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Wild yeasts of Styria – Two yeast species isolated from a spontaneous fermented wild ale in Styria and their co-fermentation characteristics

Two different species of yeast were isolated from a spontaneously fermented and barrel-aged ale, brewed in Styria (Austria). To achieve this, a simple and robust method was implemented to isolate the yeasts and to screen their most essential fermentation characteristics. Both species, which were identified as *Pichia fermentans* and *Candida boidinii* by MALDI-TOF analysis, were isolated and incubated successfully on Sabouraud Gentamicin Chloramphenicol 2 agar (SGC 2). In a fermentation experiment, which was repeated three times, the co-fermentation characteristics of both the yeast species were compared to batches with mixed fermentation, inoculated with the complete microbiome of the original wild ale. For this purpose, a wort based on 100 % Pilsener malt (Weyermann), with 10 °Plato original gravity, was produced and adjusted with one bittering hop addition of Herkules (16.2 % alpha acid) to a total of circa 20 IBU. The batches, containing 4 litres of inoculated wort each, were incubated for 28 days at a temperature of 25 °C and 70 % of relative Humidity in a constant climate chamber. Although the yeast fermented batches and the batches fermented with the complete microbiome showed a similar apparent degree of attenuation of approximately 70 % at the end of the experiment, the pH-value of the microbiome-fermented batches was lower. Additionally, the microbiome-fermented batches were more turbid than the yeast fermented batches, whereas the yeast fermented batches clarified remarkably in comparison. Taking into consideration that both *P. fermentans* and *C. boidinii* are ubiquitous yeast species and have not often been a focus of research on unconventional brewing yeasts, these results may offer new perspectives on possible future applications of these yeast species. Additionally, the introduced practical approach can be implemented by small-scale wild ale breweries with reasonable effort.

Descriptors: wild yeast, spontaneously fermented beer, non-conventional yeasts, mixed fermentation, *Pichia fermentans, Candida boidinii*

1 Introduction

Spontaneous fermentation is the fermentation without the intentional addition of fermenting organisms or enzymes. As far as beer is concerned, spontaneous fermentation can involve a variety of yeast species and a wide range of different bacteria, which in most instances are considered contaminants in conventional breweries and wineries.

The interactions of these different organisms during fermentation are complex and may include antagonistic, synergistic, and mutualistic interdependencies.

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Hence, spontaneously fermented beers like traditional Belgian Lambic are based on the successful establishment of a whole microbiome and its metabolic activities and products. There is a delicate balance between the fermentation by-products, namely alcohols, acids, and esters, which must remain within certain concentration limits to render the final product drinkable.

Historically, it is impossible to reconstruct where and when exactly spontaneous fermentation became intentional fermentation.

According to *Liu* et al., the history of (spontaneously fermented) beer-like beverages may have begun with the Natufian culture, based on archaeological findings dating to 13,000 years ago, which were excavated at the Raqefet cave in Israel [1]. These findings were followed by an extended debate about whether it was truly a beer or only the by-product of grain milling and the baking of bread [2–5].

Liu et al. based their argumentation mainly on residues found in stone mortars, such as starch granules from wheat, barley and

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other Poaceae [1]. Referring to this, Eitam, for instance, states that Liu et al. may have indeed overinterpreted mortar-like stone devices used for grain milling as mashing and fermentation vessels [1, 2]. The author comprehensibly deduces why the purpose of these stone mortars was more likely the dehusking and milling of grain only and that the intention of the Natufians thereby was rather to prepare dough to bake bread [2].

This statement notwithstanding, processed cereals can also go into fermentation without the involvement of humans, as Eitam additionally points out [2].

And this is where the intention of most modern brewers of spontaneously fermented beers comes full circle. For these brewers the quintessence of brewing traditional Lambic beers and comparable spontaneous brews is the minimisation of human involvement in the fermentation and maturing process whilst ensuring a consumable final product [6, 7].

This also implicates an extensive biodiversity of contributing microorganisms, dependent on the environmental factors of the fermentation site. In this respect, each brewery with a focus on spontaneously fermented beers features a unique collection of yeasts and bacteria with different traits and metabolic properties [8].

So, if the specific and unique local microbiome of a brewery (-environment) also becomes a unique selling proposition, it seems reasonable to further investigate the composition of this microbiome.

In this research article, we present a local case study of a spontaneous brew by a brewers' collective in Styria (Austria), in which we combined yeast hunt methods such as those proposed by Hutzler, basic methods of microbiology and simple fermentation experiments to gain insights into the traits of the main protagonists [9].

In cooperation with the MOSA culture collective, the brewers' collective, which is referred to, the following research questions were determined:

Which yeast species can be isolated from the spontaneous fermented wild ale?

What are their basic fermentation characteristics with regard to extract degradation, acidification and attenuation?

What are the most distinct differences in fermentations by the present yeasts when compared to fermentations facilitated by the whole present microbiome?

It should be mentioned in advance that the present study can only be considered as preliminary. Our main objective is to provide a practical guideline and a simple, robust, and cost-effective approach for small-scale breweries with spontaneous brews to isolate, categorise and catalogue their most important microorganisms. However, in the best case it may support the initial impulse of a long-term initiative to collect, isolate and identify wild yeast species of Styria (Austria) and to assess their potential in brewing.

2 Material and methods

The wort of the spontaneous brew where the initial sample was derived from consisted of 60 % barley malt and 40 % unmalted wheat, adjusted to 13 °P and approximately 20 IBU with aged hops from organic farming. The intention of the brewers thereby was to create their individual interpretation of a Lambic, or to be more precise, a Gueuze based on regionally available resources. Since the term Lambic enjoys legal protection within the EU by the Commission Implementing Regulation (EU) 2017/2216 of 1 December 2017, the blended and finally bottled beverage was labelled Gueuze Style Ale [10].

The sampled vintage was brewed in October 2021. The wort was cooled by a plate heat exchanger, mixed with residual amounts of the previous vintage and then transferred into an old barrique (225 L), which was deposited in the wine cellar of a Demeter-certified biodynamic winery, (Wein von Ploder Rosenberg, Unterrosenberg 86, 8093, Austria).

The wort was fermented there at ambient temperature. The cellar was neither heated during the winter months nor cooled in summer.

450 millilitres of sample were taken twice from the barrique with sterile pipettes and a sanitised food grade silicon tube and transferred into two autoclaved graduated PYREX® 1 L round media storage bottles each, which contained 450 ml of buffered peptone water (BPW, derived from bioMérieux Austria GmbH). This was done to regenerate microorganisms which are difficult to cultivate because of special nutrient requirements, but which are likely to be present in the beer. These Pyrex bottles served as stock solution and reserve sample, respectively. Both were stored at 6 °C until

Table 1 HPLC apparatus used for determining the amino acids (automated precolumn OPA derivatisation)

Apparatus	Manufacturer
HPLC	1260 Infinity II LC Systems (Agilent Technologies Österreich GmbH, Austria)
HPLC pump	1260 Infinity II Quaternary Pump (Agilent Technologies Österreich GmbH)
Detector	1260 Infinity II Diode Array Detector HS (Agilent Technologies Österreich GmbH)
Precolumn	UHPLC Guard 3PK, InfinityLab Poroshell HPH C-18 (Agilent Technologies Österreich GmbH)
Column	Poroshell HPH-C18 Column (Agilent Technologies Österreich GmbH)
Column thermostat	1260 Infinity II Multicolumn Thermostat (Agilent Technologies Österreich GmbH)
Autosampler	1260 Infinity II Vialsampler
OPA Reagent (conc.) o-phtaldialdehyde and 3-mercaptopropionic acid in borate buffer (5061-3335)	Agilent Technologies (USA)
FMOC Reagent (conc.) 9-fluorenylmethylchloroformate in acetonitrile (5061-3337)	Agilent Technologies (USA)

Table 2 Eluents and composition

Eluent	Composition
A	10 mM Na_2HPO_4
	10 mM NA_2B4O_7
	5 mM NaN_3
	Adjusted to pH of 8.2 with HCl 37 %, p. a.
B	45 % (v/v) acetonitrile
	45 % (v/v) methanol
	10 % (v/v) water

Table 3 HPLC eluent gradients

Time	Eluent A %	Eluent B %	Flowrate [ml/min]
0	98	2	1.5
0.35	98	2	1.5
13.4	43	57	1.5
13.5	0	100	1.5
15.7	0	100	1.5
15.8	98	2	1.5

further use in the refrigeration cell.

2.1 Isolation of yeasts

To isolate the yeasts, a fivefold serial dilution was prepared, initially diluting 1 ml of sample with 9 ml of BPW. 100 microlitres of each dilution was then evenly spread with a Drigalski spatula on Sabouraud Gentamicin Chloramphenicol 2 agar plates (from bioMérieux Austria GmbH). SGC media consist of peptone (10 g/L), glucose (20 g/L) and agar (15 g/L). In addition, SGC media contain two antibiotics, Gentamicin (0.04 g/L) and chloramphenicol (0.5 g/L), which both exhibit antimicrobial effects against aerobic and anaerobic gram-negative bacteria [11]. Gentamicin inhibits the protein synthesis in bacteria by affecting mRNA and tRNA interactions in bacteria, whereas chloramphenicol shows bacteriostatic effects by affecting the peptidyl transferase and thus protein synthesis [12–14]. SGC media are standard media in clinical microbiology, used to isolate yeasts and filamentous fungi from microbiomes consisting also of bacteria susceptible to gentamicin and chloramphenicol [15]. 100 µl of each dilution, again, were evenly spread on Columbia agar + 5 % sheep blood (COS, from bioMérieux Austria GmbH), a general culture medium used in clinical microbiology for bacteria of special nutrient requirements, as a control [16, 17]. The isolation steps were conducted under laminar flow. The plates were then incubated at 30 °C for 96 hours in an incubator and photographically documented afterwards.

Colonies of both the SGC2 medium and the COS medium were investigated macroscopically and using light microscopy (EVOS M5000 Imaging System, Thermo Fisher Scientific). Two macroscopically and microscopically different yeast colonies were identified and sent to the Research Center Weihenstephan for Brewing and Food Quality for species-identification by MALDI-ToF Mass Spectrometry after email correspondence with Dr.-Ing. Mathias Hutzler.

Table 4 Applied methods in the analysis of the samples

Method [20–22]	Application
MEBAK 10.11.4.2	Determination of cfu/ml
MEBAK 2.9.1	Degassing of samples
MEBAK 2.9.2.3	Original Gravity [% w/w], Extract [% w/w]
MEBAK 2.9.6.3	Alcohol by volume [% vol.], Apparent and Real degree of Attenuation [% w/w]
MEBAK 2.13	pH-value
MEBAK 2.26.1.5*	CO_2 concentration [g/L]
MEBAK 2.14.1.2	Haze/Turbidity (EBC formazin units)
MEBAK 2.6.4.1.2**	Determination of Amino Acids in Wort and Beer (sample preparation)
Steed (2010) [11]***	Determination of Amino Acids in Wort and Beer (HPLC analysis)

*Modifications in sample preparation method were necessary due to the biofilm formation in the microbiome-inoculated batches

**Modifications were necessary due to the available lab equipment

***Internal method

The colony forming units (cfu) of yeasts were determined by counting the colonies of five of the plates of the 10^{-1} and five plates of the 10^{-2} dilution. The open-source digital image processing software ImageJ, from *Schneider* et al., was used to count the colonies and determine the total viable cell count of the original sample [18].

The calculated mean of cfu was related to the initial beer before the very first dilution step (1 : 1, 450 ml of sample and 450 ml of BPW).

2.2 Fermentation experiments

Wort was prepared from 100 % Pilsener Barke® malt (Mich. Weyermann® GmbH) to assess the essential fermentation characteristics of the isolated yeasts. The malt was conditioned with water (2 % w/w of total grist) and crushed with a two-roller mill (Maltman 55, Egon Sommer Maschinenbau GmbH), adjusted to a crushing gap of 0.7 mm. A descendent single-temperature infusion mash was prepared in a 36 L brew-in-a-bag setup according to [19]. Thereto, 6 kg of malt were pre-mashed in an insulated and preheated stainless-steel pot in 6 litres of hot tap water at a temperature of 66 °C, resulting in a pre-mash temperature of approximately 62 °C. The pre-mashed malt was then added subsequently in two

Table 5 Selection of wild yeasts of the genera Candida and Pichia in context with spontaneous fermentation, beverages or food and the respective references

Yeast species	References
Candida boidinii	[25, 29]
Candida ethanolica	[30]
Candida fructus	[30]
Candida zemplinia	[31, 32]
Pichia fermentans	[33]
Pichia kluyveri	[30, 33]
Pichia membranifaciens	[8, 29]

Fig. 1 Overview of experimental design

portions within 10 minutes to 12 L of brewing water at 75 °C with continuous stirring, targeting a final mash temperature of 65 °C with a grist to water ratio of 1:3. The pH-value of the mash was adjusted to 5.6 with lactic acid E270. The mash was kept at 65 °C for 60 minutes and afterwards tested for complete saccharification with iodine solution. The decision to use solely Pilsener malt was made to reduce variable components in the experimental design and to improve reproducibility. The wort was adjusted to approximately 10 °P and 20 IBU with hop pellets type 90 of the variety Herkules (16.2 % alpha, BarthHaas GmbH & Co. KG). Four glass carboys of a total volume of 5 L were autoclaved and then filled with four litres of wort each. One glass carboy was inoculated with yeast subsequently, whereas the other three glass carboys were inoculated with the whole microbiome at the same time. The four carboys were placed in a WTB-Binder climatic chamber and fermented at a preset temperature of 25 °C with 70 % relative humidity for 28 days. 25 ml of samples were taken from each batch regularly on day 0, 1, 2, 3, 7, 14, 21 and day 28 after the inoculation with yeast and microbiome, respectively. Samples were taken with sterile pipette tips, using an Powerpette Plus pipette controller. The samples were collected in 50 ml sterile conical centrifuge tubes and the sample temperatures were then measured subsequently with a Pt-100 temperature probe.

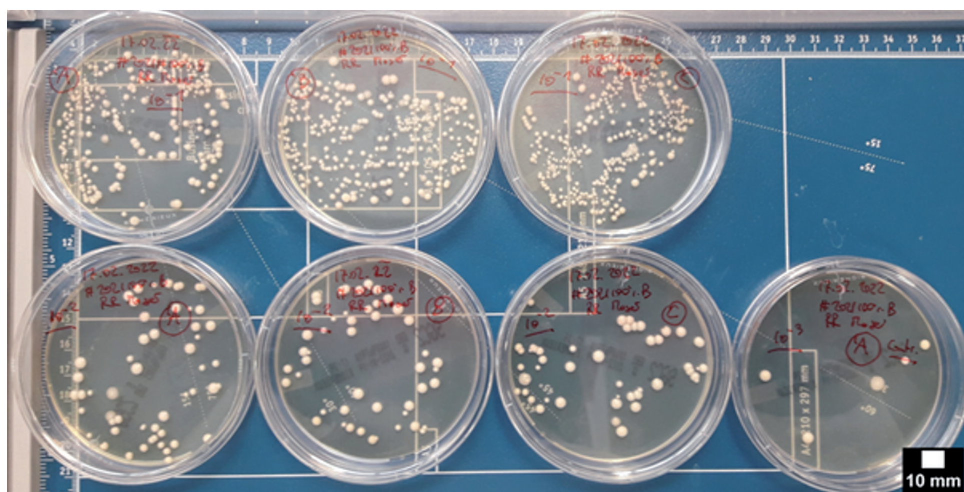


Fig. 2 Serial dilutions ranging from 10^{-1} to 10^{-2} and 10^{-3} of the original wild ale sample, showing yeast colonies on SGC2 plates

After the samples were degassed, the extract and pH-values were measured with a DMA 35 Portable density meter (Anton Paar GmbH) and an Aqualytic®

AL10pH pH-meter, simultaneously recording the sample temperatures (Pt-100). Samples for the amino acid determination by HPLC analysis were collected simultaneously, here again, sterile pipette tips and 50 ml sterile conical centrifuge tubes were used.

The fermentation experiments were repeated three times, providing one triplicate of yeast-fermented batches and three triplicates of microbiome-fermented batches in total.

Eventually, after the fermentation assay was completed, one yeast sample and one microbiome sample per fermentation experiment were randomly selected and 250 ml of sample volume each were analysed at the Sudhaus Anton Paar with the PBA-B Generation M modular beer analysis system, coupled with CarboQC, DMA 4500 M and Alcolyzer 3000 Beer (Anton Paar GmbH). These final measurements were conducted to verify the calculated apparent degree of attenuation, alcohol by volume and the pH-values. Additionally, turbidity (EBC), and residual extracts, apparent and real, were determined. Regarding the final measurements, adaptations of the sample preparation were necessary. Batches which were inoculated with the complete microbiome showed signs of biofilm formation and were pronounced ropey. Hence, all samples collected for the final measurements were centrifuged in capped 50 ml sterile tubes at 5.000 rpm for 15 minutes and 4 °C. Because the biofilm would affect the measurability of the sample, centrifugation was necessary, despite the loss of CO_2 in the process. Hence, both samples, collected from yeast-inoculated batches (YiB) and from microbiome-inoculated batches (MiB), were treated the same way. To this end, YiB was also centrifuged using the same parameters.

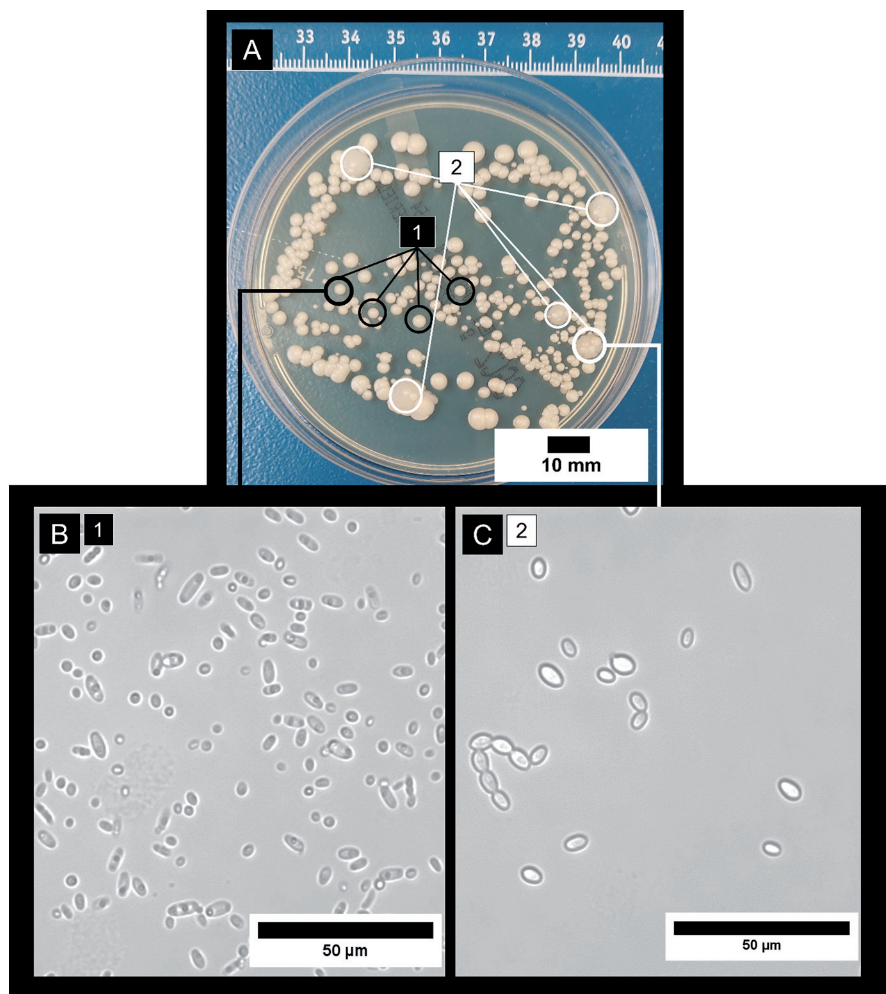


Fig. 3 Isolated and identified yeast from the spontaneously fermented Styrian wild ale. Two yeast species were originally isolated and could be distinguished macroscopically (A) and microscopically (B): *C. boidinii*, forming colonies smaller in diameter (1), and *P. fermentans*, forming larger and irregular-shaped colonies (2)

2.2.1 Preparation of the yeast inoculation cultures

The yeast inoculation culture (YIC) was prepared as follows: All visible colonies of three SGC2 plates of the 10-1 dilution were harvested and resuspended in 50 ml of BPW in a previously autoclaved wide-neck Erlenmeyer flask of 100 ml total volume. The wide-neck Erlenmeyer flask then was subsequently covered again with the aluminium foil used for the autoclavation of the flask. The YIC was resuspended in an orbital shaker-incubator for at least 3 hours. 50 ml of YIC (one wide-neck Erlenmeyer flask) was used to inoculate one batch of wort (4 L).

2.2.2 Preparation of the microbiome inoculation cultures

Initially, the same procedure as used for the yeast inoculation culture was intended to be used for the microbiome inoculation culture (MIC), but due to inhomogeneous growth of cultures on COS, the method was adapted. 10 ml of the stock solution originally sampled from the barrique was transferred into 40 ml of BPW in previously autoclaved wide-neck Erlenmeyer flasks of a total volume of 100 ml. The flasks were then covered again with aluminium foil and the MIC was incubated for 96 h in the orbital shaker. The aim of this methodical approach was to preserve the composition of the original microbiome as far as possible. Although the conditions may not be considered ideal for all microorganisms contained in the sample, this seemed the most adequate compromise available at the time. 50 ml of MIC (one wide-neck Erlenmeyer flask) was used to inoculate one batch of wort (4 L).

2.3 HPLC analysis of amino acid assimilation

An internal method of amino acid analysis by HPLC was tested in a preliminary experimental setup. This method is similar, but not identical to MEBAK 2.6.4.1.2 and was established inhouse to analyse amino acid profiles of insect larvae from Tenebrio molitor [20, 21]. The main objective was to test this internal routine application for its suitability in the amino acid analysis of wort and beer. Hence, only amino acid concentrations of the first experimental trial (one batch inoculated with yeast, three batches inoculated with the complete microbiome) were analysed.

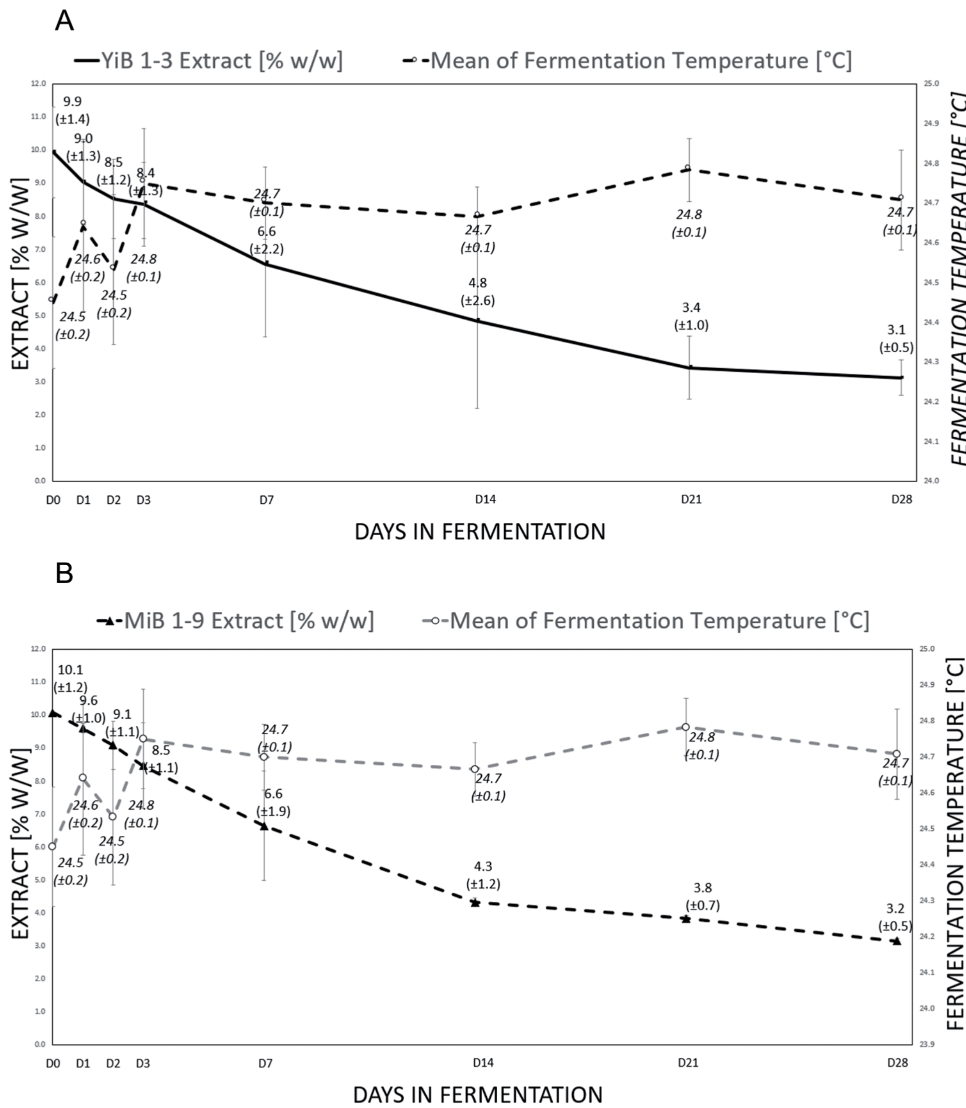


Fig. 4 **Fermentation curves. Shown are the mean values and ± SD (N = 3) of the yeast inoculated triplicate batches YiB (A) and the microbiome inoculated batches MiB (B), mean ± SD (N = 9) and the fermentation temperatures of all batches (n = 12)**

The concentrations of amino acids were also determined in the original wort by HPLC analysis, using automated pre-column OPA derivatisation. The used apparatus of HPLC is listed in table 1.

The samples were prepared where possible according to MEBAK 2.6.4.1.2, but adaptations, such as centrifugation, were necessary due to the requirements of the used equipment [20]. Additionally, an automated precolumn OPA derivatization method of HPLC, according to Steed [20, 21], was applied. Therewith the OPA solution was automatically diluted and applied to the sample vials (see also Table 1). After degassing in the ultrasonic bath (Sonorex Super, RK 103 H, derived from Bartelt Gesellschaft mbH) and adding 600 µl of internal standard DL-norvaline (analytical standard, Merck KGaA) to 400 µl of wort and beer samples respectively, the samples were stored for 30 minutes in a refrigerator at 7 °C and then centrifuged in the Centrifuge 5804 R (Eppendorf AG) at the available maximum of 11.000 rpm for 15 minutes. The eluents A and B were prepared as shown in table 2.

Both eluents were filtered through regenerated cellulose membrane filters of 0.45 µm pore size. The HPLC operating conditions showing the gradient of both eluents is given in table 3.

10 µl of the sample were injected. The column heater temperature was set to 40 °C. The samples of the first trial were

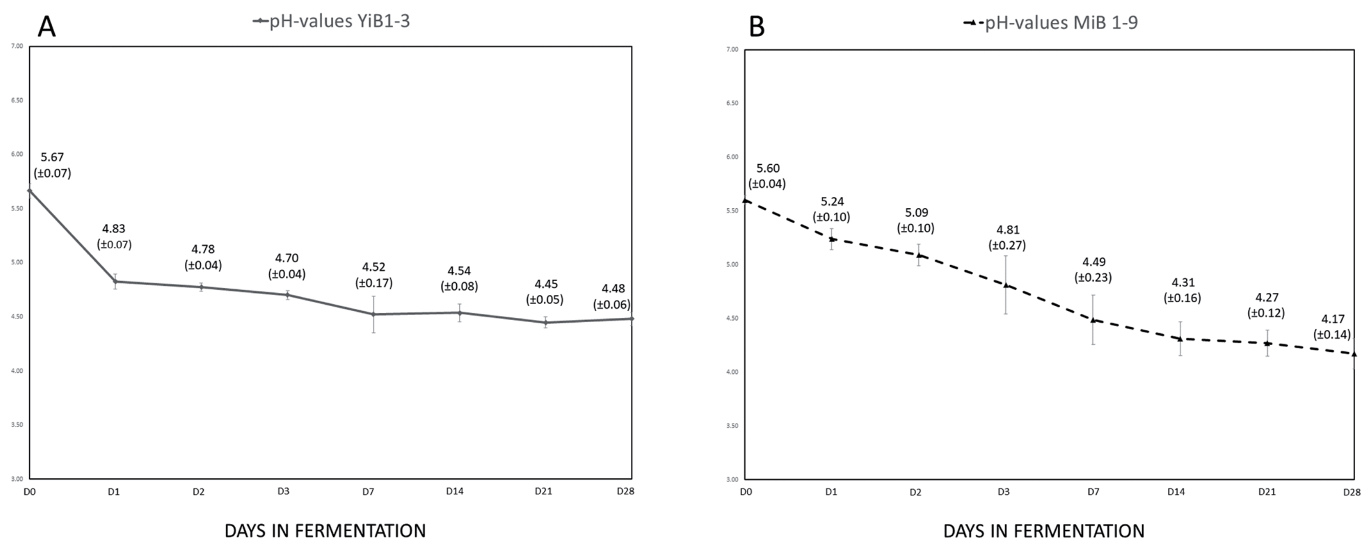


Fig. 5 **Changes in the pH-values of YiB (A), shown are mean values ± SD (N = 3) and MiB (B) during fermentation Shown are mean values, ± SD (N = 9)**

Table 6 Results of the final PBA-B measurements after the end of the fermentation experiments (mean values ± SD, n = 3)

	Alcohol by vol. (% v/v)	Apparent extract (% w/w)	Real extract (% w/w)	Apparent attenuation (% w/w)	Real attenuation (% w/w)	CO_2 conc. [g/L]	Turbidity (EBC)
YiB I-III	3.86 (± 0.55)	3.13 (± 0.47)	4.54 (± 0.66)	70.04 (± 0.86)	57.81 (± 0.62)	1.27 (± 0.08)	2.85 (± 0.91)
MiB I-IX	3.78 (± 0.39)	3.12 (± 0.45)	4.50 (± 0.56)	69.79 (± 1.93)	57.59 (± 1.49)	0.33 (± 0.13)	13.22 (± 0.99)

analysed by HPLC, comparing the amino acid assimilation in the yeast inoculated wort and the microbiome inoculated wort.

All HPLC grade amino acid reference standards, buffers and chemicals were purchased from Agilent Technologies (Santa Clara, 95051 California, USA), Merck KGaA (Sigma-Aldrich), and Carl Roth GmbH + Co. KG (76185 Karlsruhe, Germany).

2.4 List of methods

Table 4 provides an overview of all applied methods.

The methodical approach and the overview of the experimental design are given in figure 1 (created with BioRender.com) [23].

3 Results and discussion

3.1 Isolation of yeasts

Colony forming units of both determined dilutions ranged from a mean of 364 colonies per plate (dilution of 10^{-1}) to 48 cfu per plate (dilution of 10^{-2}); an exemplary image is shown in figure 1. The viable yeast cell count of the original sample was calculated at $7.4*10^5$ cfu/ml.

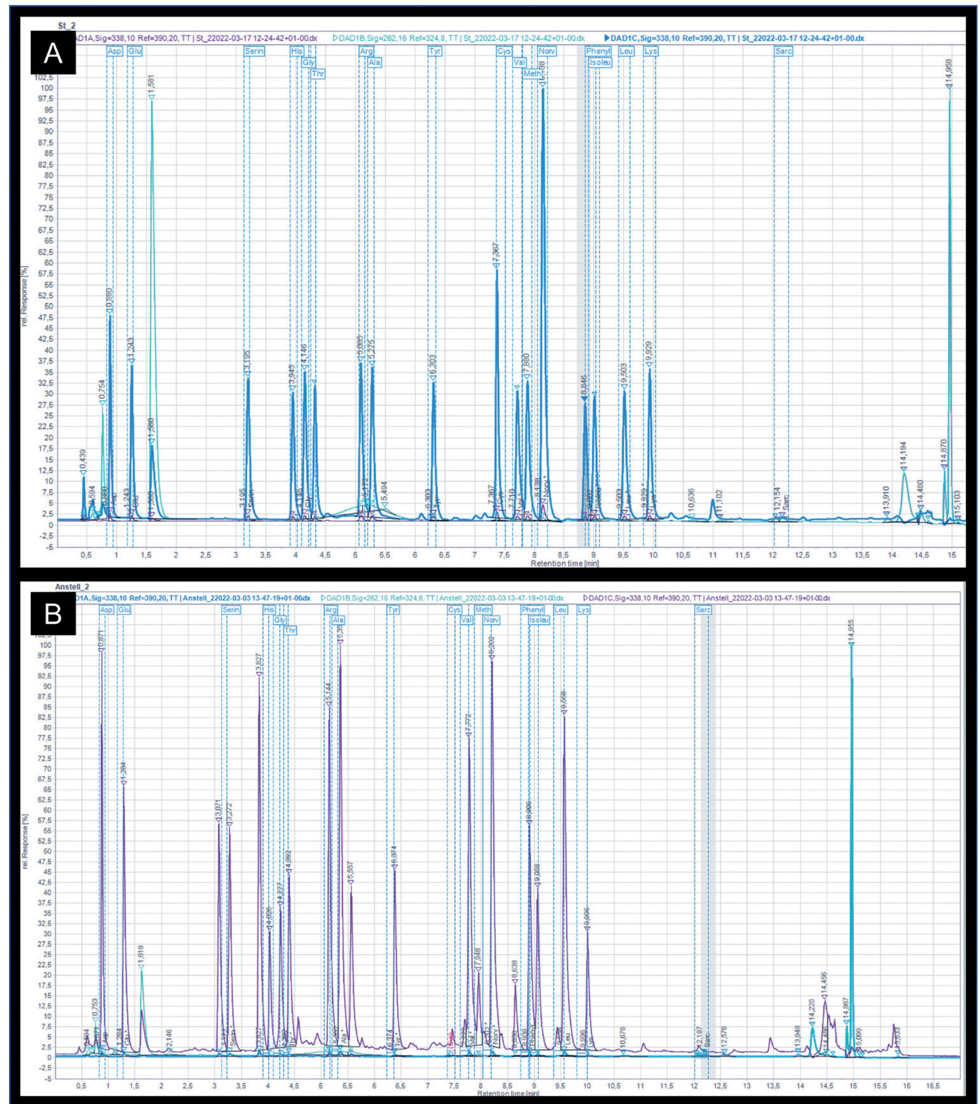


Fig. 6 HPLC chromatogram of the amino acids standards (A) and amino acids identified in the original wort sample (B) before inoculation. Norvaline (Nor) and Sarcosin (Sarc) served as internal standards

The results of the MALDI-ToF analysis conducted by the Research Center Weihenstephan for Brewing and Food Quality revealed that two different yeast species were present in the originally taken sample: *Candida boidinii* and *Pichia fermentans*. Both yeast species are ubiquitous and occur in soil, water and on leaves and can be considered anthropophilous [24, 25]. According to various authors, species of both genera, *Candida* and *Pichia*, are also typical aerobic yeast species occurring in untargeted spontaneous fermentation of beer or wort [26–28]. In contrast to targeted spontaneous fermentation, such as it is applied in the production of traditional Belgium Lambic beers, untargeted spontaneous fermentation can be considered as the unintentional inoculation of wort or beer with wild yeasts [25, 29]. Several species of *Candida* and *Pichia*, already have been mentioned in context with untargeted spontaneous fermentation and the corresponding references, are listed in table 5.

Both yeast species were clearly distinguishable macroscopically and microscopically, regarding the diameter of their colonies and their cell shapes respectively, as shown in figure 2.

P. fermentans formed colonies larger in diameter than *C. boidinii*. Where *P. fermentans* colonies reached more than 5 mm in diameter, the diameters of *C. candida* were distinctively smaller, with observed diameters smaller than 4 mm. In addition, the cells of *P. fermentans* proved to be larger as well than those of *C. boidinii*. The presence of *P. fermentans* colonies on SGC2 became perceptible

Table 7 Amino acids categorised based on the rate of their assimilation and amino acid concentrations determined in the original wort

Group [37]	Amino acid	Concentration in Original Wort [mg/L]	Common concentration ranges [mg/L] [38, 39]
Group 1 immediate and total assimilation	Arginine (Arg)	113.6 (SD ± 2.8)	61–219
	Asparagine (Asn) and Aspartic Acid (Asp)*	86.7 (SD ± 10.7)	79–282
	Glutamine (Gln) and Glutamic acid (Glu)**	95.4 (SD ± 3.1)	47–246
	Lysine (Lys)	53.2 (SD ± 11.2)	65–195
	Serine (Ser)	39.1 (SD ± 2.4)	39–140
	Threonine (Thr)	48.1 (SD ± 1.4)	39–109
Group 2 slow but continuous assimilation	Histidine (His)	42.7 (SD ± 1.3)	21–113
	Isoleucine (Ile)	59.8 (SD ± 1.7)	48–155
	Leucine (Leu)	117.4 (SD ± 3.3)	100–316
	Methionine (Met)	23.8 (SD ± 0.7)	20–67
	Valine (Val)	90.4 (SD ± 2.5)	78–214
Group 3 Assimilation after lag-phase	Alanine (Ala)	80.2 (SD ± 2.8)	62–205
	Glycine (Gly)	24.0 (SD ± 1.0)	20–59
	Phenylalanine (Phe)	104.2 (SD ± 2.8)	63–226
	Tryptophan (Trp)	n. A.	n. A.
	Tyrosine (Tyr)	78.2 (SD ± 1.2)	62–200
Group 4 No assimilation within the first 60 h	Proline (Pro)	n. A.	n. A.

*Henceforth designated Glu

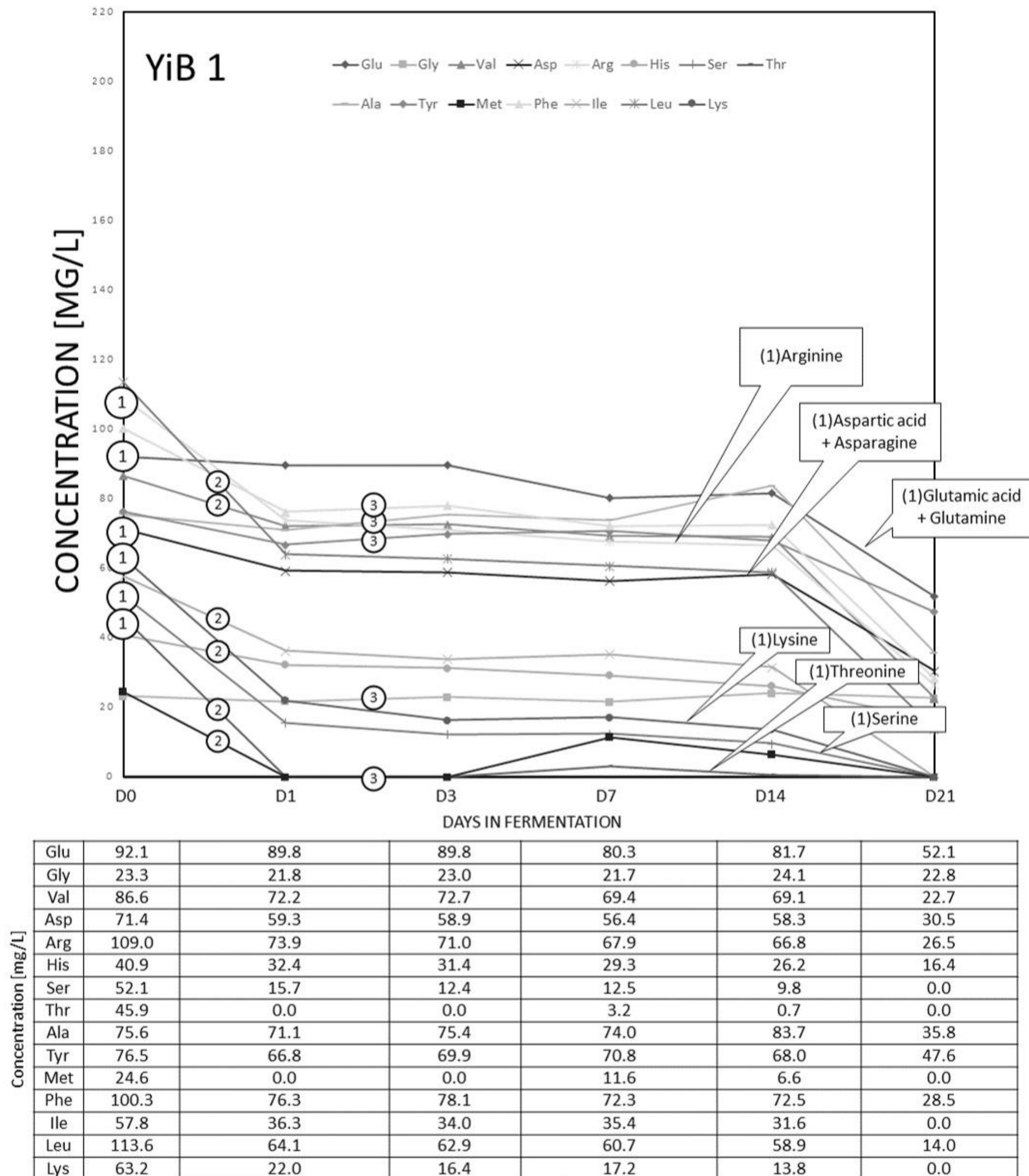
**Henceforth designated Asp

only after 48 to 72 h in the incubator. Hence, the incubation time was eventually set to 96 hours instead of 72 hours after preliminary tests. Although it was not determinable by microscopical investigations which of the two yeast species became the dominant one during the fermentation process, the most likely species may be *C. boidinii*. According to *Caputo* et al., *P. fermentans* strains probably do not assimilate maltose and sucrose and show only low expression of the invertase enzyme [33]. Hence, the main protagonist of the extract degradation in the yeast inoculated batches may likely be *C. boidinii*. Nevertheless, wort produced from Pilsener malt has been reported to contain a variety of fermentable carbohydrates: *Canonico* et al. quantified glucose in concentrations of 7.5 g/L, in addition to sucrose (6.4 g/L) and maltose (55.7 g/L) in wort produced from Pilsener malt [34]. This may explain why *P. fermentans* was present in the collected original sample and grew on the SGC2 medium, which contains 2 % (w/v) of glucose. Hence, *P. fermentans* shows itself to be capable of co-fermentation.

3.2 Fermentation curves

Remarkably, the batches inoculated with the two yeast species and the batches inoculated with the microbiome did not show remarkable differences regarding their apparent attenuation and their apparent extract. Even though the first batch inoculated with the isolated yeasts started fermentation with a delay of 24 h in comparison to the following yeast inoculated batches, its apparent degree of attenuation and its final pH-value did not differ noticeable (Fig. 3, Table 5). However, when regarding alcohol by volume and pH-values of the different batches, distinctions became evident. Whereas yeast YiB were noticeably higher in alcohol by volume and less acidic than MiB, these were expectedly more acidic and lower in alcohol by volume. On average, both MiB and YiB achieved an apparent attenuation of approximately 70 % after 28 days. The extract degradation curves over time and in comparison, are shown in figure 4.

The degradation of extract started faster in YiB, gradually slowed down on the third day of fermentation, and thenceforward converged with the extract degradation rate of the MiB. Regarding the apparent extract, there was no distinct difference between YiB or MiB. At the same time, the rate of extract degradation was approximately even. Regarding the pH-changes over the course of the fermentations, the first clear distinctions could be made. Whereas the acidification of the YiB occurred faster and more pronounced within the first three days after inoculation, the final pH-values of MiB were, as expected, noticeable lower (Fig. 5). Microbiomes which are involved in spontaneous fermentations, such as the fermentation of traditional Lambic, include a variety of acidifying bacteria. *Spitaels* et al. identified *Pediococcocus damnosus*, *Leuconostoc mesenteroides*, *Acetobacter cerevisiae* and *Acetobacter lambici* on the inside of Lambic barrels [8]. According to *Tyakht* et al., lactic acid bacteria, such as genus *Lactobacillus*, are predominant in sour and wild ales [30]. Hence, a lower pH-value of MiB was expected. In fact, it can be assumed that the acidification process will continue in the reserve sample because the original sample collected from the maturing wild ale in the barrel was measured with an apparent residual extract of 3.0 and a pH-value of 3.22 (mean ± SD 0.01, n = 12). It can be assumed that MiB will also undergo the four specific phases of fermentation of spontaneous brews, which are described by various authors: The initial fermentation



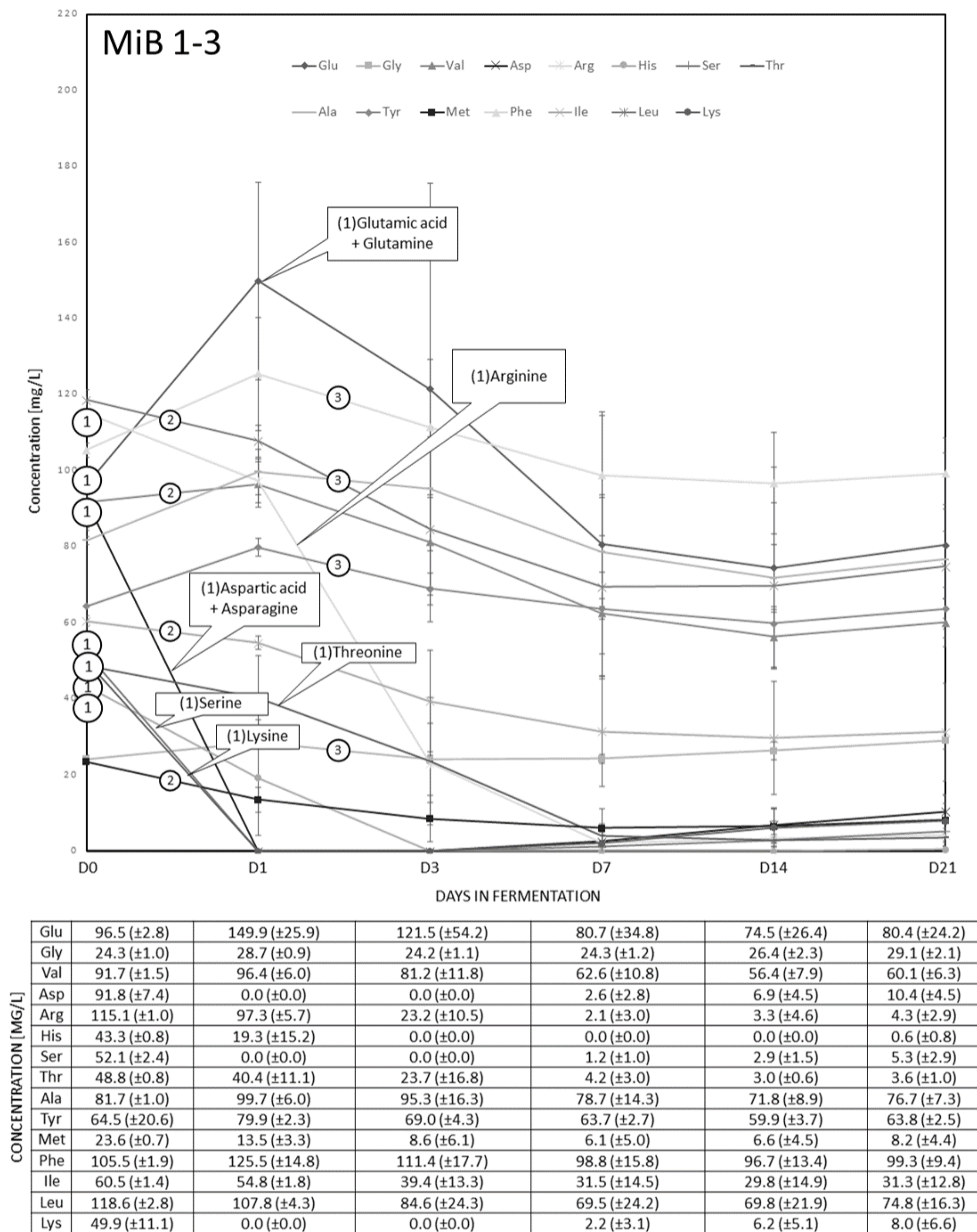
	D0	D1	D3	D7	D14	D21
Glu	92.1	89.8	89.8	80.3	81.7	52.1
Gly	23.3	21.8	23.0	21.7	24.1	22.8
Val	86.6	72.2	72.7	69.4	69.1	22.7
Asp	71.4	59.3	58.9	56.4	58.3	30.5
Arg	109.0	73.9	71.0	67.9	66.8	26.5
His	40.9	32.4	31.4	29.3	26.2	16.4
Ser	52.1	15.7	12.4	12.5	9.8	0.0
Thr	45.9	0.0	0.0	3.2	0.7	0.0
Ala	75.6	71.1	75.4	74.0	83.7	35.8
Tyr	76.5	66.8	69.9	70.8	68.0	47.6
Met	24.6	0.0	0.0	11.6	6.6	0.0
Phe	100.3	76.3	78.1	72.3	72.5	28.5
Ile	57.8	36.3	34.0	35.4	31.6	0.0
Leu	113.6	64.1	62.9	60.7	58.9	14.0
Lys	63.2	22.0	16.4	17.2	13.8	0.0

Fig. 7 Changes in amino acid concentration during the fermentation in YiB 1 by possible assimilation, degradation, or metabolism (first trial, N = 1)

phase, the alcoholic fermentation phase, the acidification phase (after approximately 4 months) and the maturation phase, beginning 12 months after the inoculation [7, 28].

In the same context, the alcohol content, real extract, turbidity, and other parameters of 250 ml of each of the YiB were determined and compared with three randomly selected samples of the same volume from the MiB, to highlight possible influences of the bacterial microbiome on these parameters. The results of the PBA-B analysis are given in table 6.

It became apparent that the clearest differences between the YiB and MiB concerned the pH-value, the CO_2 concentration, and the turbidity of the final fermentation products. Albeit that the alcohol concentration in the YiB was slightly higher in comparison with the MiB, this disparity was not a distinct one. However, the CO_2 concentration in YiB was pronouncedly higher than in MiB. Several explanations for this observation may be applicable but can only be regarded as pure speculation regarding the available results. The composition of the complete microbiome and its wide-ranging interactions and its products may be one possible reason. The observed ropey consistency of the MiB indicated the presence of biofilms which can be formed by bacteria species of the genera *Pediococcus* and *Lactobacillus*. This is another plausible reason for the different CO_2 concentrations [35, 36]. It can be assumed that the necessary adaptations in the sample preparation method,



	D0	D1	D3	D7	D14	D21
Glu	96.5 (±2.8)	149.9 (±25.9)	121.5 (±54.2)	80.7 (±34.8)	74.5 (±26.4)	80.4 (±24.2)
Gly	24.3 (±1.0)	28.7 (±0.9)	24.2 (±1.1)	24.3 (±1.2)	26.4 (±2.3)	29.1 (±2.1)
Val	91.7 (±1.5)	96.4 (±6.0)	81.2 (±11.8)	62.6 (±10.8)	56.4 (±7.9)	60.1 (±6.3)
Asp	91.8 (±7.4)	0.0 (±0.0)	0.0 (±0.0)	2.6 (±2.8)	6.9 (±4.5)	10.4 (±4.5)
Arg	115.1 (±1.0)	97.3 (±5.7)	23.2 (±10.5)	2.1 (±3.0)	3.3 (±4.6)	4.3 (±2.9)
His	43.3 (±0.8)	19.3 (±15.2)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.6 (±0.8)
Ser	52.1 (±2.4)	0.0 (±0.0)	0.0 (±0.0)	1.2 (±1.0)	2.9 (±1.5)	5.3 (±2.9)
Thr	48.8 (±0.8)	40.4 (±11.1)	23.7 (±16.8)	4.2 (±3.0)	3.0 (±0.6)	3.6 (±1.0)
Ala	81.7 (±1.0)	99.7 (±6.0)	95.3 (±16.3)	78.7 (±14.3)	71.8 (±8.9)	76.7 (±7.3)
Tyr	64.5 (±20.6)	79.9 (±2.3)	69.0 (±4.3)	63.7 (±2.7)	59.9 (±3.7)	63.8 (±2.5)
Met	23.6 (±0.7)	13.5 (±3.3)	8.6 (±6.1)	6.1 (±5.0)	6.6 (±4.5)	8.2 (±4.4)
Phe	105.5 (±1.9)	125.5 (±14.8)	111.4 (±17.7)	98.8 (±15.8)	96.7 (±13.4)	99.3 (±9.4)
Ile	60.5 (±1.4)	54.8 (±1.8)	39.4 (±13.3)	31.5 (±14.5)	29.8 (±14.9)	31.3 (±12.8)
Leu	118.6 (±2.8)	107.8 (±4.3)	84.6 (±24.3)	69.5 (±24.2)	69.8 (±21.9)	74.8 (±16.3)
Lys	49.9 (±11.1)	0.0 (±0.0)	0.0 (±0.0)	2.2 (±3.1)	6.2 (±5.1)	8.0 (±6.6)

Fig. 8 Changes in amino acid concentration [mg/L] during the fermentation in MiB 1–3 by possible assimilation, degradation, or metabolism (first trial, concentrations are mean values ± SD, N = 3)

due to the increased viscosity of the affected samples, are the most logical explanation and may suffice regarding the present results for the time being.

3.3 HPLC analysis of amino acid assimilation

The HPLC separation by automated precolumn OPA derivatization was acceptable. Peak identification and retention time matched those of the reference standards. Two exemplary chromatograms are displayed in figure 6.

A total of 16 amino acids were identified in the samples, whereas cysteine was only identified once, namely in batch YiB on day 7 of fermentation. Although all four fermentation carboys (YiB and MiB 1-3) were filled with aliquots of the same original wort, samples were collected from all four carboys before inoculation. This was done

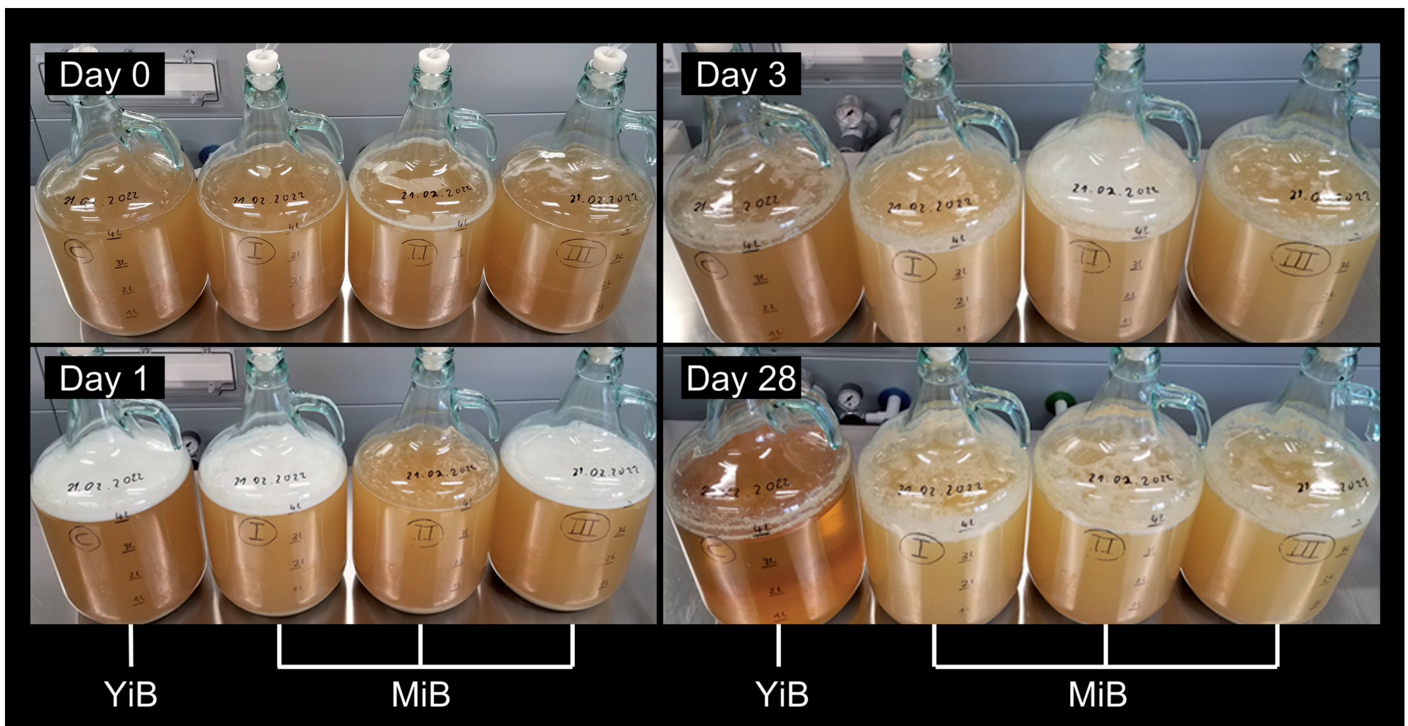


Fig. 9 Visual changes over the course of fermentation from Day 0 (day of inoculation) to Day 28 (finalisation of the experiments), YiB: yeast inoculated batch, MiB: microbiome inoculated batches

to ensure acceptable results regarding the assimilated amounts of amino acids over the course of the fermentations. The concentration of the respective amino acid identified in the original wort of the first trial is given in table 6. According to Jones and Pierce, the amino acids are listed in groups ranging from 1 to 4, based on the manner of their assimilation and are juxtaposed with known concentrations according to *Garza-Ulloa* et al. and *Schönberger* [37–39]. One drawback of the used HPLC analysis method is that glutamic acid and glutamine, as well as aspartic acid and asparagine, cannot be separated, therefore the concentrations of these amino acids are expressed as sum parameters. The given results consider the recovery rate of the internal standard norvaline and the sample dilution and were corrected accordingly.

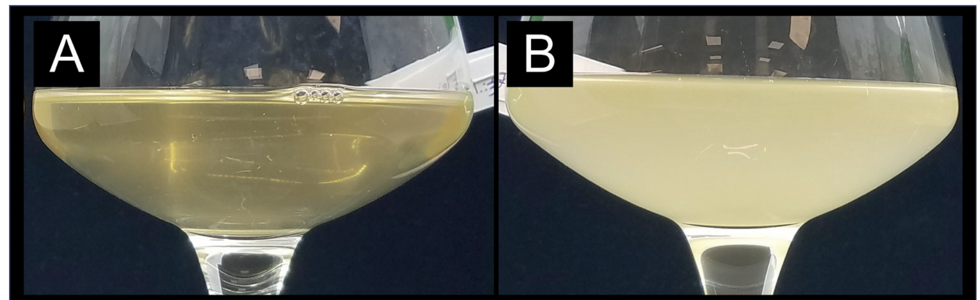


Fig. 10 Final fermentation products in comparison: Yeast inoculated product (A) and microbiome inoculated product (B)

The changes in the amino acid concentrations were much more pronounced in MiB 1-3 than in YiB, albeit the interpretation of these results is a questionable endeavor. This internal method was tested the first time in-house on wort and beer samples. Matrix effects, aside from cell autolysis by-products may have severe but yet unknown influences. MEBAK 2.6.4.1.2 would be preferable in terms of comparability [20]. However, at the given time, the described method was the only one available. Provided the analysis results presented in figures 7 and 8 are accurate, amino acids of Group 1 are assimilated fast by YiB1 (according to Jones and Pierce) but contradictory not in total, with one exception: threonine [37]. However, the fast assimilation rate of Group 1 amino acids over the first three days of assimilation fits the lag-phase and therefore seems plausible. The concentrations of certain amino acids, such as L-alanine (Group 3), increased after 14 days of ongoing fermentation, which may be due to normal metabolic yeast activity, but could also indicate a flaw in the introduced method.

Regarding Group 1 amino acids, MiB1-3 yielded expected results according to Jones and Pierce, with one major exception (Fig. 8) [37]. The concentration of Glutamic acid and glutamine (sum parameter Glu) was increased by more than 50 % after the first 24 h of fermentation. This increase within the lag-phase was observable in all the MiB batches of the first trial (MiB 1-3) and affected the concentrations of other amino acids such as Phenylalanine (Group 3) as well. Prima facie, this sudden increase in amino acids may indicate cell autolysis, which can cause an abundance of free amino acids in the fermenting wort, or by the bacterial degradation of wort proteins [40, 41]. In this context, *De Roos* et al. observed an accumulation of certain biogenic amines (cadaverine and tyramine, amino acid degradation products) caused by bacteria during the fermentation of Lambic beer [41]. However, the most probable reason is an excretion of amino acids into the medium, due to a change in yeast metabolism, as reported by *Feuillat* and

Charpentier [42]. For this reason, the increase in amino acid concentration in our findings seems at least plausible. This finding is noteworthy but requires more in-depth analysis in the next trials. The different assimilation rate of Group one amino acids between YiB and MiB is also deserving of further analysis.

3.4 Observations

In conclusion, the fermentation characteristics of YiB and MiB seemed extraordinarily similar, but the final products could not be more different. Despite the uniformity of most of the determined fermentation parameters, the optical appearance regarding colour and turbidity of YiB and MiB was completely different. In this context, the turbidity and viscosity of MiB were remarkably higher than those of YiB. YiB displayed distinct clarification, whereas MiB developed a pronounced turbidity and, colourwise, a brighter appearance within the first three days of fermentation (Fig. 9). This observation was also confirmed in the final PBA-B measurements. The turbidity of YiB was determined with 2.85 EBC, and the turbidity of MiB with 13.22 EBC. These results also matched the visual appearance of YiB and MiB after 28 days of fermentation, as shown in figure 10.

Even though not tested in scientific trials, it should be mentioned that the flavour of YiB could be described as neutral to bland, whereas the flavour of MiB was pungently citric and partly reminiscent of traditional Berliner Weiße.

4 Conclusion

The isolation of both yeast species, the setup of the fermentation trials and the measurements of the most important fermentation characteristics could be achieved with reasonable effort and performed with basic lab equipment. It is of course also favourable that to most wild ale breweries the environmental microbiome is not a risk but an asset. Submitting samples for species identification by Maldi-ToF and the analysis costs can be regarded as a minimal investment with high benefits for small-scale wild ale breweries. It can therefore be concluded that the introduced approach is a viable one to select and screen wild yeasts for their suitability for brewing. The most exceptional findings when comparing YiB and MiB were the reproducibility of the fermentation profiles and the tallying values in essential fermentations characteristics such as attenuation and alcohol content. Hence, it may be assumed that this very association of microorganisms isolated from this specific habitat is a stable one, with predictable physiological interactions and fermentation (by-)products. Though the experiments were conducted under controlled environmental conditions, the almost identical fermentation profiles were nonetheless unexpected. However, the final products of the co-fermented yeast trials and the microbiome-fermented were visually different and differed from each other in terms of flavour. The almost neutral character of the co-fermented yeast trials, the perceived distinct clarification of these batches and the assumed traits regarding sedimentation and flocculation of both *C. boidinii* and *P. fermentans* deserve a far more profound examination. As a next step, fermentation trials conducted with the isolated *C. boidinii* and *P. fermentans* strains will be conducted to compare the fermentation characteristics. Additionally, the viable cell count will be monitored throughout fermentation and the final products accompanied by sensory analysis. As a final, concluding remark: it is our assumption that not only two species of yeast, but further species can be isolated from the wild ale and barrel used in our observation. For this reason, the very composition of this yeast-association will also be an issue of future research.

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