx. For the purposes of the present invention,
- 25 reference to Brix of the alcoholic beverage refers to the measure of the Brix of the wort or must comprised in the alcoholic beverage. In particular, the Brix of the alcoholic beverage may be 5-18°Bx, 6-15 °Bx, 7-12 °Bx, 8-10 °Bx, 9-9.5 °Bx. Even more in particular, the Brix may be about 5-15 °Bx.

- The alcoholic beverage may be stored at a suitable temperature so as to maintain the
- 30 probiotic bacteria at a suitable level. For example, the alcoholic beverage may be stored at a temperature of about $\leq 20^{\circ}\text{C}$. Preferably, the alcoholic beverage may be

stored at a temperature of $\leq 10^{\circ}\text{C}$. In particular, the alcoholic beverage may be stored at a temperature of about $1-10^{\circ}\text{C}$, $5-8^{\circ}\text{C}$, $6-7^{\circ}\text{C}$. Even more in particular, the alcoholic beverage may be stored at a temperature of about $1-5^{\circ}\text{C}$.

According to a second aspect of the present invention, there is provided a method of
5 forming an alcoholic beverage of the first aspect, the method comprising:

- providing a wort or must;
- adding a probiotic bacteria to the wort or must;
- adding a yeast to the wort or must; and
- fermenting the wort or must at a pre-determined period of time and at a
10 pre-determined temperature to form the alcoholic beverage.

The wort or must may be any suitable wort or must for the purposes of the present invention. For the purposes of the present invention, a wort may include, but is not limited to, malted barley and/or its adjuncts such as wheat, corn, rye, rice and water,
15 which has undergone heating, boiling and cooling. The wort may also include added sugars. For the purposes of the present invention, a must may include, but is not limited to, fruit juices such as grape juice, which has undergone heating, boiling and cooling. The must may also include added sugars.

The probiotic bacteria added to the wort or must may be any suitable probiotic bacteria.
20 For example, the probiotic bacteria may be as described above. The adding a probiotic bacteria may comprise adding a suitable amount of probiotic bacteria to the wort or must. For example, the amount of probiotic bacteria added may be $1-9 \log \text{CFU/ml}$. In particular, the amount of probiotic bacteria added may be about $2-8 \log \text{CFU/ml}$, $3-7 \log \text{CFU/ml}$, $4-6 \log \text{CFU/ml}$, $4.5-5 \log \text{CFU/ml}$. Even more in particular, the amount of
25 probiotic bacteria added may be $5-7 \log \text{CFU/ml}$.

The yeast added to the wort or must may be any suitable yeast for the purposes of the present invention. The yeast may be *Saccharomyces* yeast, non-*Saccharomyces* yeast, or a combination thereof. According to a particular aspect, the yeast may be *Saccharomyces* yeast. Examples of *Saccharomyces* yeast include, but are not limited to,
30 to, *S. cerevisiae*, *S. paradoxus*, *S. pasteurianus*, *S. bayanus* and *S. cerevisiae* var. *boulardii*. According to another particular aspect, the yeast may be non-saccharomyces yeast. Examples of non-*Saccharomyces* yeast include, but are not limited to, *T.*

delbrueckii, *L. thermotolerans*, *P. kluyveri* and *M. pulcherrima*. In particular, the adding a yeast to the wort or must comprises adding *S. cerevisiae*.

The adding a yeast may comprise adding a suitable amount of yeast to the wort or must. For example, the amount of yeast added may be 1-9 log CFU/mL. In particular,
5 the amount may be about 5-7 log CFU/mL.

The method may further comprise adding a hop to the wort or must. The hop added to the wort or must may be any suitable hop as described above. The adding a hop may comprise adding a suitable amount of hop to the wort or must. For example, the amount of hop added may be ≤ 30 IBU. In particular, the amount of hop added may
10 be 0-30 IBU, 3-27 IBU, 5-25 IBU, 7.5-20 IBU, 9-18 IBU, 10-16 IBU, 12-15 IBU, 13-14 IBU. Even more in particular, the amount of hop added may be about 7.5 IBU.

The adding a probiotic bacteria and the adding a yeast may be carried out simultaneously or sequentially.

According to a particular aspect, the adding a probiotic bacteria and the adding a yeast
15 may be carried out simultaneously. The fermenting may then be carried out under suitable conditions. For example, the fermenting may comprise fermenting the wort or must at a suitable temperature for a suitable period of time. The temperature may be changed at any point during the fermenting. In particular, the fermenting may comprise fermenting the wort or must at a first temperature for a first period of time and
20 subsequently fermenting the wort or must at a second temperature for a second period of time. The method may further comprise adding a hop at the end of the second period of time.

According to a particular aspect, the adding a probiotic bacteria and the adding a yeast
25 may be carried out sequentially. In particular, the adding a yeast may be carried out after a third period of time following the adding a probiotic bacteria. The fermenting may then be carried out under suitable conditions. For example, the fermenting may comprise fermenting the wort or must at a suitable temperature for a suitable period of time. The temperature may be changed at any point during the fermenting. In particular, the fermenting may comprise fermenting the wort or must at a third
30 temperature for a fourth period of time and subsequently fermenting the wort or must at

a fourth temperature for a fifth period of time. The method may further comprise adding a hop at the end of the fifth period of time.

The first period of time, second period of time, third period of time, fourth period of time and fifth period of time may be any suitable amount of time. The periods of time may be
5 selected depending on the probiotic bacteria and the yeast added to the wort or must. For example, the first period of time, the third period of time and the fourth period of time may be 1-5 days, preferably 1-2 days. The second period of time and the fifth period of time may be 8-30 days.

The first temperature, second temperature, third temperature and fourth temperature
10 may be any suitable temperature for the purposes of the present invention. For example, the first temperature and the third temperature may be the same. For example, the second temperature and the fourth temperature may be the same.

In particular, the first temperature and the third temperature may be the same or different and may be any suitable temperature to provide favourable conditions for
15 probiotic bacteria growth and allow maximal probiotic bacteria cell populations to be reached. For example, the first temperature and the third temperature may be 25-35°C. Even more in particular, the first temperature and the third temperature may be the same or different and may be about 30°C.

In particular, the second temperature and the fourth temperature may be the same or
20 different and may be any suitable temperature to provide yeast growth. For example, the second temperature and the fourth temperature may be 13-25°C. Even more in particular, the second temperature and the fourth temperature may be the same or different and may be about 20°C.

In one embodiment, the method comprises adding a probiotic bacteria and adding
25 yeast into a wort simultaneously. The wort may be unhopped. The probiotic bacteria and the yeast are co-inoculated in the wort at a first temperature for a first period of time. For example, the first temperature may be about 25-35°C. In particular, the first temperature may be about 30°C. The first period of time may be about 1-5 days. In particular, the first period of time may be 2 days. The fermentation is then allowed to
30 proceed at a second temperature for a second period of time. The second temperature may be about 13-25°C. In particular, the second temperature may be about 20°C. The

second period of time may be about 8-30 days. In particular, the second period of time may be 8, 12 or 21 days. An isomerised hop extract of a suitable concentration is then added to the fermented wort at the end of the second period of time. For example, the hop extract may be a 7.5 IBU hop extract. The formed alcoholic beverage is then
5 stored at a suitable temperature such as about 1-5°C.

In another embodiment, the method comprises first adding a probiotic bacteria to a wort and adding a yeast only after a third period of time. The wort may be unhopped. The third period of time may be 1-5 days. In particular, the yeast may be added after 1 or 2 days following the adding of the probiotic bacteria to the wort. The probiotic
10 bacteria may be inoculated in the wort at a third temperature for a fourth period of time. The third temperature may be about 25-35°C. In particular, the third temperature may be about 30°C. The fourth period of time may be about 1-5 days. In particular, the fourth period of time may be 2 days. The fermentation is then allowed to proceed at a fourth temperature for a fifth period of time. The fourth temperature may be about 13-
15 25°C. In particular, the fourth temperature may be about 20°C. The fifth period of time may be about 8-30 days. In particular, the fifth period of time may be 8 or 21 days. An isomerised hop extract of a suitable concentration is then added to the fermented wort at the end of the fifth period of time. For example, the hop extract may be a 7.5 IBU hop extract. The formed alcoholic beverage is then stored at a suitable temperature
20 such as about 1-5°C.

According to a particular aspect, the formed alcoholic beverage may be stored at a suitable temperature following the fermentation. For example, the alcoholic beverage formed from the method of the present invention may be stored at a temperature of $\leq 20^{\circ}\text{C}$. In particular, the alcoholic beverage may be stored at a temperature of about
25 $\leq 10^{\circ}\text{C}$. Even more in particular, the alcoholic beverage may be stored at a temperature of about 1-5°C.

According to another aspect, there is provided the alcoholic beverage as described above for use in medicine. In particular, the alcoholic beverage of the present invention may be for use in improving the gut health and/or digestion of a consumer of the
30 alcoholic beverage.

Whilst the foregoing description has described exemplary embodiments, it will be understood by those skilled in the technology concerned that many variations may be made without departing from the present invention.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting.

EXAMPLE

All materials used in the examples provided are as follows:

Material	Source
<i>Lactobacillus paracasei</i> LAFTI L26	DSM Food Specialties Ltd., Heerlen, The Netherlands
<i>Saccharomyces cerevisiae</i> Safale S-04 dry ale yeast	Lesaffre, Lille, France
<i>Lactobacillus paracasei</i> Lpc-37	Danisco A/S, Copenhagen, Denmark
<i>Lactobacillus rhamnosus</i> HN001	Danisco A/S, Copenhagen, Denmark
<i>Metschnikowia pulcherrima</i> Flavia	Lallemand Pty, Ontario, Canada
<i>Torulaspora delbrueckii</i> Prelude	Chr. Hansen A/S, Horsholm, Denmark
<i>Saccharomyces cerevisiae</i> Saflager W34/70 lager yeast	Lesaffre, Lille, France

10

A sweet wort was prepared by reconstituting 12.2 % (w/v) dry light malt extract (Thomas Coopers Breweries, South Australia, Australia) into de-ionized water, followed by boiling for 20 minutes to achieve hot break. After which, 2.0 % (w/v) dextrose (Thomas Coopers Breweries, South Australia, Australia) and 0.2 % (w/v) Cascade hop pellets (Yakima Chief- Hopunion, Yakima, USA) were mixed into the wort and boiled for another 60 minutes. Cold Ice Mountain distilled water (Fraser and Neave, Limited, Malaysia) was then added to top up to the original batch weight, and the wort cooled using an ice bath for around 60 minutes 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 minutes to ensure wort sterility, which was verified using potato dextrose agar (PDA; Oxoid Ltd., Hampshire, UK). Hops were omitted in the preparation of unhopped wort, for use as pre-culture and for the simultaneous and sequential fermentation stages.

20

Example 1 – Antimicrobial action of iso- α -acids on probiotic bacteria

To elucidate the anti-bacterial action of hop acids on probiotic bacteria, and the difficulty in incorporating probiotic bacteria in hopped beers, hopped wort was fermented with three different probiotic strains, *L. paracasei* L26, *L. paracasei* Lpc-37 and *L. rhamnosus* HN001, and their growth and stability recorded. For the screening stage, triplicate fermentations of *L. paracasei* L26, *L. paracasei* Lpc-37 and *L. rhamnosus* HN001 were carried out in 500 mL capped glass bottles, containing 200 mL of hopped wort. Each glass bottle was inoculated with 1 % (v/v) of the respective probiotic pre-culture, and incubated statically at 37 °C for 10 days.

Figure 1 shows the survival kinetics of *L. paracasei* L26, *L. paracasei* Lpc-37 and *L. rhamnosus* HN001 in hopped wort throughout the 10-day fermentation period. All three probiotic strains cell counts gradually declined and by day 7, were not detectable. Cell death can be attributed to the presence of hop iso- α -acids, as the probiotic bacteria could reach high viable cell counts of at least around 8.50 Log CFU/mL in unhopped wort during growth in pre-cultures grown in unhopped wort (data not shown). The decline in cell numbers thus indicated that all three probiotic strains were unable to survive in hopped wort, and therefore, were not hop resistant. Probiotic cell death in hopped wort is not surprising as these bacteria do not have complex mechanisms that enable them to be well adapted to grow and survive in beers.

L. paracasei L26 showed the highest survivability, followed by *L. paracasei* Lpc-37 and *L. rhamnosus* HN001. *L. paracasei* L26 still had detectable cell numbers of 5.13 Log CFU/mL on day 4 while *L. paracasei* Lpc-37 and *L. rhamnosus* HN001 had cell numbers of 6.12 and 4.13 Log CFU/mL on day 2 respectively. *L. paracasei* L26 thus showed a better degree of resistance to hop iso- α -acids before dying out completely. Since *L. paracasei* L26 displayed the greatest survivability in hopped wort, it was used in further fermentations with yeast as shown in the examples below.

Example 2 – Probiotic beer fermentation using different inoculation strategies**2.1 Co-inoculation of *S. cerevisiae* S-04 and *L. paracasei* L26**

During co-fermentation, triplicate fermentations were carried out in 500-mL capped glass bottles, containing 400 mL of unhopped wort. *L. paracasei* L26 and *S. cerevisiae*

S-04 yeast were co-cultured at approximately 6.72 Log CFU/mL and 5.00 Log CFU/mL, respectively, to favour the growth of *L. paracasei* L26. The controls included the same inoculum of *L. paracasei* L26 and *S. cerevisiae* S-04 pre-culture, respectively, into unhopped wort. Fermentations were first carried out statically at 30°C from day 0 to day 2 to provide favourable conditions for probiotic growth and allow maximal probiotic cell populations to be reached. Fermentation then proceeded at 20°C from day 2 to day 10 to cater to yeast growth.

After 10 days of fermentation, shelf life tests for the same samples were carried out (shelf life stage). On day 10, 27 International bittering units (IBUs) of isomerised hop extract (Brouwland, Beverlo, Belgium) was added to the unhopped beers in the same hop concentration as Example 1. To assess the effect of refrigerated and ambient storage temperatures on probiotic survivability, the samples were stored at 5°C and 25°C. The end of shelf life for each fermentation set was determined when plate counts for *L. paracasei* L26 fell below 7.0 Log CFU/mL.

The results obtained are shown in Figure 2. Based on Figure 2, during the fermentation period without hop extract, *L. paracasei* L26 monoculture (in the absence of yeast) was able to achieve high stationary phase cell counts of 9.06 Log CFU/mL on day 10 in the absence of hops. The results thus reinforce the antibacterial action of iso- α -acids on *L. paracasei* L26. In the presence of yeast, *L. paracasei* L26 was still able to achieve high stationary phase cell numbers and sustain them throughout the 10-day fermentation period, with 8.77 log CFU/mL recorded on day 10. The results indicate successful co-existential fermentation between *L. paracasei* L26 and *S. cerevisiae* S-04 during the growth and stationary phase period.

As can be seen from Figure 2, after addition of hop extract on day 10, at 25°C, *L. paracasei* L26 monoculture cell counts were not detectable within a day of storage, while *L. paracasei* L26 co-culture cell counts dropped below 7.0 log CFU/mL by day 13. The viability enhancing effect of *S. cerevisiae* S-04 was thus not strongly evident at storage temperature of 25°C. However, the viability enhancing effect was more pronounced at 5°C. At 5°C, *L. paracasei* L26 monoculture cell counts fell below 7.0 log CFU/mL by day 16, while *L. paracasei* L26 co-culture managed to maintain high viable cell numbers throughout storage and only fell below the benchmark of 7.0 log CFU/mL (required to provide health benefits) by day 32. Therefore, the viability enhancing

effects are attributed to both refrigerated storage temperatures as well as the presence of yeast.

Non-volatile characteristics of the hopped beer at different storage temperatures are shown in Table 1. Based on Table 1, lactic acid produced by *L. paracasei* L26 led to low pH values (3.52-3.64) recorded in the beers inoculated with both *L. paracasei* L26 with *S. cerevisiae* S-04. Due to the contribution by lactic acid (5.08-5.15 g/L), the resulting beer will thus taste sour.

Parameter	L26 + S-04 fermented hopped beer (Day 10)	Hopped beer (end shelf life)	
		L26 + S-04 (25°C; Day 13)	L26 + S-04 (5°C; Day 32)
pH	3.64 ± 0.04a	3.54 ± 0.01b	3.52 ± 0.01b
°Brix (%)	7.48 ± 0.04a	7.32 ± 0.00b	7.43 ± 0.01ab
Sugars (g/L)			
Total mono- and disaccharides	1.41 ± 0.13a ^{&}	1.52 ± 0.15a ^{&}	0.00 ± 0.00b*
Organic Acid (g/L)			
Acetic acid	1.41 ± 0.05a	1.38 ± 0.01a	1.44 ± 0.03a
Lactic acid	5.08 ± 0.09a	5.15 ± 0.14a	5.10 ± 0.03a

[#]All values consisted of mean ± standard deviation obtained from single analysis and triplicate fermentations, using Tukey's test for significance.

^{a,b,c,d} Statistical analysis ANOVA at 95 % confidence level with same letters indicating no significant difference

[&] Indicates below LOQ but above LOD

* 0.00 ± 0.00 = not detected

Table 1: Non-volatile changes in hopped beer during 25°C and 5°C storage by co-inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04

2.2 Sequential inoculation of yeast and probiotic bacteria

During the sequential fermentation stage, the effect of adding *S. cerevisiae* S-04 yeast on the second day of fermentation, on the viability of *L. paracasei* L26 was investigated. This was to determine if probiotic viability could be improved with the late addition of yeast.

Similar procedures to co-inoculation (Example 2.1) were carried out for sequential inoculation fermentation. Triplicate fermentations were carried out in 500-mL capped glass bottles containing 400 mL of unhopped wort. *L. paracasei* L26 (1 % (v/v)

inoculum) was added to each glass bottle. On the second day of fermentation, 1 % (v/v) of *S. cerevisiae* S-04 was added into the same sample. Monocultures of *L. paracasei* L26 and *S. cerevisiae* S-04 in the same inoculum served as controls. Fermentations were first carried out statically at 30°C from day 0 to day 2 to provide
5 favourable conditions for probiotic bacteria growth and allow maximal probiotic bacteria cell populations to be reached. Fermentation then proceeded at 20°C from day 2 to day 10 to cater to yeast growth.

After 10 days of fermentation, shelf life tests for the same samples were carried out. The procedure was similar to the one for the co-inoculation experiment outlined in
10 Example 2.1. The same dosage of isomerized hop extract, storage temperatures and conditions for end of shelf life were utilized.

Growth kinetics of *L. paracasei* L26 in unhopped wort during sequential fermentation period of 10 days, followed by its survival kinetics during the sequential fermentation storage period, after addition of isomerized hop extract, are shown in Figure 3.

15 Based on Figure 3, similar growth kinetics to co-fermentation were observed. In the absence of yeast, *L. paracasei* L26 monoculture was able to achieve high stationary phase cell counts of 8.95 log CFU/mL on day 10 in the absence of hops, reinforcing that unhopped wort without hops added is a suitable growth medium for *L. paracasei* L26. In the presence of yeast, *L. paracasei* L26 was similarly able to achieve high
20 viable cell counts of 8.99 log CFU/mL on day 10, indicating the that the yeast does not impede the growth of *L. paracasei* L26.

From Figure 3, it can be seen that both *L. paracasei* L26 monoculture and sequential culture cell counts fell below 7.0 log CFU/mL within 1 day of storage at 25°C. Similar to the co-inoculation fermentation, the viability enhancing effect of *S. cerevisiae* S-04 was
25 more promising at 5°C. At 5°C, *L. paracasei* L26 monoculture cell counts fell below 7.0 log CFU/mL by day 16, while *L. paracasei* L26 co-culture cell counts only fell below by day 19. It can be seen that the viability enhancing effects may be due to both refrigerated storage temperatures as well as the presence of yeast.

The death of *L. paracasei* L26 during storage may be due to the addition of hop extract
30 as *L. paracasei* L26 monoculture without addition of isomerized extract on day 10 and

stored at 5°C still had high viable cell counts of 8.88 log CFU/mL on day 22 (data not shown).

Non-volatile characteristics of the hopped beer at different storage temperatures are shown in Table 2. From Table 2 it can be seen that significant amounts of lactic acid produced (8.25-8.39 g/L) by *L. paracasei* L26 led to low pH values (3.30-3.31) recorded in the beers inoculated with both *L. paracasei* L26 with *S. cerevisiae* S-04. The accumulation of lactic acid will result in sour tastes in the final probiotic beer product.

Parameter	L26 + S-04 fermented hopped beer (Day 10)	Hopped beer (end shelf life)	
		L26 + S-04 (25°C; Day 11)	L26 + S-04 (5°C; Day 19)
pH	3.30 ± 0.00a	3.30 ± 0.00a	3.31 ± 0.01a
°Brix (%)	9.13 ± 0.12a	8.72 ± 0.07b	8.75 ± 0.02ab
Sugars (g/L)			
Total mono- and disaccharides	9.29 ± 1.24a	6.01 ± 0.26b	4.34 ± 0.12b
Organic Acid (g/L)			
Acetic acid	1.48 ± 0.02a	1.45 ± 0.12a	1.48 ± 0.02a
Lactic acid	8.39 ± 0.17a	8.33 ± 0.28a	8.25 ± 0.16a

[#]All values consisted of mean ± standard deviation obtained from single analysis and triplicate fermentations, using Tukey's test for significance.

^{a,b,c,d} Statistical analysis ANOVA at 95 % confidence level with same letters indicating no significant difference

Table 2: Non-volatile changes in hopped beer during 25°C and 5°C storage by sequential inoculation of *L. paracasei* L26 with *S. cerevisiae* S-04

It can be seen that the synergistic effects of refrigeration storage and addition of yeasts on probiotic bacteria viability were muted in comparison to using co-inoculation strategy of Example 2.1.

Example 3 – Enhancing probiotic bacteria viability and extending shelf life

A longer shelf life would reduce losses attributed to long cold chain distribution networks, as well as mitigate losses resulting from unsold beers which are subject to high alcohol tariffs. Accordingly, the effect of hop levels on the probiotic bacteria was investigated.

As seen in Example 1, hop iso- α -acids detrimentally affected probiotic bacteria viability. Since *L. paracasei* L26 exhibited greater survivability during co-inoculation with *S. cerevisiae* S-04 compared to sequential inoculation (Example 2), co-fermentation with the probiotic bacteria and yeast was used in this example.

- 5 Methods carried out were similar to Example 2.1, but with different levels of hop extract. Triplicate fermentations of *S. cerevisiae* S-04 yeast and *L. paracasei* L26 probiotic were carried out in 1-L capped glass bottles, containing 600 mL of unhopped wort. Each glass bottle was inoculated with 0.5% (v/v) of yeast pre-culture and 1% (v/v) of probiotic bacteria pre-culture, and incubated statically at 30°C for 2 days, followed by
10 at 20°C for 8 days.

- On day 10, 7.5, 15 and 22.5 International bittering units (IBUs) of isomerised hop extract (Brouwland, Beverlo, Belgium) was added to the unhopped beers respectively. A triplicate set of beers was left unhopped. The samples were stored at 5°C and 25°C for shelf life stage. The end of shelf life for each fermentation set was determined when
15 plate counts of *L. paracasei* L26 fell below 7.0 Log CFU/mL.

- Figure 4 shows the growth and survival kinetics of *L. paracasei* L26 when co-inoculated with *S. cerevisiae* S-04 in hopped wort of different IBU levels, at 5°C. During storage with added hop extract, the survival of the probiotic bacteria improved with decreasing hop concentration. This is expected as a reduction of hop iso- α -acids, an inhibitor of
20 bacterial growth, created a less hostile environment for probiotic bacteria survival. At 22.5 and 15 IBUs, the beers had a shelf life of 6 and 10 days, respectively. At 7.5 IBUs, the probiotic bacteria maintained cell counts above 7.0 log CFU/mL for one month. This is an improvement from the 22 days of shelf life from Figure 2. In unhopped beer (0 IBUs), the probiotics maintained cell counts above 8 log CFU/mL for
25 at least four months.

- Figure 5 shows the growth and survival kinetics of *L. paracasei* L26 when co-inoculated with *S. cerevisiae* S-04 in hopped wort of different IBU levels, at 25°C. As expected, probiotic bacteria samples stored at 25°C had a poorer survival rate compared to samples stored under refrigeration. All hopped samples displayed probiotic bacteria
30 cell counts below 7.0 log CFU/mL after three days of storage, while the unhopped sample maintained probiotic cell counts above 7.0 log CFU/mL for only around 38 days

of storage, as compared to at least four months under refrigeration (Figure 4). The results indicate the importance of cold chain distribution and storage of the probiotic beers to maintain probiotic bacteria viability.

Figure 6(a) and Figure 6(b) show the changes in pH during fermentation and storage at 5°C and 25°C, respectively. pH readings were similar across different samples. The low pH (~3.5) achieved across the samples also suggest that lactic acid was produced by the probiotic bacteria, resulting in a sour taste.

Figure 7(a) and Figure 7(b) show the changes in °Brix readings during fermentation and storage at 5°C and 25°C, respectively. °Brix readings were similar across different samples, indicating that a difference in hop concentration did not affect the fermentative ability of the yeast as well as nutrient utilization significantly.

Therefore, it can be seen that the viability of the probiotic bacteria increased with decreasing hop levels, with the anti-microbial action of the hops being more pronounced at 25°C compared to 5°C.

Example 4 – Modifying fermentation procedure by addition of probiotic bacteria after yeast fermentation

Triplicate fermentations were carried out in 250-mL capped glass bottles containing 220 mL of hopped wort. The hop concentration was equivalent to addition of 27 IBUs of isomerized hop extract. *S. cerevisiae* S-04 (0.5% (v/v) inoculum) was first added to each glass bottle. After 10 days of fermentation at 20°C, 1% (v/v) of *L. paracasei* L26 was added into the same sample. Shelf life tests were carried out thereafter. The procedure was similar to that of the co-inoculation experiment outlined in Example 2.1. The same storage temperatures and conditions for end of shelf life were utilized.

Figure 8 shows the survival of added *L. paracasei* L26 during storage at 5°C and 25°C. After probiotic bacteria inoculation on day 10, followed immediately by storage at 5°C and 25°C, *L. paracasei* L26 exhibited better survivability at 5°C, compared to 25°C, with cell counts of 6.67 and 5.27 log CFU/mL on day 42 at 5°C and 25°C, respectively. This further emphasises the need for cold storage and cold chain distribution for the probiotic beers to maintain probiotic bacteria viability, regardless of inoculation strategy employed.

Figures 9 and 10 show the pH and °Brix readings during the fermentation and storage periods. °Brix values were similar to that of the co-fermentation samples in Example 2.1 (Table 1).

From Figure 8, it was observed that although *L. paracasei* L26 did not exhibit growth, its population remained static for at least one month during storage at refrigeration. This is likely due to the sequestration of hop acids by the yeast, creating a more favourable environment for probiotic bacteria survival. Further, as shown in Figure 9, a higher pH was obtained compared to the co-fermentation samples of Example 2.1 (Table 1), at 4.6-4.7 vs. 3.5. Higher pH values reduce the potency of hop acids and have an overall positive effect on probiotic bacteria survival.

Thus, based on the results obtained in Figure 8, due to the relatively unchanging probiotic cell count throughout storage at 5°C, a version of a probiotic beer could be created whereby a higher dosage of probiotics is added after the initial yeast fermentation period (such as above the minimum 7 log CFU/mL, for e.g. 8 log CFU/mL). This would also create a more favourable and acceptable flavour profile as the resultant beers would be less acidic, in contrast to allowing the probiotics to partake in the fermentation process with yeast.

Example 5 – Probiotic beer fermentation using different probiotic bacteria and yeast pairings

Examples 2 to 4 have focussed on using *L. paracasei* L26 and *S. cerevisiae* S-04 as the probiotic and yeast pairing. Other probiotic bacteria and yeast pairings were also investigated to determine if other probiotic bacteria and yeast strains could be used to form alternative versions of the probiotic beer. These probiotic bacteria and yeast pairings include *S. cerevisiae* S-04 and other *Lactobacillus* probiotic bacteria (Example 5.1), *L. paracasei* L26 and other non-*Saccharomyces* yeast (Example 5.2), and *L. paracasei* L26 and *S. cerevisiae* W34/70 lager yeast (Example 5.3).

5.1 Fermentation using *S. cerevisiae* S-04 and other *Lactobacillus* probiotic bacteria

L. rhamnosus HN001 was used to elucidate the anti-bacterial action of hops (Example 1). Alone, it is susceptible to the anti-bacterial action of hops. However, it is unknown if its survivability can be improved in the presence of yeast, similar to *L. paracasei* L26

(Example 2). Therefore, unhopped wort was fermented with *S. cerevisiae* S-04 and *L. rhamnosus* HN001, and 7.5 IBUs of hop extract added at the end of fermentation, followed by storage at 5°C and 25°C. Another probiotic bacteria strain, *L. acidophilus* NCFM was also utilized to investigate if its survivability was improved in the presence
5 of yeast.

Triplicate fermentations were carried out in 1-L capped glass bottles containing 550 mL of unhopped wort. *L. paracasei* L26, *L. rhamnosus* HN001, and *L. acidophilus* NCFM were used for co-fermentation with *S. cerevisiae* S-04. 1% (v/v) of probiotic bacteria inoculum and 0.5% (v/v) of yeast inoculum was added to each glass bottle.
10 Fermentation was carried out statically at 30°C for 2 days, followed by 20°C for 8 days.

After 10 days of fermentation, 7.5 IBUs of isomerized hop extract was added to each sample. Shelf life tests were carried out in a similar procedure to that of the co-inoculation experiment outlined in Example 2.1. The same storage temperatures and conditions for end of shelf life were utilized.

15 Figure 11 shows the growth and survival of *L. paracasei* L26, *L. rhamnosus* HN001, and *L. acidophilus* NCFM when co-inoculated with *S. cerevisiae* S-04 in unhopped wort, followed by addition of 7.5 IBUs hop extract and storage at 5°C. *L. rhamnosus* HN001 and *L. acidophilus* NCFM were both able to coexist with *S. cerevisiae* S-04 during co-fermentation, as shown by the high cell counts achieved (8.06 log CFU/mL and 8.49
20 log CFU/mL, respectively) after 10 days of fermentation.

From Figure 11, *L. rhamnosus* HN001 displayed the poorest survivability during cold storage, with cell counts below 7.0 log CFU/mL after four days of refrigeration, while cell counts of *L. paracasei* L26 fell below 7.0 log CFU/mL within one month of storage, reinforcing the results obtained previously in Example 3 (Figure 4). *L. acidophilus*
25 NCFM, displayed promising cell counts above 7.0 Log CFU/mL for two months of cold storage, falling below 7 Log CFU/mL by day 57 of storage. This is a significant improvement from one month of shelf life under refrigeration for *L. paracasei* L26 (at the same level of 7.5 IBUs for both *L. acidophilus* NCFM and *L. paracasei* L26). Therefore, *L. acidophilus* NCFM may also be used in formation of probiotic beer. *L.*
30 *acidophilus* NCFM has been shown to survive gastrointestinal transit in both healthy and diseased human populations and has a long history of safe human consumption.

L. acidophilus NCFM has been shown to induce several health benefits in human trials, including but not limited to, reducing the incidence of pediatric diarrhea, stabilization of intestinal microbiota during antibiotic therapy, and improve small bowel bacterial overgrowth symptoms. Therefore, incorporating *L. acidophilus* NCFM in probiotic beers
5 would therefore provide consumers with the alternative option of healthier beers.

Figure 12 shows the growth and survival of *L. paracasei* L26, *L. rhamnosus* HN001, and *L. acidophilus* NCFM when co-inoculated with *S. cerevisiae* S-04 in unhopped wort, followed by addition of 7.5 IBUs hop extract and storage at 25°C. It can be seen that *L. rhamnosus* HN001 displayed the poorest survivability, followed by *L. paracasei* L26
10 and *L. acidophilus* NCFM, mirroring the trends observed at 5°C (Figure 11). The anti-bacterial action of hops is also more pronounced at higher temperatures, with cell counts for *L. rhamnosus* HN001, *L. paracasei* L26, and *L. acidophilus* NCFM falling below the benchmark of 7.0 log CFU/mL on days 1, 4 and 9 of storage respectively.

Figure 13(a) and Figure 13(b) shows the pH readings obtained during fermentation with
15 *S. cerevisiae* S-04 and *L. rhamnosus* HN001, *L. acidophilus* NCFM, and *L. paracasei* L26 and storage at 5°C and 25°C, respectively, while Figure 14(a) and Figure 14(b) shows the corresponding °Brix readings at storage temperatures of 5°C and 25°C. While pH readings were similar between the samples, the °Brix values were higher for *L. rhamnosus* HN001, indicating that less sugars were utilized by the probiotic bacteria
20 and yeast during the fermentation period, as compared to *L. acidophilus* NCFM and *L. paracasei* L26.

5.2 Fermentation using *L. paracasei* L26 and other non-*Saccharomyces* yeast

The results of previous examples have demonstrated the ability of *Saccharomyces cerevisiae* S-04 to enhance the survival of *Lactobacillus paracasei* L26 in hopped beer.
25 With the increasing demand for novelty beers, this example investigates the potential of producing probiotic beers by co-culturing *L. paracasei* L26 with non-*Saccharomyces* yeasts, as well as the viability-enhancing effect of these yeasts on the probiotic bacteria.

Co-fermentation was performed as described in Example 2.1 with *Torulaspora delbrueckii* Prelude or *Metschnikowia pulcherrima* Flavia in place of *S. cerevisiae* S-04.
30 Fermentation was carried out with 300 mL of unhopped wort in 500-mL glass bottles.

For co-cultures, the unhopped wort was inoculated with 6.69 log CFU/mL of *L. paracasei* L26 and 5.63 log CFU/mL of *T. delbrueckii* Prelude, or 5.08 log CFU/mL of *M. pulcherrima* Flavia. Yeast monocultures were also prepared by inoculating the unhopped wort with the same yeast inoculums. The inoculated worts were incubated at
5 30°C under static conditions from day 0 to 2 to favour the growth of *L. paracasei* L26. Subsequently, the fermentation temperature was lowered to 20°C from day 2 to 12 to accommodate the growth of the yeasts.

After 12 days of fermentation, the beers were dosed with isomerised hop extract (Brouwland, Berverlo, Belgium) to a bitterness rating of 7.5 IBU. Shelf life testing of the
10 hopped beer was carried out as described in Example 3 at 5°C and 25°C.

Figure 15 shows the growth and survival kinetics of *L. paracasei* L26 throughout the 12-day fermentation period when it was co-cultured with either *T. delbrueckii* Prelude or *M. pulcherrima* Flavia in unhopped wort and during storage at 5°C. The growth of *L. paracasei* L26 in the presence of the two non-Saccharomyces yeasts was similar to
15 when the probiotic bacteria was cultured with *S. cerevisiae* S-04 (Example 2.1; Figure 2). After the fermentation, viable probiotic cell counts of 8.83 – 8.97 log CFU/mL in the beer was achieved. This indicated that *T. delbrueckii* Prelude and *M. pulcherrima* Flavia did not exert inhibitory effects on *L. paracasei* L26 during fermentation.

At 5°C storage, the *L. paracasei* L26 count of co-cultured beers remained above 7.0
20 log CFU/mL for at least 8 days during storage at 5°C, after which it declined to 6.69 – 6.76 log CFU/mL on day 27. As reported in Example 1, the probiotic bacteria cell count of the *L. paracasei* L26 monoculture in hopped beer was less than the minimum therapeutic level (7.0 log CFU/mL) by the 5th day of storage. Hence, the presence of non-Saccharomyces yeasts could also improve the survival of the probiotic bacteria in
25 beer. Nevertheless, the probiotic viability-enhancing effect of *T. delbrueckii* Prelude and *M. pulcherrima* Flavia were not as pronounced as *S. cerevisiae* S-04, as *L. paracasei* L26 cell counts remained above 7.0 log CFU/mL for 1 month (Example 3, Figure 4; Example 5.1, Figure 11) when co-cultured with the latter yeast.

The changes in viable cell counts of *L. paracasei* L26 in hopped beer when co-cultured
30 with either *T. delbrueckii* Prelude or *M. pulcherrima* Flavia and subsequent storage at 25°C are shown in Figure 16. When beers were stored at 25°C, the viable probiotic

counts declined from 8.83 – 8.97 log CFU/mL to less than 3 log CFU/mL within four days. This was in agreement with the results collected thus far, whereby the survival-enhancing effect of yeasts on *L. paracasei* L26 in hopped beer was minimal at room temperature (Figures 2, 3, 5, 8, and 12).

- 5 Figure 17(a) and Figure 17(b) shows the pH readings obtained during the 12-day fermentation period and storage period at 5°C and 25°C, respectively, while Figure 18(a) and Figure 18(b) shows the corresponding °Brix readings at storage temperatures of 5°C and 25°C. While pH readings were similar between the samples, the °Brix values were higher for *L. rhamnosus* HN001, indicating that less sugars were
10 utilized by the probiotic bacteria and yeast during the fermentation period, as compared to *L. acidophilus* NCFM and *L. paracasei* L26.

5.3 Fermentation using *L. paracasei* L26 and other *S. cerevisiae* W34/70 lager yeast

Previously, *S. cerevisiae* S-04 ale yeast was used together with *L. paracasei* L26 during co-fermentation and sequential fermentation in unhopped wort (Example 2).

- 15 During refrigerated storage with added isomerized hop extract, *L. paracasei* L26 in the co-culture maintained high viable cell counts above 7.0 log CFU/mL for 22 days, compared to the sequential culture (9 days).

As mainstream commercial beers are lager beers, this example investigates the use of a lager yeast strain, *Saccharomyces cerevisiae* W-34/70, during co- and sequential
20 inoculation with *L. paracasei* L26.

- Methods were similar to Example 2.1. Triplicate fermentations were carried out in 500-mL capped glass bottles, containing 350 mL of unhopped wort. For the co-fermentation samples, unhopped wort was inoculated with *L. paracasei* L26 (1 % (v/v)) and *S. cerevisiae* W-34/70 yeast (1 % (v/v)). For the sequential fermentation samples, the
25 same inoculum of *L. paracasei* L26 and *S. cerevisiae* W-34/70 yeast was added into unhopped wort, but *S. cerevisiae* W-34/70 was only added on day 1 of fermentation. Mono-cultures of *S. cerevisiae* W-34/70 served as controls. Fermentations proceeded statically at 30°C from day 0 to day 1 to provide favourable conditions for probiotic bacteria growth. The temperature was then lowered to 20°C from day 1 to day 22 to
30 allow for yeast growth.

After 22 days of fermentation, a dosage of 7.5 IBUs of isomerized hop extract (Brouwland, Beverlo, Belgium) was added into the unhopped beers. The hopped beers were then stored at 5°C and 25°C and the survivability of *L. paracasei* L26 assessed.

Figures 19 and 20 show the growth and survival of *L. paracasei* L26 during fermentation and storage with *S. cerevisiae* W-34/70 at 5°C and 25°C, respectively. It can be seen that *L. paracasei* L26 was able to co-exist with the lager yeast *S. cerevisiae* W-34/70 during the 22 day fermentation period, maintaining high cell counts above 8.60 log CFU/mL for both co-inoculation and sequential inoculation samples.

After addition of 7.5 IBUs of isomerized hop extract and subsequent storage, *L. paracasei* L26 in the co- and sequential inoculated samples still maintained cell counts above 8.60 log CFU/mL within 4 days of storage when stored at 5°C. In contrast, *L. paracasei* L26 cell counts fell below 7.0 log CFU/mL for all samples stored at 25°C within the same duration, reinforcing the importance of cold chain distribution and storage of the probiotic beers to maintain probiotic viability.

Data from Figure 20 also suggests that co-inoculation method may be a better technique in prolonging probiotic viability rather than sequential inoculation, with no detectable probiotic bacteria cell counts for the sequentially inoculated sample, but 6.63 log CFU/mL was still detected for *L. paracasei* L26 in the co-inoculated sample at 25°C on day 4 of storage. The findings echo that obtained in Example 2, where *L. paracasei* L26 co-cultured with *S. cerevisiae* S-04 maintained high viable cell counts above 7.0 log CFU/mL for 22 days, compared to the sequential culture (9 days). Such effects are less pronounced for samples stored at 5°C, with no significant difference in probiotic cell counts between the co- and sequentially inoculated samples on day 31 of storage, although probiotic cell counts via sequential inoculation was slightly lower than via co-inoculation method (Figure 19).

Under refrigeration, probiotic bacteria cell counts fell below the 7.0 log CFU/mL benchmark by day 31 of storage. This indicates that *S. cerevisiae* W34/70 provided a similar extent of probiotic bacteria viability enhancing effects to *S. cerevisiae* S-04, which possessed a shelf life of 1 month (Example 3, Figure 4; Example 5.1, Figure 11). Thus, this shows that lager type of probiotic beer may be produced.

Figure 21(a) and Figure 21(b) shows the pH readings obtained during the 22-day fermentation period and storage period at 5°C and 25°C, respectively, while Figure 22(a) and Figure 22(b) shows the corresponding °Brix readings at storage temperatures of 5°C and 25°C. pH readings for samples containing *L. paracasei* L26 were 3.55-3.56, similar to readings obtained with probiotic beer fermentation using *L. paracasei* L26 and *S. cerevisiae* S-04 (Example 2, Tables 1 and 2).

From Figures 22(a) and 22(b) show that the °Brix readings declined slowly in all samples, reaching a plateau at the end of the 22-day fermentation period. The absence of further decreases in °Brix values in all samples indicates complete sugar utilization by yeasts, and justifies the start of storage period after 22 days.

Claims

1. An alcoholic beverage comprising probiotic bacteria.
2. The beverage according to claim 1, wherein the alcohol content of the beverage
5 is $\geq 0.5\%$ by volume.
3. The beverage according to claim 1 or 2, wherein the beverage further comprises a hop.
- 10 4. The beverage according to claim 3, wherein the hop or its derivative has a bitterness of ≤ 30 IBU.
5. The beverage according to any preceding claim, wherein the probiotic bacteria comprises lactobacilli, bifidobacteria, or a combination thereof.
- 15 6. The beverage according to any preceding claim, wherein the probiotic bacteria comprised in the beverage is a lactobacilli selected from: *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, or a combination thereof.
- 20 7. The beverage according to any preceding claim, wherein the probiotic bacteria has a cell count of ≥ 5.0 log CFU/mL.
8. The beverage according to any preceding claim, wherein the beverage has a pH of 2-6.
- 25 9. The beverage according to any preceding claim, wherein the beverage has a Brix of 4-20 °Bx.
10. The beverage according to any preceding claim, wherein the beverage is beer.
- 30 11. A method of forming an alcoholic beverage according to any of claims 1 to 10, the method comprising:
 - providing a wort or must;
 - adding a probiotic bacteria to the wort or must;

- adding a yeast to the wort or must; and
- fermenting the wort or must at a pre-determined period of time and at a pre-determined temperature to form the alcoholic beverage.

5 12. The method according to claim 11, further comprising adding a hop or its derivative to the wort or must.

13. The method according to claim 11 or 12, wherein the hop has a bitterness of $\leq$ 30 IBU.

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14. The method according to any of claims 11 to 13, wherein the probiotic bacteria comprises lactobacilli, bifidobacteria, or a combination thereof.

15. The method according to any of claims 11 to 14, wherein the probiotic bacteria
15 comprised in the beverage is a lactobacilli selected from: *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, or a combination thereof.

16. The method according to any of claims 11 to 15, wherein the yeast is *Saccharomyces* yeast, non-*Saccharomyces* yeast, or a combination thereof.

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17. The method according to any of claims 11 to 16, wherein the yeast is *S. cerevisiae*, *S. pasteurianus*, *T. delbrueckii*, *M. pulcherrima*, *P. kluyveri*, *L. thermotolerans*, or a combination thereof.

25 18. The method according to any of claims 11 to 17, wherein the adding a probiotic bacteria and the adding a yeast is carried out simultaneously.

19. The method according to claim 18, wherein the fermenting comprises
fermenting the wort or must at a first temperature for a first period of time and
30 subsequently fermenting the wort or must at a second temperature for a second period of time.

20. The method according to claim 19, wherein the method further comprises adding a hop or its derivative at the end of the second period of time.

21. The method according to any of claims 11 to 17, wherein the adding a probiotic bacteria and the adding a yeast is carried out sequentially.

5 22. The method according to claim 21, wherein the adding a yeast is carried out after a third period of time following the adding a probiotic bacteria.

23. The method according to claim 22, wherein the fermenting comprises fermenting the wort or must at a third temperature for a fourth period of time following
10 the adding a yeast and subsequently fermenting the wort or must at a fourth temperature for a fifth period of time.

24. The method according to claim 23, wherein the method further comprises adding a hop at the end of the fifth period of time.

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25. The method according to any of claims 11 to 24, wherein the formed alcoholic beverage is stored at a temperature $\leq 20^{\circ}\text{C}$.