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The Role of Adjunct Milled Rice, Barley Malt, and different Koji Variations in Alcoholic and Non-Alcoholic Beer Production

This study explores the feasibility of using adjunct milled rice in brewing non-alcoholic beer (NAB) traditionally made with barley malt. By substituting barley malt with varying proportions of rice the impact on wort quality and fermentation performance were assessed. Practically, increasing rice content required adjustments, such as adding rice husks for filtration and pre-gelatinising the rice. Despite these modifications, wort characteristics, including original gravity, remained suitable for NAB production. Fermentation with *Saccharomycodes ludwigii* at 16 °C revealed that higher rice content led to slightly increased alcohol levels (up to 0.45 % v/v when using 100 % rice) within the trials and faster fermentation times (15 days down to 5 days) until a steady ethanol content was reached. Sensory evaluation indicated that higher rice content shifted the flavour profile from "worty" to a more “vanilla” and “buttery” profile. Additionally, Koji (rice inoculated with some species of *Aspergillus*) was tested as a substitute for barley malt and exogenous purified enzymes. Yellow Koji had higher α-amylase activity, and surpassed white Koji in terms of original gravity and alcohol content. However, Koji-only worts were challenging to filter, suggesting the need for further optimisation. Overall, the integration of adjunct milled rice and Koji offers potential for reducing production time and enhancing flavour profiles in NAB brewing, though practical applications require additional refinement.

Descriptors: non-alcoholic beer, *Saccharomycodes ludwigii*, rice, grain, adjunct, Koji

1 Introduction

In recent years, consumer preferences have notably shifted towards healthier lifestyle choices, leading to an increased consumption of beer with reduced alcohol content [1]. Non-alcoholic beers or beverages are typically defined as having less than 0.5 % ABV [2–4], while "alcohol-free" denotes the complete absence of alcohol [2]. Germany, the leading NAB consumer in the EU [1], saw production volumes rise from less than two million hectolitres in 2005 to 4.8 million hectolitres by 2022 [5]. The increasing popularity and economic significance of NAB are also observed in North and South America and other continents [6]. The production of non-alcoholic beers employs sophisticated techniques to maintain flavour integrity while minimising alcohol content and involves precise management to enhance both mouthfeel and flavour complexity. Despite the low alcohol levels, non-alcoholic beers strive to replicate the taste, aroma, and mouthfeel of traditional beer styles. There are primarily two approaches for producing non-alcoholic beer: a microbiological method that suppresses ethanol production during fermentation [6–8] and a physical method that removes ethanol from fully fermented beer [4, 8]. Additionally, hybrid techniques combining different methods can be used to closely mimic the flavour profile of their alcoholic counterparts in non-alcoholic beers [4]. The production of NABs encompasses a wide range of engineering options for optimising the brewing process. This flexibility makes NAB brewing an excellent field for investigating the use of different starch-containing raw materials.

Moreover, the increasing prevalence of drought and heat periods is having a detrimental effect on global yields of major crops, including those crucial for brewing [9, 10]. Additionally, international conflicts are further reducing the availability of brewing raw materials [11], exacerbating the need for adaptive strategies in NAB production. This highlights the need for innovative approaches in NAB production to mitigate the impact of climate change on the availability and cost of raw materials. By exploring alternative starch sources to barley, the NAB industry can enhance resilience and sustainability in the face of environmental challenges.

Rice (*Oryza sativa* L.), a staple crop of global importance, ranked as the third most produced crop in 2023/2024, with a production totalling 513.54 million metric tons [12]. Rice offers positive aspects for brewing such as being a gluten-free cereal with a dry matter

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composition of approx. 70 % starch, 5 – 8 % protein, 0.2 – 2.2 % oil and some inorganic compounds [7, 13, 14]. In the United States, rice production in 2023 amounted to 218,291 thousand centum weight (cwt), equivalent to approximately 11,090 metric tons [15]. From a more local perspective, Arkansas leads rice production within the United States, producing 106,968 centum weight (cwt) in 2023, which translates to approximately 5,434 metric tons [16].

Recent research has demonstrated that rice malt can be effectively utilised in the brewing of various alcoholic products [13, 14, 17, 18]. Research by *Guimaraes* et al. [14] has confirmed the suitability of rice for malting, highlighting its brewing quality attributes. Guimaraes et al. [14] showed enhanced limit dextrinase and the self-saccharifying power in most of the rice malts they studied, underscoring the enzymatic capabilities of rice during malting. Similarly, *Mayer* et al. [13] explored the production of an all-rice malt beer without relying on added enzymes, highlighting the potential for utilising inherent enzymatic activity in rice malt. However, given the resource-intensive nature of traditional malting processes in terms of cost and energy, incorporating adjunct milled rice with exogenous enzymes represents a promising avenue for advancing NAB production techniques. This approach not only offers potential cost savings but also introduces opportunities to explore other natural enzyme sources beyond commercially available purified enzymes. Such innovations could significantly impact the efficiency and sustainability of the mashing process in brewing.

From an enzymatic perspective Koji can offer an opportunity in producing NAB with a high portion of rice. In Asia the use of Koji in producing beverages and food has a long tradition [19]. There are three main types of Koji in use for producing alcoholic beverages in Asia a) yellow Koji (*Aspergillus oryzae*) used for Sake production b) white Koji (*Aspergillus luchuensis* var. *kawachii*) used for the production of Shochu as well as black Koji (*Aspergillus luchuensis* mut. *awamori*) also for Shochu brewing [19, 20]. Koji moulds synthesise a variety of enzymes, including cellulase, protease, amylase, and lipase, which decompose complex grain structures to acquire nutrients necessary for their growth. These degradation products subsequently serve as essential nutrients for yeast in subsequent brewing processes, promoting alcoholic fermentation, while the limited vitamins produced by Koji function as growth-enhancing factors [19–21]. Current research of *Lee* et al. [21] shows the strength of Koji in combination with sorghum to replace 30 % of barley malt in producing alcoholic beer.

This manuscript aims to analyse the brewing potential of locally grown Arkansas rice (ARoma 22) for NAB production by increasing the proportion of adjunct milled rice in the brewing grist bill from 0 % – 100 %. The fermentation process of the NABs were carried out using *S. ludwigii* [7]. Physical-chemical and sensory characteristics of the final worts and beers were evaluated. Overall, the potential benefits and challenges of using adjunct milled rice, specifically ARoma 22, as a supplement in NAB brewing is highlighted. As the proportion of adjunct milled rice increases, the use of exogenous enzymes becomes essential to break down starch and other biomolecules, supporting fermentation and supplying yeast with necessary nutrients. In light of recent insights, the potential of utilizing white or yellow Koji as an alternative enzyme source in beer production was also explored.

2 Materials and methods

2.1 Brewing process

NAB production was performed by increasing the proportion of adjunct milled rice (ARoma 22; Delaplaine Specialty Rice, Delaplaine, AR, USA) in the brewing grist bill from 30 % – 100 %. The brews were carried out on a 4 vessel (2 heating vessels, 2 mash vessels) 75 L Ss Brewtech (Wildomar, CA, USA) plant producing 30 L of cooled wort. The Ss Brewtech Grain Mill was used in roller setting 0 to mill the barley malt (American Pale Two-Row; Rahr Malting Co., Shakopee, MN, USA) and roller setting -2 for milling the rice. The rice was pre-gelatinised (rice:water ratio of 1:4) in one heating vessel. The rice pre-gelatinisation was done by mashing in at 70 °C (adding 0.1 ml/kg total grist Termamyl®SC 4X, Novozymes A/S, part of Novonesis Group, Bagsværd, Denmark) followed by a heating up to 90 °C with a rest of 20 minutes. After the rest the pre-gelatinised rice was transferred to the second mashing vessel. The main grist as well as unprocessed rice husks (10 % of total grist bill – increasing lauter ability) were mashed into 74 °C water (1:4 ratio, adding 0.05 ml/kg total grist Ultraflo®Prime, Novozymes). After adding all raw materials and the pre-gelatinised rice the final rest temperature of 72 °C was reached. After combining the mash its pH was adjusted to 5.40 (± 0.10) using concentrated lactic acid (88 %; BSG, Shakopee, MN, USA). Temperature for mashing was hold for 60 min. At the end the saccharification was tested by applying the iodine method (0.02N iodine; Merck KGaA, Darmstadt, Germany).

The lautering and sparging was done until a kettle full concentration of 6.5 °P was reached using an Anton Paar EasyDens with Brew Meister Software (iOS version 4.5.0; Anton Paar GmbH, Graz, Austria) for checking the concentration. When the boiling started, CTZ hops (14.2 % α-acid; BSG Hops, Wapato, WA, USA) was added to reach a final bitterness of 20 bitterness units (BU). Furthermore, 1 g/L yeast nutrient (mixture of diammonium phosphate and food-grade urea; LD Carlson Company, Kent, OH, USA) was added at the beginning of boil. After boiling for 60 min the final gravity (7.0 °P ± 0.2) and pH (4.65 ± 0.05) was adjusted. While starting to rotate the hot wort one Whirlfloc tablet (Kerry group P.L.C, Tralee, Ireland) was dosed. After 15 min of whirlpool rest the wort was cooled in a single stage heat exchanger and transferred to the Ss Brewtech Unitanks (volume 53 L). Prior to pitching 2 g/hL Kerry Biomatex L (alpha-acetolactate decarboxylanase) and 2 g/hl Brewers Clarex® (DSM Food Specialties B.V., Delft, Netherlands) were added. Fermentation was performed with *S. ludwigii* at a pitching rate of 5 million cells/mL (WLP618; White Labs Inc., San Diego, CA, USA) at 16 °C until reaching final ethanol concentration (0.40 % v/v ± 0.05 % v/v). The beers were kegged (19.5 L stainless steel KEG) and pasteurised in boiling water to achieve 400 PU. After cooling, the beers were carbonated and stored at 4 °C until serving for sensory evaluation.

2.2 Physical-chemical analysis

Beer analyses were conducted using standardised methods outlined by the European Brewery Convention (EBC) [22]: density, original gravity, apparent and real extract (EBC 9.4; EBC 9.43.2) as well as alcohol content (EBC 9.2.6) and ASBC standard methods [23] Beer Method 9. pH (Hydrogen Ion Concentration), Beer Method 10. Color, and Beer Method 23. Bitterness has been applied. Analysis of free amino nitrogen (FAN) was carried out at the Hartwick College Center for Craft Food & Beverages (Oneonta, New York, USA) using the ASBC standard methods Wort 12. Free Amino Nitrogen (International Method) [23].

Table 1 **Starch containing raw material base parameters. Data of carbohydrate concentration, α- amylase (U/g), and acid protease (U/g) amount as stated by accompanying certificate**

	Yellow Koji	White Koji	Barley Malt	Rice
Carbohydrate [%]	80 – 90	75 – 85	78 – 80	> 75
α-amylase [U/g]	≤ 1000	≤ 60	70.5 (Unit – DU)	N/A
Acid Protease [U/g]	≤ 2000	≤ 15,000	N/A	N/A
Moisture [%]	9.53	12.28	9.80	11.00

Table 2 **Starch and enzyme containing raw material amounts used in micro mashing trials. YK = yellow Koji trial; WK = white Koji trial**

Trial name	Ref	Barley/ Rice	YK1 resp. WK1	YK2 resp. WK2	YK3 resp. WK3	YK4 resp. WK4	YK5 resp. WK5
Barley Malt [%]	100	50	50	50	50	0	0
Rice [%]	0	50	37.5	12.5	0	50	75
Koji [%]	0	0	12.5	37.5	50	50	25

For quantifying the sugar profiles of glucose, fructose, maltose, and maltotriose a high-pressure liquid chromatography (HPLC) method was adapted and optimised by the use of different application notes [24, 25]. The analysis was performed on a Waters ArcHPLC (Waters, Milford, MA, USA) equipped with a Waters Sample Manager, Quaternary Solvent Manager-R, column heater, and was coupled to a Waters Acquity QDA. The column installed was a Rezex ROA-Organic Acid H+ (8 %), 300 mm x 7.8 mm x 8 µm (Phenomenex, Torrance, CA, USA), heated to 65 °C, guarded by a SecurityGuard™ cartridge Carbo-H-4 x 3.0 mm ID (Phenomenex), 0.1 % formic acid at 0.4 mL/min was used as the mobile phase (40 min total runtime). Sample injection consisted of 0.1 µL. Calibration curve was performed from 50 to 1500 ng/µL for all compounds.

2.3 Sensory

Descriptive sensory was performed according to previously published sensory references [26, 27]. The panel consisted of ten judges (aged 19 – 38 years; 3 female, 7 male) who were trained in a single session on the following descriptors: a) aroma/flavour (worty; cereal; corn/dimethyl sulphide/cooked vegetables; buttery, vicinal diketones; hoppy; vanilla; phenolic/medicinal/Band-Aid/plastic), b) taste (sweetness intensity and aftertaste; sourness intensity and aftertaste; bitterness intensity and aftertaste), and c) mouthfeel (palate fullness/viscosity; mouthcoating/linger; metallic). The substances and concentrations used for panel training are detailed in supplemental table 1.

The sensory evaluations were conducted at the Food Science Department of the University of Arkansas over two days. The study protocol (IRB project number 2309492758) was exempted by the internal regulatory board. For testing, 30 mL of each sample was poured into a 5.5 oz Brandy tasting snifter (Acopa Corp., Miami, FL, USA), covered with plastic lids (Walmart, Bentonville, AR, USA), labelled with a random three-digit code, and left at room temperature for 60 minutes to warm from 4 °C to 20 °C. Data collection was performed using custom-designed Qualtrics Expert-Designed Templates (Qualtrics LLC, Provo, Utah, USA). For data analysis, XLSTAT statistical and data analysis software (Lumivero, New York, USA) was used.

2.4 Micro Mashing and bench top fermentations for Koji experiments

The potential of using white or yellow Koji as enzyme sources in NAB production were assessed. In brief, the laboratory milling was done using a cyclone sample mill with a 0.25 mm screen (Model 3010-080P; UDY Corporation, Fort Collins, CO, USA). The basic analytical data on the brewing raw materials (i.e. Rahr premium pale-2 row barley malt, rice, and white/yellow Koji) is reported (Table 1). The amounts of each used for each trial are shown (Table 2). The laboratory performance of the Koji (1-70A - "yellow" / MKS-S – "white" Koji; Iida Trading Co., Ltd., Osaka, Japan) were assessed using the standardised methods as described in Mitteleuropäische Brautechnische Analysenkommission (MEBAK) "extract content in adjuncts – method with malt addition" [28] followed by the "congress mash procedure" [29]. Trials were carried out for white and yellow Koji each in duplets with no further exogenous enzyme addition. Moisture content was determined using American Society of Brewing Chemists (ASBC) Method Malt 3. Moisture [23]. For getting information on the gelatinisation temperature of the raw materials differential scanning calorimetry (DSC) had been used with same instrumentation and under equal conditions as published by Guimaraes et al. [14].

Bench-top fermentations were carried out by using 100 ml of congress wort in 500 ml Erlenmeyer flasks with air locks. Fermentation was carried out 24 h (using magnetic stirrers for agitation) at 25 °C by adding 2 g of yeast (SafAle™ S-04, Fermentis, Marquette-lez-Lille, France).

2.5 Data collection and statistical data analysis

For data collection Microsoft Excel (Microsoft 365, Microsoft Corporation, Redmond, Washington, USA) was used. The data analysis was done using XLSTAT statistical and data analysis software (Lumivero, New York, USA).

3 Results and discussion

3.1 NAB production – Brewing and Fermentation

One objective of this study was to demonstrate how increasing

Table 3 **Basic chemical parameters of the wort brewed for NAB fermentation**

	Original gravity [% w/w]	Free amino nitrogen [mg/L]	Iodine normality [Yes/No]
100 Barley Malt	6.71	121.17	Yes
70 Barley Malt/30 Rice	6.82	99.35	Yes
50 Barley Malt/50 Rice	6.88	92.91	Yes
30 Barley Malt/70 Rice	6.96	74.84	Yes
100 Rice	7.15	48.85	No
Measurement precision	0.18[$]	n/a	n/a

[$] Measurement precision based on RSD or %RSD from 12-fold repeat analysis of a non-alcoholic beer (ethanol < 0.5 % v/v)

proportions of adjunct milled rice can be processed in a similar manner to 100 % barley malt. Practically, as the proportion of barley malt decreases, so does the quantity of barley husks. Husks serve as a filtration aid during lautering. To compensate for the reduced barley husks in the treatments with a higher proportion of rice, 10 % unprocessed rice husks were added [18]. Rice has been shown to contain antioxidants such as phenolic acids [30]. Therefore, rice hulls were added to all brews to ensure there were no unaccounted flavour impacts. In general, no issues were observed with lautering the NABs made with higher proportions of rice.

Generally, when considering basic beer chemistry, the brewing trials produced comparable results in wort production, including original gravity (OG), sugar composition, and wort yield (Table 3, Fig. 1). All the worts ended in comparable OG. The NAB made with 100 % barley malt had the lowest OG (6.71 % w/w), while the 100 % rice NAB had the highest OG (7.15 % w/w). Previous research exploring various potential yeast strains for non-alcoholic beer production, including *S. ludwigii*, has demonstrated adequate control over ethanol concentration, maintaining levels below 0.5 % ABV when the OG ranged between 6.0 – 8.1% wt/wt [6, 7]. Therefore, the OG in this study was ideal for producing NABs with *S. ludwigii*.

Notably, the NAB produced with 100 % rice was challenging to brew (Table 3). The wort did not become iodine normal during mashing. Therefore, the mashing process for 100 % rice treatment was slightly adjusted. As there was no barley malt addition, after pre-gelatinisation (rice water ratio 1:4) the pre-mash was cooled with water to 72 °C. After reaching this temperature the rice husks were added in a similar manner to the other trials. However, at this temperature, in addition to adding 0.05 ml/kg total grist Ultraflo® Prime, an extra 0.1 ml/kg total grist Termamyl® SC 4X and 0.06 ml/kg total grist Attenuzyme® Key (Novozymes) were added to facilitate starch breakdown. Amyloglucosidase (also glucoamylase) can help to break down amylose and amylopectin resulting in more fermentable sugars [31]. Therefore, it should be used with great caution in NAB production to avoid generating excessive low-molecular-weight sugars, which could lead to alcohol concentrations exceeding 0.5 % v/v.

The temperature activity profile of Attenuzyme® significantly decreases when used above 60 °C [31]. To assist with only limited breakdown of starch a higher then recommended temperature range was used. The amounts of glucose (18.7 g/l) and fructose (9.5 g/l) in the 100 % rice treatment (Fig. 1) were higher compared to the other brews. This indicates that the addition of glucoamylase resulted in variation in the carbohydrate spectrum and indicated that residual enzymatic activity was present at this elevated temperature. As mentioned this can be problematic because *S. ludwigii* is able to metabolise these simple sugars into alcohol [32]. Despite this, the sugar distribution percentage aligns with findings from previous studies [6]. Crucially, to prevent ethanol production, the predominant sugar in these worts should be maltose, as *S. ludwigii* does not effectively metabolise this disaccharide.

From a yeast nutrition perspective (i.e. amino acids / free amino nitrogen "FAN") it is important to mention that the overall protein content of rice is lower compared to barley and barley malt [14]. Therefore, changing the amount of barley malt by supplementing with rice should also affect the FAN content, which is a crucial nutrient for yeast. As expected, the 100 % barley brew presented with the highest FAN content (121.17 mg/L) while all rice wort only presented with 48.85 mg/L (Table 3). This is in agreement with how brewers have used adjuncts like rice in the past [33], but it is an important reminder that rice can be used to dilute protein

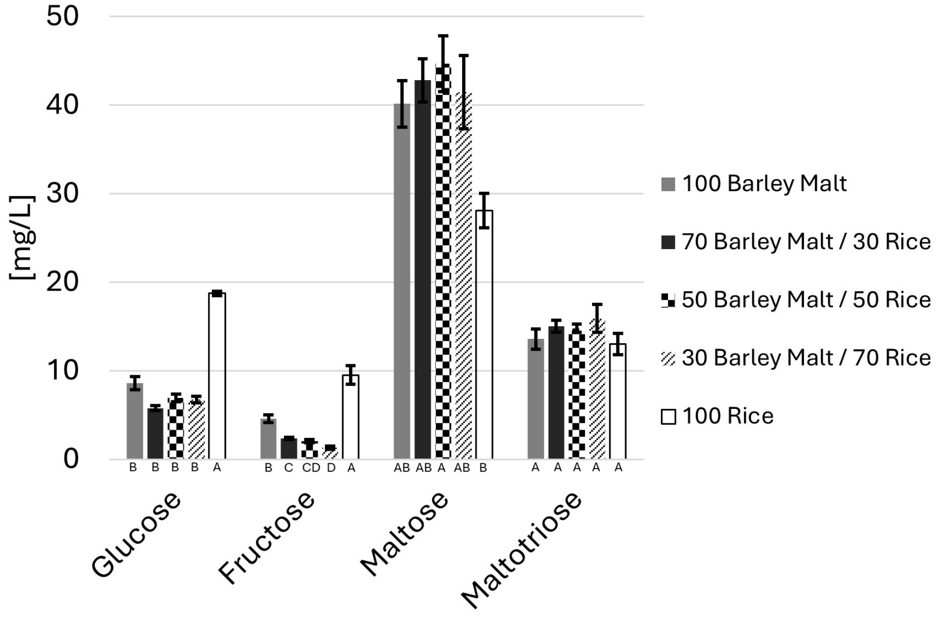

Fig. 1 **Sugar composition distribution of glucose, fructose, maltose and maltotriose per each wort produced. Error bars are expressed as standard errors. Letters under bars are indicating LS mean groupings based on Tukey (HSD). Significant differences had been shown on Glucose ($p < 0.05$) and Fructose ($p < 0.05$) while no significant differences were observed for Maltose ($p = 0.056$) and Maltotriose ($p = 0.246$)**

Table 4 **Physical-chemical basic characterisation of the NABs brewed**

	Alcohol [% v/v]	Apparent extract [% w/w]	Apparent degree of fermentation [%]	Bitterness [BU]	Colour [EBC]
100 Barley Malt	0.38	6.30	10.42	21	10.8
70 Barley Malt/30 Rice	0.33	6.17	9.44	21	4.8
50 Barley Malt/50 Rice	0.43	6.05	11.88	20	3.9
30 Barley Malt/70 Rice	0.34	6.28	9.34	22	4.1
100 Rice	0.45	5.07	14.68	20	3.3
Relative standard deviation (RSD) [%]	0.85[$]	0.12[$]	0.85[$]	0.00[&]	0.00[&]

[$] Measurement precision based on RSD or %RSD from 12-fold repeat analysis of a non-alcoholic beer (ethanol < 0.5 % v/v)

[&] Measurement precision expressed as average standard error of all samples

and FAN given the impact of climate change on barley protein levels. When German brewers emigrated to the USA in the 19th century, they encountered six-row barley, which had a higher protein content compared to the two-row barley they used in Europe, making it difficult to brew beers that met the preferences of American consumers. To manage this excess protein and produce clearer, more stable beers, they began using adjuncts like rice to dilute the protein and FAN levels. This approach remains significant today as climate change is increasing barley protein content [34], further complicating protein management and necessitating the continued use of adjuncts like rice to maintain beer stability and clarity. Given that the yeast used in this investigation is a non-maltose metabolising strain with limited fermentation capabilities, the FAN amount in the produced worts was adequate to achieve the target ABV concentration (i.e. 0.3 – 0.5 %) even with 100 % rice [32].

The fermentation of all the worts as described in section "Materials and methods – Brewing process" was conducted at 16 °C, a temperature that falls within the ideal range for the yeast's fermentation spectrum as indicated by the supplier [6]. The resulting beers exhibited comparable basic physical-chemical data related to fermentation (Table 4). All beers showed alcohol concentrations lower than 0.5 % v/v and can in turn be classified as non-alcoholic [2–4]. This suggests that the non-normal iodine staining observed in the 100 % rice brew may be attributed to other sources. Like barley, rice starch is composed of amylose and amylopectin; however, their chemical structures and iodine affinities differ [35]. Without a sufficient amount of limit dextrinase from the barley malt or exogenous addition, the result will be an incomplete starch breakdown. Since Attenuzyme® Key was added at a temperature of 72 °C, its activity decreased over the first few minutes of mashing, leading to inactivation and insufficient cleavage of α-1-6 glycosidic bonds. As a result, it is hypothesised that the remaining α-1-6 bonds contribute to the iodine staining, as the iodine affinity of rice amylopectin is higher than that of barley amylopectin [35]. From a brewing and fermentation perspective, the use of *S. ludwigii* in this study did not present any challenges related to non-iodine normality in wort, as the fermentation performance was satisfactory for NAB production (Table 4). However, when employing alternative yeast strains under similar production and raw material conditions, this outcome may not be as predictable. Variability in strain behaviour could potentially lead to complications, particularly regarding the non-iodine normality of the wort, which may impact the overall fermentation efficiency and beer quality with different yeast strains.

Notably, rice percentage in the grist also significantly affects colour (Table 4). The 100 % barley malt NAB had a colour of 10.8 EBC. Adding just 30 % rice reduced the colour by a factor of 2.25. This pale colour introduced by rice in NAB production presents new opportunities for producers to create NABs with distinct colours. A recent study by Guimaraes et al. [14] reported intriguing visual wort colours (golden-reddish) from pigmented rice, which could be a viable option for NAB brewing without the need for food colouring agents.

Interestingly from a cellar management perspective increasing rice amounts significantly decreased the time of fermentation (Fig. 2). This effect was most pronounced in the 100 % rice brew and can be attributed to the amyloglucosidase and the fermentable sugars present as there is a significant higher amount of glucose and fructose in this wort. Even though significance was not that clear for maltose (p = 0.056) the concentration is lower in the 100 % rice brew. Nevertheless, the NAB brewed with 70 % rice and 30 % barley malt had a shorter fermentation time compared to those with a higher malt content. The carbohydrate spectrum in the wort was largely similar across the brews (Fig. 1). It can also be observed that, starting from the addition of 30 % rice, the amounts of glucose and fructose are reduced compared to the 100 % barley malt brew (Fig. 1). As a result, the yeast does not require as much time to metabolise these sugars, reaching the final degree of apparent fermentation more quickly. The reduced fermentation duration can be further explained by the increased availability of fermentable sugars. The OGs are comparable (Table 3) as are the alcohol concentrations (Table 4). Literature indicates a minimum of approximately 130 mg/L FAN is required for satisfactory fermentation in full-strength wort (OG 10 – 12 % w/w) and underscores the differing nitrogen uptake across various yeast strains [36]. Another study on NAB production using different yeast strains found varying FAN metabolism across the strains, starting with a FAN concentration of 110 mg/L [32]. Our results show, considering the FAN contents (Table 3), that the free amino nitrogen amount was satisfactory and not limiting

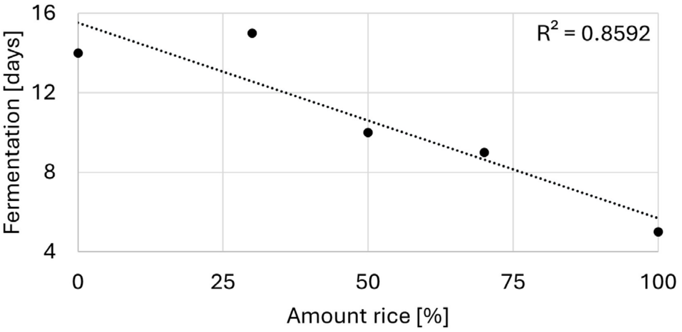

Fig. 2 **Correlation of rice amount used [%] and fermentation performance [days until stable ABV was reached] of the NAB trials**

even when the concentration drops to 48.85 mg/L in the 100 % rice brew. Although FAN concentrations should not be overstated due to the addition of yeast nutrients (diammonium phosphate and food-grade urea) in all trials, the results still highlight the positive impact of rice in brewing NABs. Rice can reduce production and fermentation time, ultimately increasing productivity when use as a supplemental raw material in NAB brewing.

3.2 Sensory evaluation

When tasting NABs, one of the main descriptors for products brewed with microbiological techniques, particularly using *S. ludwigii*, is the attribute "worty" [6, 26, 37]. Research has established a clear link between "worty" impressions and the aldehydic profile of NABs. Specifically, methional (3-Methylsulfanylpropanal – associated with a cooked potato aroma) is identified as a primary driver of worty impressions [38]. The aldehydic and worty characteristics in NABs, as well as in beer generally, are influenced by the raw materials, particularly the malt used [39]. Malt production involves Maillard reaction products that contribute to the aldehyde content in malt, wort, and the final beer. By using unmalted rice, these aldehyde compounds can be reduced in NABs made with variations of barley malt and rice.

Although this publication does not discuss the aldehydic profile of the beers, the sensory analysis indicates that rice has an impact on the flavour profile of the NABs produced (Fig. 3). As illustrated in figure 3, the sensory profile of the 100 % barley malt NAB is characterised by descriptors such as “worty”, “cereal”, and “metallic”. As the amount of rice in the NABs produced increases, the sensory profile shifts significantly in the principal component analysis (PCA) from the bottom right corner, which is associated with wort-like attributes, to the upper left corner, driven by more “vanilla” and “buttery” impressions. Meanwhile, the NAB with 50 % barley and 50 % rice, as well as the one with 30 % barley and 70 % rice, are more described as hoppy in the performed sensory analysis. This movement indicates a substantial sensory change, with the 100 % rice beer positioned at the complete opposite end of the spectrum from the 100 % barley malt beer. This shift highlights a clear sensory difference, suggesting that increasing rice content alters the sensory profile of the NABs. Moreover, the 100 % rice NAB is more strongly associated with descriptors like “vanilla” and “buttery”, evident in both ortho- and retronasal impressions. ARoma 22 rice is an aromatic rice variety that was expected to yield this type of flavour based on a high amount of 2-acetyl-1-pyrroline [40] which is often linked to a “popcorn” like flavour [41]. It is believed that the panel identified a “popcorn” flavour and associated it with “buttery” and “vanilla” impressions, as these were the descriptors most closely matching those used in the sensory evaluation. This is an important point because often brewers add rice and want to not have an impact on flavour.

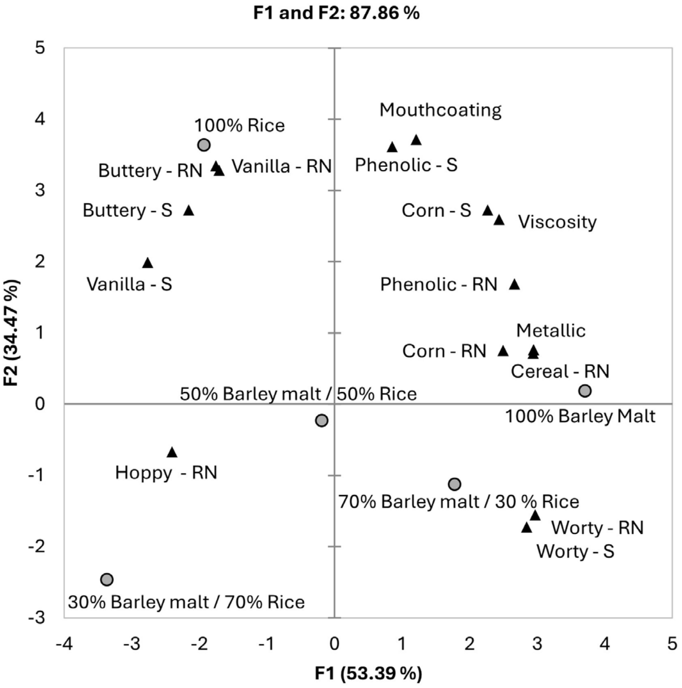

Fig. 3 **Principal component analysis showing the significant sensory results of the NABs (grey circle) and the aroma and flavour ratings (black triangle). The abbreviation “S” is showing results from smelling the sample while “RN” represents the retronasal findings**

Consumer preference studies have shown that NABs with pronounced worty characteristics are less favoured, while those with more hop-forward profiles receive higher ratings [26]. Also, significant work has been done to develop techniques to remove this flavour [42]. However, the current work shows that as wort-related impressions decrease by increasing rice percentages, other sensory attributes become more prominent, with “hoppy” being one such attribute. Despite maintaining consistent hop additions and BU results (Table 4), the higher hop ratings associated with increased rice amounts can be attributed to the way rice positively influences (decrease) covering flavour impressions. Overall, the shift away from wort-like sensory profiles with increased rice content clearly demonstrates the positive impact of rice on the sensory attributes of NABs. This change suggests that incorporating higher rice portions in brewing NABs can result in a more desirable flavour profile, enhancing overall product appeal and a good cost alternative to more downstream methods to remove wort character.

3.3 Koji as a beer brewing enzyme source

In the production of a fully rice based NAB, using adjunct milled rice poses a challenge due to its insufficient enzymatic potential for complete starch breakdown (see above). The lack of necessary enzymes means that adjunct milled rice alone cannot effectively convert its starches into fermentable sugars required for successful fermentation and desirable product characteristics. Additionally, for individuals with celiac disease, the use of barley malt is not an option due to its gluten content. This condition necessitates the exploration of alternative brewing methods and ingredients that avoid gluten while still ensuring the production of a high-quality NAB.

Currently, in beer production, when using high amounts of unmalted starch-containing raw materials, brewers typically employ purified enzymes to facilitate various biomolecular breakdowns [43]. However, another option can be the use of Koji. This enzyme-containing raw material has been used in Asian cultures for thousands of years [19]. Another benefit of Koji is that, while it can grow on different grains, rice is the most commonly used [19, 21], making it a perfect ingredient for gluten-free, natural based beer production.

Table 5 **Mashing trial worts produced with different amounts of barley malt (B), rice (R), yellow Koji (YK) and white Koji (WK). Analytical results are showing fructose – FRU / glucose – GLC / maltose – MAL / maltotriose – MTR, the original gravity – OG and free amino nitrogen – FAN. Variation expressed as standard errors. Superscript letters on the OG are indicating LS mean groupings based on Tukey (HSD) ($p < 0.05$)**

Trial	B [%]	R [%]	YK [%]	WK [%]	FRU [g/L]	GLC [g/L]	MAL [g/L]	MTR [g/L]	OG [% w/w]	FAN [g/L]
Ref	100	–	–	–	3.9 ± 0.2	13.6 ± 0.7	111.0 ± 5.3	24.0 ± 1.2	8.42^{CD} ± 0.00	211.37
Malt/Rice	50	50	–	–	2.1 ± 0.0	10.7 ± 0.1	74.2 ± 1.7	19.6 ± 0.5	9.07^{AB} ± 0.00	103.41
YK1	50	37.5	12.5	–	3.4 ± 0.1	12.2 ± 0.2	71.7 ± 1.2	19.5 ± 0.1	9.22^{A} ± 0.01	118.21
YK2	50	12.5	37.5	–	3.1 ± 0.2	17.1 ± 0.6	68.8 ± 1.0	18.5 ± 0.2	9.41^{A} ± 0.02	144.14
YK3	50	–	50	–	3.9 ± 0.3	18.0 ± 0.9	70.6 ± 0.7	18.9 ± 0.1	9.53^{A} ± 0.00	157.17
YK4	–	50	50	–	4.7 ± 0.4	12.2 ± 1.0	41.2 ± 0.1	11.8 ± 0.1	6.83^{F} ± 0.28	68.96
YK5	–	75	25	–	0.6 ± 0.0	16.4 ± 0.1	32.4 ± 1.2	20.5 ± 0.2	7.41^{D} ± 0.02	41.76
WK1	50	37.5	–	12.5	11.9 ± 0.6	10.5 ± 0.5	42.7 ± 0.3	26.8 ± 0.2	9.42^{A} ± 0.05	198.02
WK2	50	12.5	–	37.5	12.6 ± 1.1	10.7 ± 0.2	26.0 ± 0.2	9.5 ± 0.0	8.56^{CD} ± 0.01	259.92
WK3	50	–	–	50	13.2 ± 0.6	9.9 ± 0.9	19.3 ± 1.3	5.3 ± 0.3	8.38^{CD} ± 0.02	278.21
WK4	–	50	–	50	13.9 ± 0.7	13.0 ± 1.3	19.1 ± 0.5	4.4 ± 0.1	8.67^{BC} ± 0.06	163.03
WK5	–	75	–	25	11.9 ± 0.3	10.5 ± 0.4	17.4 ± 0.2	4.3 ± 0.1	8.10^{E} ± 0.00	123.03

Micro mashing trials demonstrated the effectiveness of different Koji varieties (white and yellow) in starch breakdown compared to a barley malt reference, which used the same standard 2-row barley as in previous brewing trials. It is important to mention, that the congress mash method was applied in these trials [29]. Consequently, the congress wort does not perfectly meet the requirements for producing NABs, particularly concerning the sugar spectrum, as this method is optimised for the temperature ranges suitable for starch degradation in standard alcoholic beer brewing. Nonetheless, this section provides an initial glimpse into the potential opportunities that Koji can offer in the brewing of beer. Additional bench-top fermentations further emphasise the unique aspects of each Koji variety in brewing beer (Fig. 4).

Overall, maltose was the predominant sugar produced in all the trials (Table 5). From a brewing perspective, this is advantageous, as maltose is the primary carbohydrate required for producing alcoholic beers. From an NAB perspective, using *S. ludwigii*, this is also a positive outcome, as it results in a majority of non-fermentable sugars for this yeast strain, leading to low or no alcohol concentrations [6, 7, 32].

Another important observation is that when replacing 50 % of barley malt with rice, the OG increases and, consequently, the capacity of the brewing process is increased (Table 5). In addition, the overall OG is further increasing when yellow Koji is added to the barley malt and rice grist load. This can be linked to the higher carbohydrate amount of this ingredient (Table 1). Overall, the trials show that using only Koji and rice, without barley malt, results in the lowest OGs. This highlights the positive extract yield from a mixture of barley and rice, with Koji serving as an additional enzyme source. At this stage, while it remains speculative, the original gravity (OG) demonstrates a significant increase based on the Tukey HSD statistical analysis. The data presented in Table 5 suggest that the optimal combination for maximising OG consists of approximately 20 – 25 % yellow Koji, 12.5 % white Koji, and rice, particularly when 50 % of barley malt is substituted. This blend appears to effectively enhance the overall OG. Among the Koji varieties used, yellow Koji outperforms white Koji in terms of extract and sugar composition (Table 5). This is due to the significantly higher overall amount of α-amylase in yellow Koji compared to white Koji (Table 1). In all trials involving white Koji, there was a shift in the sugar spectrum from maltose to higher amounts of fructose and glucose. From a NAB production perspective using *S. ludwigii*, white Koji might be undesirable since the yeast can metabolise these monosaccharides [32].

The final congress worts were fermented in bench-top trials using S-04 yeast (laboratory standard procedure). However, this

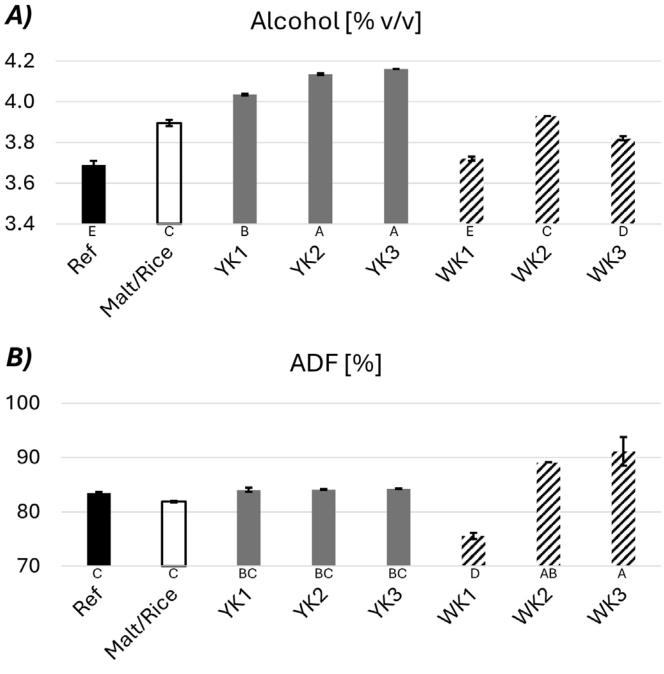

Fig. 4 **Results of final alcohol content (A) and apparent degree of fermentation (ADF - B) of the congress wort bench-top fermentation trials. Ref – 100 % barley malt / Malt = barley malt / YK – yellow Koji / WK – white Koji, error bars are expressed as standard error. Letters under bars are indicating LS mean groupings based on Tukey (HSD) ($p < 0.05$)**

procedure was not possible for the rice and Koji-only trials (YK4 / WK4 and YK5 / WK5) due to the non-filterability of the congress mash. For these trials filtration resulted in only 50 ml of wort, which was barely sufficient for basic wort analysis. The use of Koji and rice for producing gluten-free NABs needs to be adjusted and further investigated. The Koji used is described as "pre-gelatinised," meaning that the rice was steamed before inoculating it with Koji moulds [19, 21]. However, upon further investigation using DSC the gelatinisation temperature of the yellow Koji was 55.4 °C while the white Koji presented 57.4 °C. When mixed with pre-gelatinised rice, the resulting mash was sticky and highly viscous. During the congress mash procedure, this viscosity decreased significantly due to enzymatic reactions. However, the final wort remained non-filterable, preventing fermentation from being carried out. From a brewing perspective, this represents a major drawback of using only rice and Koji. Technologically, adding rice husks to the mash could improve filterability and help avoid issues in the lauter tun or mash filter [18] but this was not attempted in this study.

Compared to a 100 % barley malt reference, most Koji ferments resulted in higher alcohol concentrations after 24 hour forced bench top fermentations with S-04 (Fig. 4A), and varying ADF (Fig. 4B). The yellow Koji achieved the highest alcohol production, which can be attributed to the higher OG of the worts (Table 5). Additionally, the concentration of fermentable sugars is greater in the yellow Koji worts than in the white Koji worts, leading to higher alcohol levels in the yellow Koji trials. In contrast, the ADF increased with higher amounts of white Koji in the grist load. As the white Koji exhibits higher protease activity (Table 1), the amount of FAN is also greater in all worts produced with white Koji compared to those produced with yellow Koji or no Koji (Table 5).

As previously shown increasing the rice content in the grist bill leads to a decrease in free amino nitrogen (FAN). With barley malt alone, 211.37 mg/L FAN was detected. However, when substituting 50 % of the barley malt with rice, FAN levels drastically decreased to 103.41 mg/L. This reduction can be attributed to the lower protein content of rice compared to barley malt [14] and a lower proteolytic activity as the rice had been unmalted and pre-gelatinised (boiled for 30 min before mixing with barley malt [28]). This is further evidenced by the low amounts of FAN in the YK4, YK5, WK4, and WK5 trials, which did not include any barley malt. Since the white Koji exhibits higher proteolytic activity (Table 1), the amount of FAN is greater in all worts produced with white Koji compared to those produced with yellow Koji or no Koji at all. These FANs are essential yeast nutrients that contribute to faster fermentation. Consequently, the increase in the apparent degree of fermentation (ADF) observed in the trials (Fig. 4 B) can be attributed to the higher FAN levels provided by the white Koji. Reducing the amount of ARoma 22 rice in the white Koji trials led to increased FAN levels, surpassing those in the barley-only wort (WK2 – 259.92 mg/L and WK3 – 278.21 mg/L). This impact of the Koji used is not observed in the yellow Koji trials (YK2 – 144.14 mg/L and YK3 – 157.17 mg/L).

Since amino acids and FAN are essential yeast nutrients during fermentation [36], using Koji can help balance the reduction in FAN that occurs when the amount of barley is decreased. As these trials were conducted under laboratory conditions (e.g., 25 °C fermentation temperature, 24 hour fermentation, no pressure, 100 ml wort, and ale yeast), it is advisable to adjust these parameters in future studies to better investigate the brewing potential of Koji. Based on the initial results, a combination of yellow and white Koji may offer the best opportunity to achieve high OG, resulting in higher ethanol concentrations, and improved fermentation performance by adjusted FAN concentrations.

4 Summary

As the brewing industry faces potential issues with raw material supply [9–11] in the coming years, it must reconsider product design. Additionally, changes in well-being and alcohol consumption trends are expected to drive an increase in NAB sales [1, 6]. These factors highlight the importance of exploring the potential of rice as a raw material in NAB production. The data presented demonstrates the positive effects of using increasing amounts of adjunct milled rice in brewing. A two-stage mashing process can be employed to pre-gelatinise the rice and make the starch accessible to enzymes. Although increasing the rice content leads to a decrease in enzymatic potential, this can be addressed by using exogenous enzymes. The worts produced showed comparable basic results (Table 3), with expected decreases in FAN concentrations as barley malt was reduced. Despite the lower FAN levels, fermentation was not negatively impacted. In fact, increasing rice content was found to shorten fermentation time, which is advantageous for increasing production volumes (Fig. 2). No sensory defects related to fermentation were detected, and the overall beer ratings indicated a reduction in wort-like characteristics when rice was used, which aligns with consumer preferences for NABs [26].

The issue of abnormal iodine tests in 100 % rice NABs was not resolved in this study, and adjustments to exogenous enzymes did not yield satisfactory results. To address this, alternative enzyme sources were investigated. Koji (Aspergillus spp.), a traditional brewing ingredient in Eastern cultures [19–21], was explored for its potential in biomolecule breakdown. Two Koji varieties (yellow and white) were evaluated for their degradation abilities. Mini mashing trials revealed differences in carbohydrate and FAN profiles (Table 5), with yellow Koji showing higher maltose and maltotriose levels, beneficial for NAB production, while white Koji resulted in higher glucose and fructose levels with increased FAN. This makes white Koji a good candidate for brewing 100 % (non) alcoholic rice beers.

In summary, rice demonstrates promising potential for brewing NABs, offering benefits in both brewing efficiency and flavour enhancement. For optimal starch conversion and yeast nutrition without the need for additional supplements, a blend of yellow and white koji may provide the most effective solution, although further trials are required to assess the influence of koji combinations on overall beer quality.

Furthermore, labelling beers (including NABs) made exclusively with rice can be challenging due to regulatory issues related to ingredient usage (e.g. Sake production [19]) and local labelling practices. In some regions, such products may not be allowed to be labelled as "beer," and terms like "fermented cereal" or "grain-based product" could be more appropriate.

Disclosure statement

The authors report there are no competing interests to declare.

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Supplemental material:

Supplemental Table 1 **Standards for panelists training for sensory attributes evaluated and their recipes**

Sensory/Aroma attribute	Recipe of standard used
Worty	Congress wort using 100 % munich malt in accordance to: MEBAK online. Method R-206.00.002. Congress Mash Method. Rev. 2016-03. Mitteleuropäische Brautechnische Analysenkommission (MEBAK®) e.V., Freising, BY, Germany.
Cereal	2 g Cheerios® (General Mills, Minneapolis, MN, USA)
Corn / dimethyl sulfide / cooked vegetables	30 g of canned cooked corn (Great Value Golden Sweet Whole Kernel Corn, Walmart Inc., Bentonville, AR, USA)
Buttery / vicinale dicetone	2,3-Butandiol (purity 97 %, Merck KGaA, Darmstadt, Germany) in a 20 mL glass vial with screw cap
Hoppy	Myrcene (purity technical grade, Merck KGaA, Darmstadt, Germany) in a 20 mL glass vial with screw cap
Vanilla	8 g/L vanillin (purity 99 %, Merck KGaA, Darmstadt, Germany) in water
Medicinal/ Band-Aid®/ plastic	1 cm x 2 cm piece of Band-Aid® (Johnson & Johnson Consumer Inc., New Brunswick, New Jersey, USA) in a 20 mL glass vial with screw cap
Taste	
Sour	6 g/L 88 % Lactic acid (LD Carlson Co., Kent, OH, USA)
Sweet	12 g/L household sugar (Walmart Inc., Bentonville, AR, USA)
Bitter	45 mg/L 30 % iso-extract (ISO 30 %, Hopsteiner, Mainburg, Germany)
Mouthfeel	
Thin body	Water
Palatefullness / viscosity	20 g/L yeast (SafAle™ S-04, Fermentis, Marquette-lez-Lille, France) in water
Metallic	90 mg/L iron (New Spring Valley vitamins, Philadelphia , Pennsylvania, USA) in water
Cleansing water	
Carbonated pectin water	1 g/L Sure jell premium fruit pectin (Kraft Heinz Foods Company, Chicago, IL, USA) in water at ~ 25 psi with CO_2