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Functional Non-Alcoholic Beer Fermented with Potential Probiotic Yeasts

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Functional Non-Alcoholic Beer Fermented with Potential Probiotic Yeasts

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Abstract

The development of innovative non-alcoholic beer (NAB) with health benefits, with the use of non-conventional potential probiotic yeasts offers an interesting alternative to standard NAB brewing strategies. In this study, potential probiotic non-*Saccharomyces* yeasts *Pichia manshurica*, *Kluyveromyces lactis*, and *Kluyveromyces marxianus*, along with commercial probiotic yeast *Saccharomyces boulardii*, were characterized and tested for functional NAB production, whereas *P. manshurica* was used in NAB production for the first time. Growth and viability were assessed across a range of temperatures, pH, and iso-α-bitter acids. The tested yeasts withstood conditions typical of the beer matrix and human digestive tract and had positive phenolic off-flavour phenotype. Two strains, *K. lactis* and *K. marxianus*, showed strong β-glucosidase activity, which may enhance beverage aroma complexity. Ethanol levels in beers fermented with non-*Saccharomyces* yeasts remained below the NAB limit (≤0.5% v/v). Analysis of volatile organic compound profiles revealed the potential of these yeasts to produce higher alcohols and esters valuable from a brewer's perspective. Sensorial evaluation of prepared NAB produced with potential probiotic non-*Saccharomyces* yeasts revealed floral and clove-like aroma traces. This study provides valuable insight into novel probiotic fermentations and the potential application of unconventional yeasts in functional, aromatic, and health-oriented non-alcoholic beverages.

Keywords: non-*Saccharomyces* yeasts; probiotic yeasts; non-alcoholic beer; functional beer

1. Introduction

The traditional beer markets are thriving, and brewers aim to meet emerging consumer trends [1]. Although the NAB market was valued at $36.7 billion, it represents only 4.3% of global beer production [2]. Rising interest in healthier foods has led to functional beers, such as probiotic or gluten-free options [3]. The probiotic market, worth $61.2 billion in 2021, is projected to grow by 7.7% by 2030 [4]. Beverages as functional matrices are becoming more common, yet adding probiotics to beer is challenging due to its harsh environment [5]. Consumers increasingly prefer beers with lowered ethanol content aligned with healthier lifestyle [6], while breweries preserve capital from lower tax burdens [7]. According to EU Regulation 1169/2011, beverages over 1.2% vol. are alcoholic, but most countries define NAB as ≤0.5% vol. [8]. NAB can be made by limiting ethanol during fermentation or removing it post-production, but this can cause off-flavours and aroma loss [9,10]. Use of non-maltose fermenting yeasts helped improve NAB sensory qualities [10–12].

Probiotics are live microorganisms with health benefits when consumed in sufficient amounts [13]. As ethanol is a drug, probiotic beers should be alcohol-free [14]. Labels may state CFU levels, but high counts don't always mean better health effects [15]. Probiotics can colonize the gut, compete with pathogens, and produce beneficial compounds [16]. Though only few probiotic yeasts (PYs),

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like *Saccharomyces boulardii* or *Kluyveromyces fragilis B0399*, are used, other genera also show probiotic potential, including tolerance to low pH, bile salts, and antimicrobial activity [17].

S. boulardii, a GRAS organism [18], is a subtype of *S. cerevisiae* and produces ethanol, CO_2, and bioactives like GABA (γ-aminobutyric acid) and B vitamins [19]. *K. lactis*, studied since the 1960s [20], ferments lactose using LAC12 and LAC4 genes [21] and can produce ethanol even anaerobically [22]. *K. marxianus*, a thermotolerant, Crabtree-negative yeast, survives at 52 °C and can produce ethanol above 40 °C, but its inability to ferment maltose makes it suitable for NAB [23]. *Pichia manshurica*, found in fermented foods and wines, is associated with biofilm and volatile phenol production [24]. Though never used in beer, it showed survival potential under stress factors [25] and successfully enhanced vinegar aroma profile in one study [26].

This study applies *S. boulardii*, *K. lactis*, *K. marxianus*, and *P. manshurica* as sole fermentation cultures in functional NAB production. Results support the concept of using probiotic yeasts to develop next-generation health-promoting beverages.

2. Materials and Methods

2.1. Yeast Strains

Yeast strains used in this study (Table 1) were maintained on YPDA medium [10 g.L^{-1} yeast extract (Oxoid, ThermoFisher Scientific, USA), 10 g.L^{-1} peptone (Thermo Scientific™, USA), 20 g.L^{-1} glucose (Merck, Darmstadt, Germany) and g.L^{-1} agar (Carl Roth, GmbH, Germany), pH 6.2] and stored at 4 °C.

Table 1. Yeast strains used in this work with their abbreviation and short description.

Yeast	Abbreviation	Description
Pichia manshurica 1 CCY* 039-063-001	PM1	Potential probiotic strain
Pichia manshurica 2 CCY* 039-063-004	PM2	Potential probiotic strain
Kluyveromyces lactis CCY* 026-012-002	KL	Potential probiotic strain
Kluyveromyces marxianus CCY* 029-008-010	KM	Potential probiotic strain
Saccharomyces boulardii HANSEN CBS** 5926	SBL	Control probiotic strain

* CCY = Culture Collection of Yeasts (Bratislava, Slovakia), ** CBS = Central Bureau of Fungal Cultures (The Netherlands).

2.2. Preparation of Yeast Starters

Yeast starters used in experiments were prepared by 24h submerse cultivation of individual yeast strains in liquid YPD medium (10 g.L^{-1} yeast extract, 20 g.L^{-1} glucose, 10 g.L^{-1} peptone (Thermo Scientific™, USA) pH 6.2; 20 ml in 100 ml Erlenmeyer flasks) on an orbital shaker (Biosan ES-20, Riga, Latvia) at 2 Hz, 28 °C.

2.3. Fermentation of Saccharides

The ability of yeast strains to ferment saccharides (glucose, maltose, lactose) was tested as previously described by [27] in glass tubes containing inverted Durham tubes. Production of CO_2 indicating the saccharide fermentation was evaluated after 7-day static cultivation at 25 °C. Experiments were performed in triplicates.

2.4. ß-Glucosidase Activity

Yeast strains with positive *ß*-glucosidase activity are capable of hydrolyzing aesculin as the sole carbon source to glucose and aesculetin, which reacts with present iron ions to form a dark compound. Tested yeast strains were inoculated onto plates with medium containing: aesculin 1.0 g.L^{-1} (Fisher Scientific, USA), iron (III) citrate 3-hydrate (Acros Organics®, USA), 0.5 g.L^{-1}, yeast extract 8.0 g.L^{-1}, agar 15 g.L^{-1}, and were incubated for 24h at 25 °C. The intensity of *ß*-glucosidase

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activity was evaluated based on the formation of the dark zone and the intensity of diffusate colouring. Experiments were performed in triplicates.

2.5. Phenolic Off-Flavour (POF) Phenotype

Yeasts capable of decarboxylating ferulic acid, into the formation of 4-vinyl guaiacol (4-VG) which imparts beer with a clove-like aroma, can be characterized by their positive (POF^+) or negative (POF^-) phenotype. Yeast starters used in experiments were prepared by 24h submerse cultivation of individual yeast strains in liquid YPD medium. For each yeast strain, 20 ml of pure and sterile YPD medium was poured into glass tube with the addition of 0.2 ml of 1% (v/v) ferulic acid solution prepared by adding ferulic acid (Merck, Darmstadt Germany) into 96% (v/v) of ethanol (CentralChem®, Slovakia). Glass tubes were inoculated by the tested yeast strains in triplicates. Tubes were then sealed and statically incubated at 25 °C for 24h. Evaluation was done by six people and was performed by sensorial analysis comparison of glass tubes containing tested yeast strains against controls, where as a positive control (POF^+) yeast SafBrew™ LA-01 was used. As a negative control (POF^-), yeast LalBrew® LoNa™ was used.

2.6. Tolerances of Different Conditions

To determine sensitivity of strains to different temperature conditions, 1×10^6 Cells.mL^{-1} of liquid yeast starters were cultivated at 4 °C, 20 °C and 37 °C for 24h in sterile glass tubes each containing YPD medium.

Sensitivity of strains to different pH (3; 4; 5 and 6) was evaluated by cultivating 1×10^6 Cells.mL^{-1} of fresh liquid yeast starter at 37 °C for 24h in sterile glass tubes each containing YPD medium, where pH was adjusted by adding 35% (v/v) of HCl (CentralChem®, Slovakia). Sensitivity of strains to different concentrations of iso-α-bitter acids in terms of IBU (international bitterness unit) (0; 10; 30; and 50) was evaluated by cultivating 1×10^6 Cells.mL^{-1} of fresh liquid yeast starter at 25 °C for 24h in sterile glass tubes each containing YPD medium, where IBU units were adjusted by the addition of iso-α-bitter acid solution (Brewferm®).

The tolerance of different temperatures, pH, concentrations and iso-α-bitter acids was evaluated based on the growth of the yeast culture, which was determined by measuring the optical density of the biomass suspension at a wavelength of 600 nm (ΔOD_{600nm}) against pure YPD medium used as a blank. Viability of yeast cells was determined microscopically using the staining with 0.1% (w/w) methylene blue solution. Experiments were performed in triplicates.

2.7. Fermentation and Maturation

For beer production, 480 ml of wort (8°P made from Pilsen malt and Žatecký poloraný červenák hops (Saaz)) prepared in 25 L Laboratory Microbrewery (Braumeister, Speidel, Germany) in 500 ml fermentation PET flasks was inoculated with yeast starters of pitch rate of 1×10^6 Cells.mL^{-1}. Flasks were closed and fermentation proceeded at 20 °C for 2 days after which maturation proceeded at 3 °C for 3 weeks. Beers were then stabilised by pascalisation procedure at 400 MPa for 3 min. Finally, fermented beer samples were analysed for composition of residual saccharides, organic acids, glycerol, ethanol and profile of main volatile organic compounds (VOCs). Viability of cell cultures in final beer samples was determined after stabilisation procedure. Fermentation experiments were performed in triplicates.

2.8. Beer Analyses

Basic Beer Parameters

Ethanol concentration and pH of beer and wort samples was determined using a density meter DMA 4500M coupled with Alcolyzer Beer ME, Haze QC ME Turbidity Measuring Module and pH ME Beverage Measuring Module (Anton Paar, GmbH, Graz, Austria). Prior to analysis, fermented

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final beer samples were centrifuged (10 min, 2524 ×g) and degassed by ultrasonication for 30 min and analysed in triplicates.

Organic Compound Analysis by HPLC-RID-DAD

Before HPLC analysis, the samples were centrifuged (10 min, 2511 ×g) and supernatant was diluted with deionized water if needed. Agilent 1260 HPLC system (Santa Clara, CA, USA) coupled to RI (refractive index) and DAD (diode array detector) using Aminex HPX-87H column (300 mm, 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) was used for HPLC measurements. Sulfuric acid (5 mmol.L^{-1}) was used as the mobile phase with the flow-rate of 0.6 mL.min^{-1}. Separation was performed at 25 °C, injection volume was 20 µL. The signal was detected by RID and DAD detectors. Accurate concentrations of glucose, maltose, glycerol, acetic, lactic and citric acid were determined using the single standard addition method. The standards with purity ≥99.5% were obtained from Merck (Darmstadt, Germany). Beer samples were analysed in triplicates.

Volatile Organic Compound Analysis by HS-SPME-GC-MS

Prior to analysis, beer samples were cooled and stored at 4 °C. 50 mL of each beer sample was centrifuged (10 °C, 5054 ×g, 10 min) and supernatant was poured into 50 mL flask and enclosed. Flasks were shaken for 3 min to remove the CO_2. In the meantime, 2 g of NaCl with (≥99.9% purity, Pentachemicals, Czech Republic) were put into 20 mL dark vial together with 10 mL of beer sample and 100 µL of internal standard (IS) solution, which contained: ethyl heptanoate (≥ 99% purity, Sigma Aldrich, DE) and 3-octanol (≥ 99% purity, Sigma Aldrich, USA). Vial was vortexed for 30s to dissolve the NaCl and homogenise the sample VOCs were identified and quantified according to a method described in [27]. VOCs of beer samples were analysed in triplicates.

Sensorial Evaluation of Beer Samples

Final beer samples were analysed sensorially by six-person taste panel where attributes of beer aromatic profiles were evaluated and resulting data describing aromatic profile were visualized as a radar chart (aromagram).

3. Results and Discussion

3.1. Yeast Characterisation

Fermentation tests showed that all strains were able to ferment glucose (Table 2). Unlike the probiotic strain *S. boulardii*, the other four non-*Saccharomyces* yeast strains of *Kluyveromyces* and *Pichia* genus were unable to ferment maltose – the most abundant saccharide in wort (Table 2) and making them proper candidates for non-alcoholic beer (NAB) production by strategy of using maltose-negative yeast strains [28]. Potential probiotic strains *K. lactis* and *K. marxianus* were able to ferment lactose, as was previously confirmed by [29]. *S. boulardii* and both yeast strains *P. manshurica* have not fermented lactose (Table 2). Determination of the *ß*-glucosidase activity of tested yeasts proved, that both strains of *P. manshurica*, as well as *S. boulardii*, had weak/delayed *β* -glucosidase activity after 24h, whereas *K. lactis* and *K. marxianus* showed strong *β* -glucosidase activity (Table 2). Several studies have reported that yeasts with increased *β*-glucosidase activity play an important role in releasing aromatic aglycones from hops during fermentation [30] and thus, enhancing aroma complexity of the final beverage. Positive phenolic off-flavour (POF$^+$) phenotype was sensorially evaluated by the production of clove-like aroma (4-vinyl guaiacol (4-VG)) by all tested yeasts (Table 2). Besides diacetyl and sulfur compounds, 4-VG is mostly unwanted compound during beer production with the exception of few beer styles e.g. German Hefeweizen and Belgian Wit beers where 4-VG is considered as a part of an aromatic profile [31]. Even though the tested yeasts were POF$^+$ they might serve as a fermentation starter culture for brewing specific non-alcoholic wheat beers with clove-like aroma.

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Table 2. Determination of saccharide fermentation tests, *ß*-glucosidase activity and phenolic off-flavour (POF) phenotype tests with studied yeasts after 24h incubation at 25 °C.

Yeast (Abbreviation)	*Saccharide Fermentation			**ß-Glucosidase Activity	***POF Phenotype
	Glucose	Maltose	Lactose		
Saccharomyces boulardii (SBL)	+	+	-	positive	POF^+
Pichia manshurica 1(PM1)	+	-	-	w/d	POF^+
Pichia manshurica 2 (PM2)	+	-	-	w/d	POF^+
Kluyveromyces lactis (KL)	+	-	+	positive	POF^+
Kluyveromyces marxianus (KM)	+	-	+	positive	POF^+

„+": positive formation of CO_2 – yeast was able to ferment saccharide, „-": negative formation of CO_2 – yeast was unable to ferment saccharide; ** "w/d": weak or delayed *ß*-glucosidase activity; *** "POF^+": positive formation of phenolic off-flavours.

3.2. Tolerance of Different Temperature

Growth of yeast at different temperatures represented as ΔOD_{600nm} values, showed that the potential probiotic yeasts *Kluyveromyces* were unable to grow at 4 °C (Figure 1), however, the highest growth represented as ΔOD_{600nm} values were observed in a medium incubated at 37 °C (Figure 1). According to [32], the yeast *K. marxianus* can withstand 45 °C, but as the human body temperature equals 37 °C, it was not our goal to test growth at ≥37 °C. According to [33], the optimal growth temperature for the probiotic yeast *S. boulardii* is 37 °C. This was supported by our results and *S. boulardii* was able to grow sufficiently at whole range of temperatures (Figure 1). The maturation of beer is performed at temperatures close to 0 °C. Growth of *S. boulardii* at low temperatures (4 °C) (Figure 1) might potentially lead to unexpected fermentation of wort saccharides such as maltose or glucose due to positive fermentability of these saccharides (Table 2), hence the ethanol limit for NAB production should be maintained. Growth of both *P. manshurica*, represented as ΔOD_{600nm} values was observed at 37 °C (Figure 1), whereas no growth was observed at 4 °C and minimal at 20 °C. Results supported the suitability of tested yeast to survive the human body temperature. Overall, the highest viability (Table 3) was determined when cultivating yeast at 37 °C, support the probability of survival of the tested yeasts in a human body gastrointestinal tract. As for the 20 °C which is a temperature in temperature interval used for brewing ale style beers, the viability for both *Kluyveromyces* and both *Pichia* yeast strains has decreased for more than 10% except for *S. boulardii*, which was the most viable yeast strain (97%).

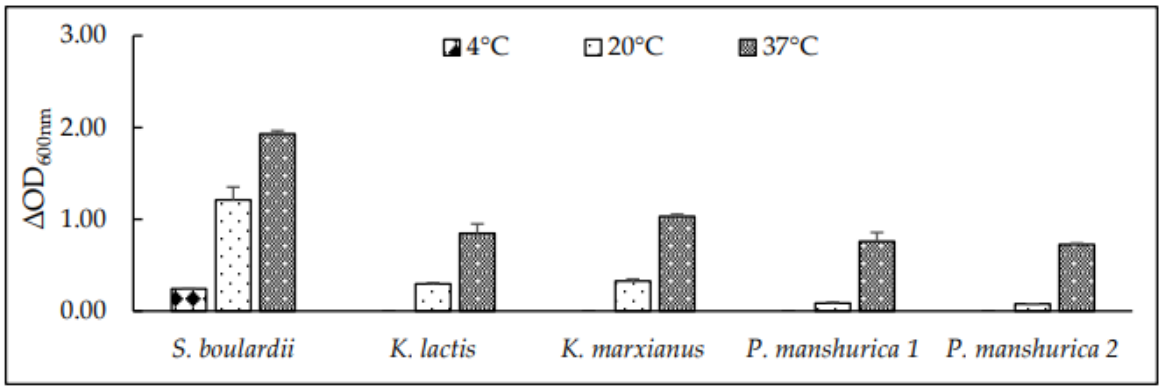

Figure 1. Temperature tolerance of tested yeasts in 2% YPD medium at 3 different temperatures (4, 20 and 37 °C) after 24h by Optical density (ΔOD_{600nm}) measurements. Results are presented as Average values of 3 OD_{600nm} measurements.

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Table 3. Viability of tested yeasts after 24h incubation of yeasts in a 2% YPD medium at 3 different temperatures (4°C, 20 and 37°C).

Yeast	*S. boulardii* (SBL)	*P. manshurica 1* (PM1)	*P. manshurica 2* (PM2)	*K. lactis* (KL)	*K. marxianus* (KM)
Viability at 4°C	83%	27%	30%	35%	37%
Viability at 20°C	97%	82%	83%	80%	85%
Viability at 37°C	98%	97%	98%	96%	96%

3.3. pH Tolerance

Determination of pH tolerance was used to identify growth behaviour of the studied yeasts to withstand harsh conditions of human stomach (pH 3), fermented medium – beer (pH 4 – 5), and fermentation medium – wort (pH 6). Yeast growth for *Pichia* and *Kluyveromyces* sp. was strongly inhibited when exposed to highly acidic environment close to pH in human stomach (pH = 3), only yeast *S. boulardii* was able to withstand this acidic environment and with the highest determined ΔOD_{600nm} values (Figure 2). According to [34] the probiotic yeast *S. boulardii* can survive under stomach conditions (pH 3), which was confirmed by our results. As for other four non-*Saccharomyces* yeasts of *Pichia* and *Kluyveromyces* sp., increased cell count (above the initial inoculated pitch rate of 10^6 Cells.ml^{-1}) in values of ΔOD_{600nm} was detected at pH ≥3 and was generally of rising characteristics to pH 6. The viability of a traditional brewery yeast should normally found to lie in the range of 90 – 99% [35] and it is generally accepted that the live cell content of the yeast slurry used for subsequent fermentation should contain >95% of live cells, whereas high yeast viability allows for the production of high-quality beer [36]. However, our viability results revealed that *Kluyveromyces* and *Pichia* yeasts were not as viable as *S. boulardii* (69%) in acidic media (pH =3) after 24h at 37°C due to their low percentage of viability which was under 21% (Table 4). Different results were obtained when several *K. marxianus* strains were investigated by [37] under harsh acidic environment (<pH 3), results showed initial reduction of cell counts from 10^9 to 10^7 Cells.ml^{-1} and afterward growth of *K. marxianus* strains during the incubation period of 96h. Stable intracellular pH is crucial for yeast growth and metabolic activity, as enzymatic functions depend on an intracellular pH environment; whereas significant deviations in extracellular pH can disrupt this balance, impairing enzyme activity and cellular function [38].

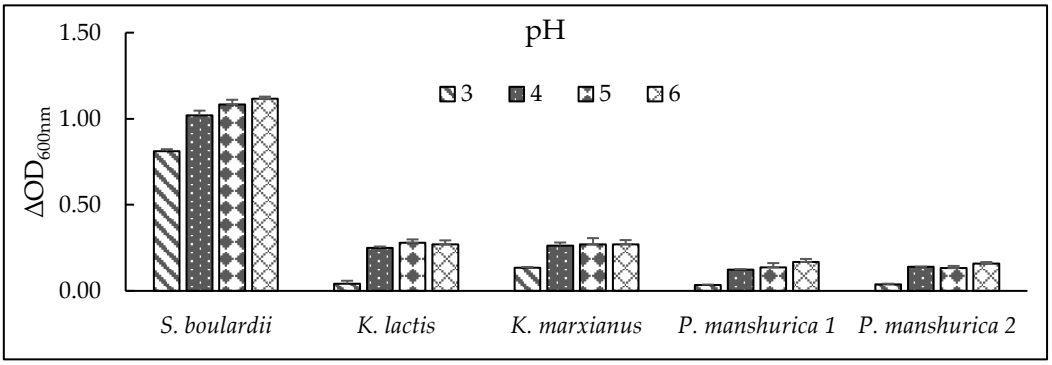

Figure 2. pH tolerance of tested yeasts in 2% YPD medium with different pH (3 – 6) after 24h, at 37 °C by Optical density (ΔOD_{600nm}) measurements. Results are presented as Average value of 3 OD measurements.

Table 4. Viability of tested yeasts after 24h incubation at 37 °C of yeasts in a 2% YPD medium at 2 different pH 3 and 6.

Yeast	*S. boulardii* (SBL)	*P. manshurica 1* (PM1)	*P. manshurica 2* (PM2)	*K. lactis* (KL)	*K. marxianus* (KM)
Viability at pH 3	69%	17%	16%	21%	18%
Viability at pH 6	97%	84%	86%	83%	87%

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3.4. Tolerance of Iso-α-bitter Acids

Hops are traditionally added during beer brewing as a bittering and flavouring agent [39]. Hops contain α-bitter acids which isomerize during boiling step to form iso-α-bitter acids – compounds responsible for bitterness of beer [40]. These substances report antimicrobial properties and protect the beer against the most common spoilage bacteria [41]. However, [42] reported that iso-α-bitter acids can affect not only the growth of lactic acid bacteria but can also inhibit the growth of yeast *S. cerevisiae* at the concentration of iso-α-bitter acids above 500 $mg.L^{-1}$ (equal to 500 International Bitterness Units – IBUs), which is approximately ten times higher than the concentrations required to inhibit bacterial growth. According to [14], most alcohol-free beers do not tend to exceed 30 IBU. The growth of tested yeasts was probed in the presence of 10,30 and 50 IBU (values characteristic for most of the beer styles) where no exceptional effects of different concentrations of iso-α-bitter acids in terms of different (IBU units) on yeast growth was detected (Figure 3). Viability of all strains in tested media with different IBUs remained above 95% (data not shown).

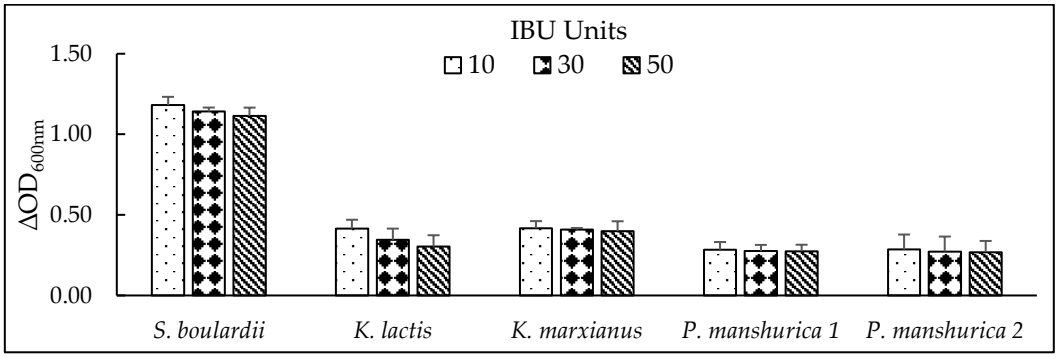

Figure 3. Iso-α-bitter acid tolerance of tested yeasts in 2% YPD medium with different IBU concentrations (10, 30 and 50) after 24h, at 25 °C by Optical density (ΔOD_{600nm}) measurements. Results are presented as Average value of 3 OD measurements.

3.5. Basic Beer Parameter Analysis

An important analytical parameter of beer is the ethanol concentration, which influences the sensory properties of beer, especially the fullness of flavour, but also colloidal and biological stability of the beer [3]. The ethanol concentration in produced beers ranged from 0.04 ± 0.01% (v/v) (PM1) to 1.52 ± 0.06% (v/v) (SBL) (Table 5) and from all the analysed beers except the beer produced with *Saccharomyces boulardii* none displayed ethanol concentration ≥0.5% (v/v) which is the limit concentration in alcohol-free beers [43]. Authors [44] also studied use of probiotic yeast *S. boulardii*, influence of hops on its propagation and fermentation performance and produced beers with 4.67 – 3.26% (v/v) of ethanol. Important note should be considered as term "probiotic beer" (containing 1 x 10^9 CFU, beneficial for health) [15] might be in conflict with the term "alcoholic beer" (generally containing more than 1.2% v/v of ethanol) where ethanol is considered as a drug. In the characterisation of beers, pH is an important quality indicator, which influences the foaminess, clarity, microbiological and colloidal stability of the beer [45]. During fermentation of wort, organic acids are produced by yeast, which leads to a decrease of the pH value of the product. The average pH of common beers ranges from 4.3 to 4.7 [46], while the pH of the produced beers ranged from 4.83 ± 0.02 (SBL) to 5.80 ± 0.01 (PM1) (Table 5) which was supposedly due to the short-used fermentation times (2 days). The viability of the tested yeasts with methylene-blue staining (data not shown) showed that pascalisation procedure successfully inactivated the yeasts in all beer samples fermented with tested yeasts (Table 1) to prevent further fermentation activity.

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Table 5. Ethanol concentration, pH of the beer samples prepared from 8°P Wort, fermented with tested yeasts (1x10^6 Cells.mL^{-1}) at 20 °C (2 days) and maturated at 3 °C (3 weeks) and pascalised at 400 MPa (3 min).

	Sample					
Basic Parameters	**8°P Wort**	**SBL**	**PM1**	**PM2**	**KL**	**KM**
Ethanol % (v/v)	n.d.	1.52 ± 0.06	0.04 ± 0.01	0.07 ± 0.01	0.13 ± 0.02	0.14 ± 0.01
pH	6.00 ± 0.06	4.83 ± 0.02	5.80 ± 0.01	5.73 ± 0.02	5.41 ± 0.01	5.36 ± 0.03

„n.d." = not detected. Values are presented as (Average ± Standard Deviation) from 3 parallel analyses. Abbreviation of beer sample correspond to a yeast abbreviation used for beer production. SBL= *S. boulardii*, PM1 and PM2 = *P. manshurica 1* and *2* KL1 = *K. lactis*, KM = *K. marxianus*.

3.6. Organic Compound HPLC Analysis

The saccharide composition of beer can greatly affect the resulting taste of beer. Saccharide such as maltose strongly contributes to the body of the beer while the glucose and sucrose contribute to the sweet taste of the beer [47]. In comparison with used wort, minimal decrease of glucose and maltose were observed in beers fermented with *Kluyveromyces* and *Pichia* yeasts (Table 6). It is known that many yeasts can assimilate certain mono- or oligosaccharides aerobically, but not anaerobically (fermentation) whereas this phenomenon is known as the Kluyver effect [48]. Oxygen availability plays a key role in determining the fermentation pattern of *K. lactis*. As oxygen availability decreases, overall glucose metabolism slows down, resulting in reduced fermentation activity [49]. This might be one of the answers to results concluded in Table 6, where only a small proportion of glucose and maltose were consumed by the non-*Saccharomyces* yeasts *K. lactis*, *K. marxianus* and both strains of *P. manshurica*. In comparison, beer fermented with probiotic yeast *S. boulardii* (SBL) had no residual glucose which was presumably utilized during the first 2 days of beer fermentation (Table 6) which is supported by the obtained results from saccharide fermentation tests where after 24h the glucose was already being fermented (Table 2). As for maltose, the most abundant saccharide present in the beer wort [39], results showed that its final concentration decreased strongly during fermentation from 36.8 ± 0.5 g.L^{-1} (wort 8°P) to 24.1 ± 0.3 g.L^{-1} in the beer fermented by *S. boulardii* (SBL) as was expected (maltose-positive strain) (Table 2) and ethanol (1.52 ± 0.06% (v/v)) was produced. Glycerol concentrations were below the perception threshold level which in beer is 10 g.L^{-1} [39]. Organic acids not only have a significant effect on the sour taste of beer but also lower the pH of beer, which affects the quality and stability of beer flavour [50]. Concentration of citric acid in beers produced by non-*Saccharomyces* yeasts was 0.1 ± 0.0 g.L^{-1}(Table 6). Beer prepared with *S. boulardii* contained (0.3 ± 0.0 g.L^{-1} of citric acid and unlike beers fermented with four other non-*Saccharomyces* yeasts (*K. lactis*, *K. marxianus* and both *P. manshurica*), also contained acetic acid (0.1 ± 0.0 g.L^{-1}) which is not desired. *S. boulardii* unique mutations cause accumulation of higher amounts of acetic acid which on the other hand might inhibit bacterial growth [51] but acetic acid affects the taste of beer in a drastic way, with its sharp acidity and vinegar notes when present in beer above the threshold concentration 200 mg.L^{-1} [52,53].

Table 6. Concentration of organic compounds (g.L^{-1}) in the beer samples prepared from 8°P Wort, fermented with tested yeasts (1x10^6 Cells.mL^{-1}) at 20 °C (2 days) and maturated at 3 °C (3 weeks) and pascalised at 400 MPa (3 min), determined by HPLC-RID-DAD.

	Sample					
Compound (g.L^{-1})	**8°P Wort**	**SBL**	**PM1**	**PM2**	**KL**	**KM**
Glucose	7.3 ± 0.2	n.d.	5.3 ± 0.2	5.4 ± 0.1	5.4 ± 0.1	5.4 ± 0.1
Maltose	36.8 ± 0.5	24.1 ± 0.3	35.5 ± 0.3	35.1 ± 0.3	34.3 ± 0.3	35.5 ± 0.6
Glycerol	n.d.	1.2 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Citric acid	n.d.	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

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Acetic acid	n.d.	0.1 ± 0.0	n.d.	n.d.	n.d.	n.d.
Lactic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Abbreviation of beer sample corresponds to a yeast abbreviation used for beer production. SBL= *S. boulardii*, PM1 and PM2 = *P. manshurica* 1 and 2, KL = *K. lactis*, KM = *K. marxianus*. Values are presented as (Average ± Standard Deviation) from 3 parallel analyses. "n.d." = not detected.

3.7. Volatile Organic Compound Analysis by HS-SPME-GC-MS

Among the most important factors influencing the organoleptic quality of beer is the presence of higher alcohols, esters and carbonyl compounds. Our study revealed that during submerse fermentations, non-*Saccharomyces* yeasts of probiotic potential *Kluyveromyces lactis*, *K. marxianus* and both *Pichia manshurica* displayed potential in production of fermentation by-products, interesting from the brewer´s perspective, namely esters and higher alcohols.

The synthesis of higher alcohols via the Ehrlich pathway involves brewing yeasts absorbing amino acids from the wort, where the amino acids serve as carriers of essential amino groups that act as building blocks for forming yeast structural component after which the remains of amino acids (α-keto acids) are irreversibly converted to higher alcohols [54]. Increase of fermentation temperature strongly affects transport of amino acid into the yeast cell and thus favouring the increase of higher alcohol production [55]. As the formation of higher alcohols is temperature dependent, it also strongly influences final ester formation where higher alcohols are necessary for ester formation [56]. Our study revealed that yeasts *P. manshurica*, *K. lactis* and *K. marxianus* were able to introduce higher alcohols such as 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol into the beer, however concentrations of these alcohols were several times lower in comparison with the fermentation led by the probiotic strain *S. boulardii* (Table 7). Authors [14] worked with probiotic strain of *S. boulardii* described influence of main fermentation parameters (temperature, pitch rate, wort composition) on content of higher alcohols and esters and revealed that increasing fermentation temperature and pitch rate increases higher alcohol and ester formation.

Esters might be implemented into a beer during fermentation, acetate esters (ethyl, 2-phenylethyl and 3-methylbutyl acetate) and ethyl esters (ethyl hexanoate, octanoate and decanoate) [57]. The formation of acetate esters involves higher alcohols and the ethyl esters are formed by condensation reaction of ethyl alcohol and acyl-CoA [58]. Final concentration of esters in beer is closely related to composition of used wort and fermentation conditions [59]. Ethyl acetate and 2-phenylethyl acetate were the only ethyl esters detected in beers prepared in this study (Table 7). According to [60], yeast *K. marxianus* and *K. lactis* might produce increased concentrations of volatile organic compounds such as esters, higher alcohols during the fermentation process.

Positive POF^+ phenotype for all tested yeast (Table 2), was supported by the presence of 4-vinylguaiacol (clove aroma) in final beers (Table 7). Diacetyl (2,3-butanedione), an unwanted yeast metabolite (buttery aroma) was not detected in the final beers. These results favour in the use of novel potential probiotic yeasts which might tailor aromatic profile of final non-alcoholic beer (e.g. wheat style beers) in a positive way and boost its functionality as a novel beverage with health benefits.

Table 7. Concentration of VOCs (volatile organic compounds ($\mu g.L^{-1}$) of the beer samples prepared from 8°P Wort, fermented with tested yeasts ($1x10^6$ $Cells.mL^{-1}$) at 20 °C (2 days) and maturated at 3 °C (3 weeks) and pascalized at 400 MPa (3 min), determined by HS-SPME-GC-MS.

	Beer Sample				
Compound ($\mu g.L^{-1}$)	**SBL**	**PM1**	**PM2**	**KL**	**KM**
Ethyl acetate	530.5 ± 85.8	18.6 ± 6.7	24.4 ± 7.9	212.0 ± 6.3	373.8 ± 54.1
2-Phenylethyl acetate	73.1 ± 18.4	n.d.	n.d.	358.1 ± 7.1	166.7 ± 11.2
3-Methylbutyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.

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2-Methyl-1-propanol	981.4 ± 199	160.6 ± 26.5	983.5 ± 87.2	371.1 ± 92.7	212.4 ± 46.8
2-Methyl-1-butanol	2867.3 ± 66.7	363.5 ± 35.7	828.2 ± 13.1	803.5 ± 42.4	488.2 ± 50.0
3-Methyl-1-butanol	6125.8 ± 21.5	615.0 ± 57.1	1195.5 ± 38.5	992.8 ± 20.2	775.2 ± 48.9
2-Phenylethanol	7955.3 ± 163.1	1730.0 ± 220.4	2377.2 ± 78.9	1372.9 ± 93.2	1197.5 ± 112.1
Ethyl hexanoate	274.2 ± 21.6	162.9 ± 18.9	146.0 ± 7.6	139.0 ± 3.0	149.4 ± 7.6
Ethyl octanoate	307.5 ± 44.3	n.d.	n.d.	n.d.	n.d.
Ethyl decanoate	366.1 ± 91.6	n.d.	n.d.	n.d.	n.d.
Hexanoic acid	3855.8 ± 488.0	332.4 ± 30.3	304.3 ± 51.6	239.1 ± 15.0	274.7 ± 16.2
Octanoic acid	2736.1 ± 484.2	827.3 ± 140.6	524.4 ± 50.7	381.9 ± 23.8	448.1 ± 15.2
Decanoic acid	1420.8 ± 273.5	n.d.	n.d.	n.d.	n.d.
4-Vinylguaiacol	3033.9 ± 48.3	650.11 ± 46.4	628.10 ± 36.6	629.65 ± 44.7	681.30 ± 50.1
Butane-2,3-dione	n.d.	n.d.	n.d.	n.d.	n.d.

Abbreviation of beer sample corresponds to a yeast abbreviation used for beer production. SBL= *S. boulardii*, PM1 and PM2 = *P. manshurica* 1 and 2, KL = *K. lactis*, KM = *K. marxianus*. Values are presented as (Average ± Standard Deviation) from 3 parallel analyses. "n.d." = not detected.

3.8. Sensorial Evaluation of Beer Samples

Beer fermented with *S. boulardii* displayed sour and alcoholic character (Figure 4) with the noticeable notes of acetic acid with ethyl acetate. Throughout the beer fermentation process, *Kluyveromyces lactis* and *K. marxianus* yeasts were able to implement strong clove and burned apple-like aroma notes (phenolic) (Figure 4) into the beer which might be beneficial for producing wheat-based functional non-alcoholic beers. Both strains of *Pichia manshurica* had no negative impact on the final beer flavour profile, beers fermented with these two strains were very sweet with the notes of rose scent (floral) (Figure 4).

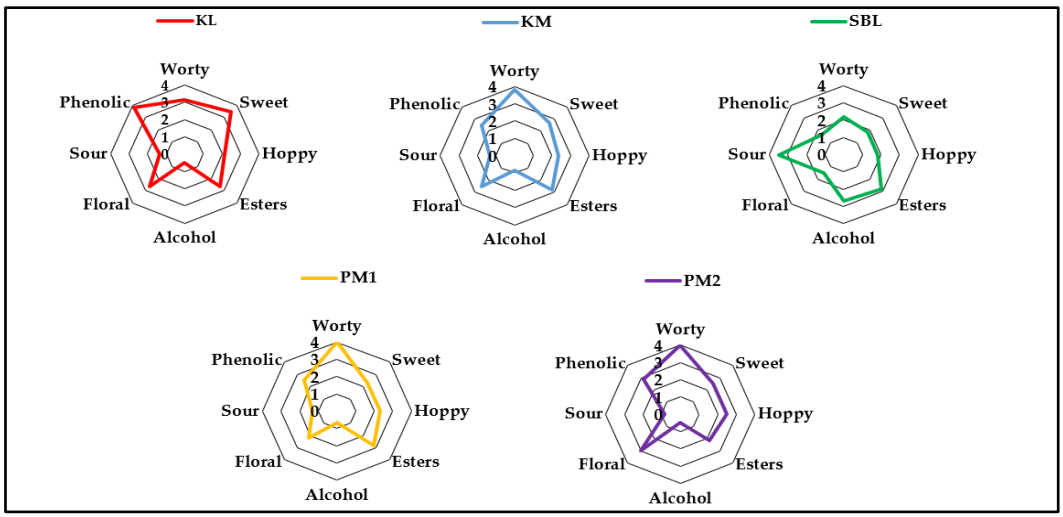

Figure 4. Sensorial evaluation of the beer samples represented as aromagram (radar chart). Abbreviation of beer sample corresponds to a yeast abbreviation used for beer production. SBL= *S. boulardii*, PM1 and PM2 = *P. manshurica* 1 and 2, KL = *K. lactis*, KM = *K. marxianus*. Each aroma attribute of the beer profile represents the mean score obtained from a panel of six evaluators. Scoring scale: was set from 0 (not perceptible) to 6 (strongly perceptible).

4. Conclusions

In recent years, rising interest in producing functional beers using yeasts with potential probiotic attributes in the beverage industry is taking place. In the presented study, we focused on the

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potentially probiotic yeasts *Kluyveromyces lactis*, *K. marxianus* and *Pichia manshurica* and their application in the non-alcoholic beer (NAB) production also using *Saccharomyces boulardii* as a control probiotic strain. The characterisation of yeast strains demonstrated the survival in the simulated conditions of the human digestive tract (human body temperature and stomach acidic pH) after 24 hours. On top of that, tested yeasts were able to ferment the beer matrix (wort), sustained different IBU (iso-α-bitter acid concentrations) as a sole fermentation culture with targeted conditions to produce NABs. Stabilisation of beer achieved inactivation of yeast, but the yeast cells remained intact – which might serve for functionality of the beer as postbiotics. The final NABs prepared using non-*Saccharomyces* potential probiotic yeasts *K. marxianus*, *K. lactis* and *P. manshurica* (first time used in the brewing) showed a potential in tailoring final beer sensory profile by producing higher alcohols (2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol, 2-phenylethanol,) and esters (ethyl acetate and ethyl hexanoate), and no acetic acid, making them a suitable alternative to the commercially available probiotic yeast *S. boulardii*. All tested yeast strains exhibited production of 4-vinylguaiacol (clove) which was supported by a POF^+ phenotype suited for wheat style beer. Sensorial analysis of the final non-alcoholic beers fermented with *K. marxianus, K. lactis* and *P. manshurica* revealed their sweet character (residual saccharides) with the notes of clove and rose aroma traces. This work provides insights into further applications in functional beer production using novel non-*Saccharomyces* potential probiotic yeast strains.

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