Chapter 10

Probiotic Beer

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Abstract

The demand for functional beverages that provide health benefits to consumers has increased in recent years. In this sense, several studies investigate the addition of probiotics in beers. However, there are several challenges to overcome when adding probiotics to beer, such as the presence of alcohol and hop compounds that prevent the maintenance of a higher viable number of microorganisms. Thus, traditional beer production routes may not be recommended for this kind of product. Here, we provide a guideline on how to prepare a probiotic beer that can be used for researching new probiotic microorganisms and highlight essential points to be considered when developing probiotic beers.

Key words Functional beer, Functional beverage, Brewing, Yeast, *Levilactobacillus brevis*, *Saccharomyces cerevisiae*

1 Introduction

Beer is one of the oldest fermented beverages, dating back to the Neolithic age, and, nowadays, the most consumed alcoholic beverage in the world. Beer was considered food for several years, and sometimes, the only beverage safe to drink [1]. The beer industry has grown remarkably as time passed, especially during the Industrial Revolution, with technological improvements in equipment, ingredients, and implementation of scientific principles. In the last two decades, craft beers have experienced exponential growth, driven by consumers who seek unique drinking experiences. The current demand for health benefits or awareness about the importance of a healthy diet has driven the beer market to develop health-oriented beverages, like low/no alcohol beer, low-calorie beer, gluten-free beer, and functional beers [2].

Before it was known that microorganisms were responsible for transforming sugars (from grains) into ethanol, carbon dioxide, and a variety of volatile compounds, early beers were soured to some degree due to acidification by wild yeast and bacteria during

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spontaneous fermentation. Therefore, some traditional sour beers, which are intentionally acidified through wild lactic acid bacteria (LAB) and/or acetic acid bacteria (AAB), are considered a classic style of craft beer. Beer styles like Belgian Lambics and Flanders Red Ales represent some of the oldest commercial sour beers, which have recently seen a strong revival [3]. To avoid inconsistencies in aroma, flavor, quality, and long fermentation periods by using wild yeasts and wild LAB, pure or mixed commercial LAB cultures are preferred by brewers to control the brewing process, making a fast and reproducible biological acidification of wort [4].

Drinking unfiltered and unpasteurized beers rich in live probiotics is related to health benefits that regular beers might not provide. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [5]. However, if the wild LAB and yeast involved in the spontaneous fermentation are not isolated and defined, and, if there is no evidence from well-designed clinical trials that suggests a possible health benefit, this undefined microbial consortium cannot be considered a probiotic [6].

Probiotic beer can be defined as a beverage obtained using probiotic microorganisms during the fermentation process [7]. The fermentation process can be conducted in one step, fermenting with one probiotic microorganism or co-fermenting using more than one microorganism [8, 9], or in two steps, fermenting with *Saccharomyces cerevisiae*, followed by fermentation with probiotic microorganisms [10]. However, producing probiotic beer is challenging. To guarantee high cell counts of live probiotics, the recommended minimum dosage is 9 Log colony-forming units (CFUs) per serving of product [5]. It is recommended to use yeasts as starter cultures to produce ethanol and carbon dioxide or to be cultured with probiotic microorganisms because probiotic LAB is incapable of fulfilling this primary purpose [11]. At the same time, the antimicrobial characteristic of hops, specifically iso-**α**-acid (17–55 ppm), can impair the growth and survival of probiotic LAB in beers [12]. In this case, using other hop derivatives such as hop essential oil is an alternative for allowing the growth of probiotic *Lactobacillus* spp. [11].

The *Bifidobacterium* and the strains of LAB—*Lactobacillus acidophilus, Lacticaseibacillus rhamnosus, Enterococcus,* and *Streptococcus*—are the most known probiotic microorganisms. The only commercial yeast used as a probiotic is *S. cerevisiae var. boulardii* [7]. Publications about probiotic beer are recent. Table 1 depicts the main probiotics being investigated to produce probiotic beers, including the beer style.

Table 1
Main probiotic microorganisms that are being investigated to produce probiotic beers

Probiotic	Beer style and process characteristics	Comments	References
L. acidophilus LA-5 and Bifidobacterium lactis BB-12	Low and alcohol-free beer Wort prepared in a commercial brewery	Starters: *Saccharomyces cerevisiae* 70,424 or *Saccharomyces rouxii* 2531 Probiotics were inoculated into the freshly made beer. After storage, cell counts were reduced, with greater losses in low-alcohol beer	[10]
Saccharomyces cerevisiae var. boulardii T1 compared with *Saccharomyces cerevisiae* Safbrew (T58)	No specific style Wort prepared with wheat and barley malts Each wort was fermented, maturated, and in-bottle refermented	After the process, cell counts were above 5 Log CFU/mL	[13]
Free and encapsulated *L. rhamnosus* GG (LGG) in alginate or alginate silica microcarriers	Pale lager Pasteurized Heineken	Encapsulation boosts the protection of the probiotic's cells during storage	[14]
S. cerevisiae var. boulardii in single and mixed cultures with *S. cerevisiae* 17 strains were isolated from natural matrices, then five were selected	No specific style Malted wort	*S. cerevisiae var. boulardii* (*Sb*) showed the ability to overcome stresses such as ethanol content, and it was dominant in almost all mixed cultures Mixed cultures increased the antioxidant capacity and total phenolic content	[15]
S. cerevisiae var. voulardii	Alcohol-free beer Synthetic medium containing dextrin, glucose, maltose, maltotriose, isomerized hop extract, and ethanol	Probiotic yeast was able to grow on a synthetic medium (highest specific growth rate on glucose) The effect of iso--acids on growth rate was not significant until 30 IBU The specific growth rate decreased at an ethanol level of 5% ABV	[16]

(continued)

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Probiotic	Beer style and process characteristics	Comments	References
S. cerevisiae var. boulardii (CECT 1474) compared with *S. cerevisiae* (SF-04)	India Pale Ale Wort was made with a kit containing 100% hopped malt extract	Beer prepared with *S. cerevisiae var. boulardii* as a single yeast starter produced higher acidification, higher antioxidant activity, lower alcohol content, similar sensory attributes, and higher yeast viability after 45 days compared with the beer prepared with a commercial *S. cerevisiae*	[8]
L. paracasei L26 in coculture with *S. cerevisiae* S-04, and single cultures as control	Sour beer Sweet unhopped wort co-fermented with both microorganisms Late addition of isomerized hop extract	Unhopped wort was used as pre-culture and for co-fermentation. Lactic acid production and satisfactory growth of L26 were reported Storage in cold temperatures and live yeast enhanced the survival of L26 in hopped wort	[11]
Lactobacillus delbrueckii pure culture and *S. cerevisiae*	Pito—sour sorghum A portion of the wort was conducted in spontaneous lactic acid fermentation A portion was fermented in pito pure culture followed by S. cerevisiae fermentation	The sensory evaluation found no difference between pito brewed with starter cultures and the traditional pito. Application of the commercial starter cultures in fermenting pito extends shelf-life by 2 days over spontaneous pito	[17]
S. cerevisiae var. boulardii 17	Yeast extract-peptone-dextrose (YPD) agar broths with different concentrations of ethanol	*S. cerevisiae var. boulardii* had great resistance to alcohol and gastrointestinal conditions	[18]
S. cerevisiae var. boulardii CNCM I-745	Yeast extract-peptone-dextrose (YPD) agar broth Yeast was tested in YPD broth with 0 to 10% vol. of ethanol	*S. cerevisiae var. boulardii* CNCM I-745 was not able to grow at a concentration above 8% vol. at 28 °C and above 5% vol. at 37 °C Mathematical modeling of yeast stress resistance is a useful tool	[19]

Levilactobacillus brevis and *S. cerevisiae* SafAle S-3	Ale beer Pale malt extract and hopped wort After 2 weeks of fermenting, probiotic cells and dextrose were added to the beer	Free and immobilized cells remained viable at the end of the storage period (24 days) Cell counts, around 5 log CFU/mL after simulated gastric and intestinal fluids	[20]
Lacticaseibacillus paracasei subsp. *paracasei* DTA 81 (isolated from stools of infants 1 to 3 weeks old), *S. boulardii* 17, *S. cerevisiae* S-04	Wheat beer Sour beer Wheat beer was tested with hopped wort fermented with *S. boulardii* 17 Sour beer was fermented with *L. paracasei* DTA 81 and *S. cerevisiae* S-04 in an unhopped wort	*L. paracasei* DTA 81 survivability was compromised when dry hopping method was applied	[9]
43 wild yeast strains belonging to different genera such as *Lachancea*, *Kluyveromyces*, *Torulaspora*, *Metschnikowia*, *Kazachstania*, *Brettanomyces*, *Pichia*, *Candida*, *Hanseniaspora*, *Rhodotorula*, *Rodosporidobolus* and *Saccharomyces* Commercial *S. cerevisiae* US-05 and *S. boulardii* were used as control strains	Pilsner Pilsner with lentil Pilsner with chickpea flour	Pilsner with chickpea flour had an unpleasant aroma and taste. Pilsner with lentil had an effective fermentative character and pleasant aromatic notes. *S. cerevisiae*, *K. unispora*, and *L. thermotolerans* increased the main aromatic compounds in pilsner with lentils. These yeasts have the potential to produce a beer with high nutritional and functional characteristics	[7]
Saccharomyces cerevisiae var. boulardii and Commercial *S. cerevisiae W34/70* as first fermentation	Pilsen Wort from Heineken Brewery	According to the study, *S. boulardii* presented satisfactory tolerance to bile acid, pH, and ethanol Sensorial analysis showed good acceptance of the probiotic beer	[21]

Since beer is a complex liquid, obtained from a variety of raw materials and brewing routes, some variability can be expected in terms of characteristics, even when prepared at a laboratory scale. Thus, we present a protocol for the preparation of a probiotic beer based on a clear beer, with a refreshing taste, low alcohol content, clean lactic acidity, and a high level of carbonation, inspired by the sour beer styles (characterized by intentionally high acidity in beer) [22].

2 Materials

Probiotic beer is made from water, fermentable carbohydrates, hops, yeast, and probiotic microorganisms. The amount of each ingredient can be calculated by hand [23] or using free online calculators (for instance, BeerSmith2 and Brewer's Friend), considering the volume of beer and the values chosen for each vital characteristic of the sour beer style. In this protocol, we consider the preparation of 1 L of beer with the following characteristics: OG 1038 g/L, FG 1009 g/L, IBU 0, SRM 10, and ABV 3.7% v/v. The material can be acquired from local commerce (*see* **Note 1**). All the materials can be used at room temperature.

Water

1 L of fresh, filtered, and chlorine-free water (*see* **Note 2**). The recommended profile for this beer style is 50–60 ppm of calcium, 0–40 ppm alkalinity, 0–50 ppm sulfate, and 0–100 ppm chloride [24] (*see* **Note 3**).

Carbohydrate Source

0.3 kg of extra-light dry malt extract (DME) (*see* **Note 4**).

Hops

0.1 g of highly concentrated hop oil extracts (*see* **Note 5**).

Microorganisms

0.8 g of dry yeast *S. cerevisiae* for alcoholic fermentation. Keep the yeast refrigerated until using it (*see* **Note 6**).

1 mL of hydrated *L. brevis* for acid fermentation. This is the probiotic strain used in beer production (*see* **Note** 7). Keep the LAB refrigerated until using it.

Equipment

The main equipment required are presented in Table 2.

Table 2
Equipment required for preparing 1 L of probiotic beer

Item	Reason
2 units of 2 L glass autoclavable container/bottle	Wort preparation
1 unit of stove or heating plate	Wort heating
1 m of ¼″ food grade silicone hose	Transfer beer from the fermentation vessel
1 unit of a rubber stopper with an airlock	Releases the CO_2 from the fermentation bottle
Bowl	Fill with cold water to refrigerate the wort
Temperature chamber (0–25 °C)	Fermentation and maturation
Refractometer	Measure wort and beer densities (OG and FG)
4 units of 330 mL amber glass bottle	Packaging
4 units of metallic caps for the glass bottles	Packaging
Bottle capper	Packaging

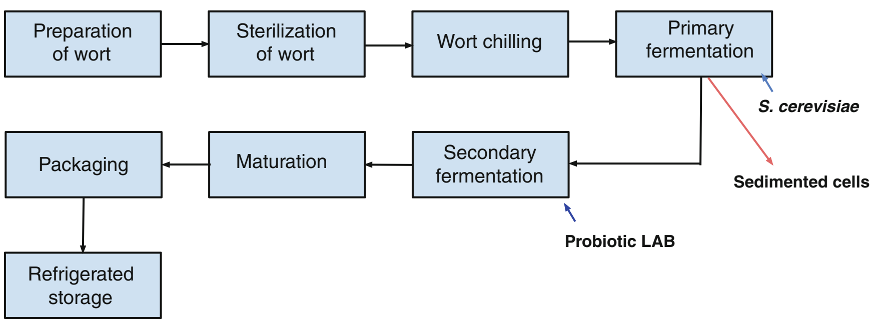

Fig. 1 Flowchart of the steps required to prepare the sour beer

3 Methods

The preparation of the sour beer follows the flowchart presented in Fig. 1 (*see* **Note 8**). Each step is described separately in this protocol.

3.1 Preparation of Wort

1. Prepare the wort using the 2 L glass container. Slowly, add the extra-light dry malt extract (DME) to 1.0 L of the previously prepared water. The mixture can be performed by using a magnetic stirrer.

3.2 Sterilization of Wort

1. After full solubilization of the DME, close the flask with aluminum foil or a cap (*see* **Note 9**).
2. Take the glass container to an autoclave. Set the temperature to 121 °C and let sterilize for 20 min [16].

3.3 Wort Chilling

1. Remove the container from the autoclave and let the temperature decrease to around 10 °C in the air (ambient condition).
2. Place the container in the water-cooling bath and let the temperature decrease to 18 °C (*see* **Note 10**). From here, be careful handling the wort, because it is susceptible to contamination.
3. After reaching the desired temperature, aerate the wort with 8–9 ppm oxygen [25]. The wort oxygenation process in the laboratory can be done by shaking the wort in the closed container for 10 min or until the minimum concentration is reached (*see* **Note 11**). Measurement of dissolved oxygen in wort can be performed by using an oximeter or other analytical chemical method.

3.4 Primary Fermentation

1. Remove the yeast from the refrigerator 1 h before using so that the cells are at a temperature close to the fermentation temperature. 0.8 g of the dry yeast *S. cerevisiae* is used for alcoholic fermentation (*see* **Note 12**).
2. Progressively sprinkle the dry yeast directly in the fermentation vessel on the surface of the wort, ensuring the yeast covers all the wort available to avoid clumps. Let the yeast be hydrated by the wort.
3. Close the container and shake it slowly to homogenize the yeast with the wort.
4. Remove the cap and attach the rubber stopper containing the airlock. This airlock relieves positive pressure due to the production of CO_2 during fermentation.
5. Place the container in the temperature chamber at 18 °C. The fermentation process continues until the final extract (FG) reaches 1009 g/L or it doesn't change in 48 h.
6. Cool the beer to 2 °C and keep it for 24 h to optimize the yeast sedimentation.
7. Slowly and carefully remove the supernatant liquid using the silicone hose and transfer it to a second fermentation vessel.

3.5 Secondary Fermentation

1. Remove the *L. brevis* from the refrigerator 1 h before using.
2. Prepare the beer for lactic fermentation (*see* **Note 13**) by heating it to 37 °C.
3. Shake the sachet with the microorganisms and inoculate 10 mL of the *L. brevis* directly into the fermentation vessel (*see* **Note 14**).
4. Cover the container and shake it to homogenize. Fermentation continues until a count of 9 Log CFUs is reached.

3.6 Maturation

1. After the second fermentation, cool the beer to 2 °C and keep it for 72 h for the maturation process (*see* **Note 15**).
2. To provide the probiotic sour beer with the flavor of hops, add 0.1 g of hop oil extracts directly to the liquid. After the addition, shake to homogenize the product (*see* **Note 16**).

3.7 Packaging

1. Pre-wash the glass bottles with mild soap and water and sanitize them with peracetic acid for 15 min (*see* **Note 17**). The sour beer should be bottled without going through a filtration and pasteurization process to ensure the permanence of living cells.
2. Pack the beer at a temperature of 2 °C and immediately cover it (*see* **Note 18**).

3.8 Refrigerated Storage

1. Keep the bottles at 5 °C to avoid the aging process and contain the fermentation [26]. The storage time should be as short as possible, a longer time will always have a negative influence on the quality and cellular viability of the probiotic beer.

4 Notes

1. Since the ingredients are from natural sources, they may have slight variations in their characteristics depending on the brand or even on the batch used. It is advisable to ask the supplier company for the specific analysis data for the batch of ingredients used.
2. Potable (tap) water can be treated by a series of filters; usually, a polypropylene filter (nominal pore size from 5–20μm) is followed by one or two steps of filtration through activated carbon to remove chlorine from water.
3. This water profile is a suggestion, based on the beer style. However, for research purposes, it is strongly recommended to use purified deionized water and analytical reagents to adjust the salt content and the required pH. Calcium sulfate, calcium chloride, magnesium sulfate, sodium bicarbonate, magnesium chloride, and sodium chloride can be used to adjust calcium (Ca^{+2}), magnesium (Mg^{+2}), bicarbonate (HCO_3^{-1}), sulfate (SO_4^{-2}), sodium (Na^{+1}), chloride (Cl^{-1}), and sulfate (SO_4^{-2}) ions [27].
4. Malted barley is the main carbohydrate source used for preparing beer, but other carbohydrate sources can also be used. The grain bill is calculated from the gravity units and color each carbohydrate source can offer to reach the desired beer OG and SRM [23].

However, to simplify laboratory studies, we suggest using the Dry Malt Extract. Using DME eliminates brewing steps (grain milling, mashing, and filtration).

5. Hops are used in beer production for impairing the aroma, flavor, and bitterness. In addition, they provide antimicrobial and antioxidant properties, which can impair probiotic growth [28]. Thus, replacing the addition of hops in the boiling stage with the use of concentrated hop extract in the maturation stage is an alternative to providing the beer with the hop aroma without harming the development of probiotics. We recommend using a concentrated hop extract with the aromatic character of grapefruit and tropical fruits.
6. Prefer using ale yeast with fast fermentation characteristics and the ability to form a compact sediment at the end of fermentation, which helps to improve the clarity of the beer.
7. There is a wide variety of probiotic species that can be used, such as *L. acidophilus, L. helveticus, L. delbrueckii subsp. bulgaricus,* and *L. paracasei*.
8. This is a proposed procedure, intended to facilitate the preparation of the sour beer at a laboratory scale and reach the desired characteristics, essential for research purposes.
9. If using a screw cap, be careful not to close the flask completely when autoclaving.
10. Ice can be used in the water bath to help decrease the temperature. The wort temperature should be just right for the specific yeast strain. For the sour beer style, the first fermentation with *S. cerevisiae* S-04 is performed at 18 °C.
11. In breweries, the wort is oxygenated using a medical oxygen cylinder with a flow meter. This equipment indicates the volume of air that dissolves in the liquid with scales from 0–15 L/min.
12. The dosage recommended in this protocol is 80 g/hL for fermenting at a temperature from 18 to 26 °C [29]. If using another yeast, follow the manufacturer's recommendation for dosage and fermentation temperature.
13. For producing probiotic sour beer, the acidification process is conducted after the primary fermentation (alcoholic fermentation) rather than the kettle sour process (common for traditional sour beer). This procedure avoids removing probiotic bacteria with the spent yeast from primary fermentation. Then, beer souring occurs after primary fermentation and removal of yeast [30].
14. The recommended concentration of the probiotic is 200 mL for 20 L of beer [31]. If using another variety of probiotic species, follow the manufacturer's recommendation for dosage and fermentation temperature.

15. During maturation, beer is saturated with carbon dioxide, and part of the turbidity-forming components of the beer settles (clarification) [25].
16. We recommend testing a dosage of 10 g/hL (range 5–40 g/hL) [32]. If using another variety of hop oil, follow the manufacturer's recommendation for dosage.
17. Follow the manufacturer's recommendation regarding the dilution of the peracetic acid.
18. There will probably be an accumulation of carbon dioxide and an increase in pressure in the bottle due to the production of carbon dioxide by the heterofermentation of *Lactobacillus* spp. [30]. In this case, the glass bottle is the most suitable because it supports a higher pressure compared to the plastic bottle. A low temperature of the beer in the bottle increases the solubility of carbon dioxide, it is recommended to pack at a temperature of 2 °C [26].

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