Title

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From lab to table: expanding gastronomic possibilities with fermentation using the edible fungus *Neurospora intermedia*

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Introduction

Fermentation – the use of microorganisms to transform ingredients – is a powerful tool for enhancing flavor, improving sustainability, and expanding creative possibilities in the kitchen (Melton, 2022, Jahn et al., 2023). Fermentation is an ancient and culturally widespread practice that dates back to early human evolution, and diverse food companies and restaurants are now building on the traditional techniques and approaches of the past to drive new innovation in food production (Liu et al., 2019, Melton, 2022, Humpenoder et al., 2022, Aro et al., 2023). In particular, solid-state fermentation using filamentous fungi – a diverse group of microorganisms that includes molds and mushrooms – has gained notoriety for its ease of use, sustainability impact, and ability to unlock flavors and textures from diverse locally available ingredients (Javourez et al., 2022, Wang et al., 2016, Yamashita, 2021). For example, *Aspergillus oryzae* (koji mold), a fungus traditionally grown on grains and legumes in the production of sake, miso, and shoyu in East Asia, is now a mainstay of many of the world's most innovative kitchens, offering chefs new opportunities to push boundaries in flavor and seasonality by applying traditional techniques with new substrates (Redzepi and Zilber, 2018, Yamashita, 2021). Commercially available cheese-derived *Penicillium* fungi and Tempeh-derived *Rhizopus* strains have been similarly used for innovative gastronomic applications (Guixer et al., 2017, Sindhu et al., 2019, Gmoser et al., 2020). However, a majority of fungal fermentations frequently used in gastronomic contexts are restricted to a small set of readily available species and strains, which have inherent limitations in their growth profiles, enzymatic activities, and sensory appeal. Expanding beyond this limited biological diversity of frequently used fungal strains is critical to unlock new gastronomic innovation.

We recently biochemically and genetically characterized strains of the edible fungus *Neurospora intermedia* (Maini Rekdal et al., 2023). This fungus is traditionally used to make oncom merah (red oncom), a fermented food produced through solid state fermentation of soymilk residue (okara) in Java, Indonesia (Andayani et al., 2020). In the traditional process, steamed okara is inoculated by back-slopping from a previous batch, followed by a brief 1–2-day fermentation period that transforms the substrate into a firm cake covered by the characteristically orange *N. intermedia* spores (Maini Rekdal et al., 2023). Similar to tempeh, red oncom can be used as a meat substitute and is usually served fried or roasted and added to soups and stews in the local cuisine. The previous study revealed that unlike commonly used edible *Penicillium* and *Aspergillus* strains, *N. intermedia* does not encode for any known mycotoxins, supporting a promising safety profile. Additionally, we found that okara fermented with *N. intermedia* was well-liked among Western customers, indicating that the fungus may have broad sensory appeal. Finally, we found that *N. intermedia* can grow on diverse plant-derived byproducts, including spent grain, oilseed presscake, fruit and vegetable pomace, and waste from plant-based milk production. Our previous work (Maini Rekdal et al., 2023) did not investigate any culinary applications of *N. intermedia* beyond the traditional substrate and fermentation process, and even though *N. intermedia* has demonstrated use in a potential burger application from stale bread and grow on other food-grade substrates in separate studies (Gmoser et al., 2020, Starzyńska-Janiszewska et al., 2017, Beuchat, 1976, Hellwig et al., 2020), new gastronomic applications and sensory evaluation of novel foods produced with *N. intermedia* are lacking.

We reasoned that, as a relatively unexplored, fast-growing edible fungus, *N. intermedia* would be ideally suited to expand the chef's toolbox and unlock new gastronomic possibilities with fermentation. Here we sought to explore the broader culinary potential of *N. intermedia* and

establish protocols and methods for its application in new gastronomic contexts. Our main objective was to evaluate the application of *N. intermedia* in fermented foods with new substrates and formats beyond the traditionally substrate and fermentation process.

1. Materials and methods

2.1 Preparation of standard *N. intermedia* inoculum

For these studies, we used *N. intermedia* #2613, which was originally isolated from oncom. The strain was obtained from the Fungal Genetics Stock Center (FGSC). *N. intermedia* was kept as a spore suspension in a solution of 30 % v/v glycerol at -80 ºC. To prepare the inoculum a small portion of *N. intermedia* was transferred from the glycerol stock into an agar slant of Potato Dextrose Agar. Culture tubes were kept in the incubator at 30 ºC for 5 days. After 5 days, 4 mL of autoclaved water was transferred to the slant and mixed with vortex, to suspend the spores. Then, the solution was transferred into a new tube with 6 mL of autoclaved water and the inoculum was ready to use in the restaurant.

2.2 Preparation of oncom with alternative substrates

To prepare the different samples of oncom, 250 g of peanut, cashew and pine nut were soaked with 4 L of water at 4°C overnight and then rinsed with water before processing. The rinsed substrates were briefly processed in the Thermomix TM6 (high speed; 20 s) to diminish the grain size and steamed in the oven for 30 min at 100 °C. They were cooled to 30 °C before inoculating with *N. intermedia*. To inoculate, a standard inoculum of *N. intermedia* was used (12 mL per 300 g of steamed grains). The inoculated substrate was covered slightly with a sterile cloth and incubated for 24 h at 29 ºC and 60 % of relative humidity. After 24 hours, the spores germinated, and the substrate was then fermented without cloth for 24 h. After 24 h the aerial orange mycelium covered all the surface (Maini Rekdal et al., 2023). The samples were kept at -80 °C until analysis.

99 **2.3 Preparation of fermented rice and amazake**

100 1 kg of raw polished rice was soaked in 2 kg of water overnight at 4 °C and then steamed at 100
101 °C for 90 min after rinsing. Rice was cooled to 30 ºC before inoculating with 0.3 % (w/w) koji
102 spores (*Aspergillus oryzae* BF-3*; Higuchi Matsunosuke Shoten Co. Osaka, Japan)*. Then, the
103 material was tightly packed and wrapped in a sterile cloth. The temperature was kept at 30 ºC and
104 the relative humidity around 95% during the ~36 h koji fermentation. The koji was completed
105 when the mycelial growth completely covered the grains (Feng et al., 2013). The same process
106 was developed to inoculate the rice with *N. intermedia* #2613, but instead of the dried koji spore
107 inoculum, 12 mL of standard *N. intermedia* inoculum was added to 300 g of the steamed rice. The
108 inoculated rice was incubated at 29 ºC and 60 % relative humidity. After 24 h the spores
109 germinated, and the rice was uncovered. The fermented rice was kept for 24 h until the aerial
110 orange mycelium covered all the surface. The RH was determined by measuring the humidity with
111 a data logger and keeping it constant as much as possible. The 60% was used to ferment rice with
112 *N. intermedia*, and 95% to make koji with *A. oryzae*. After different trials, we discovered that *N.*
113 *intermedia* needed less humidity than *A. oryzae*. Amazake was prepared following the traditional
114 recipe with some modifications. The rice that had been fermented with *A. oryzae* and *N. intermedia*
115 was mixed with cooked rice and tap water, in a proportion of 1:2:1 (fermented rice:steamed rice:tap
116 water). The steamed rice was prepared as described above. To make amazake, the mixture was
117 incubated at 50 °C for 10 hours in a sealed vacuum bag (Oguro et al., 2019). Then, the product
118 was kept at 4 °C until analysis. Rice fermented with *A. oryzae* produces 'koji'. Therefore, we
119 named the rice fermented with *N. intermedia* as 'noji'.
120

2.4 Analysis of free glucose using High Performance Anion Exchange Chromatography (HPAEC)

All samples were lyophilized prior to analysis. For extraction from solid, samples were ground into a fine powder using a mortar and pestle and then approximately 30 mg was transferred to tubes for homogenization (Lysing Matrix Z, MP Biomedicals, catalog#: 116961050-CF). 1 mL of 20% methanol with 0.1% formic acid was added and samples were subjected to bead beating for 2x1 minutes. Following bead-beating, samples were spun down at 12,000 RCF for 10 minutes to separate the solids. 500 µL of the supernatant was transferred to a centrifugal spin filter to remove any particulates and larger molecules (3 kDa cutoff) (Amicon Ultra, Sigma-Aldrich, Catalog # UFC500324). The flow-through was collected and subjected to analysis by High Performance Anion Exchange Chromatography (HPAEC) for sugar profiling. The method was adapted from (Rautengarten et al., 2019) with minor modifications. High-performance anion exchange chromatography with pulsed amperometric detection was performed on an ICS-6000 (Dionex Corporation, Sunnyvale, CA) using a CarboPac PA20 (3 150 mm, Dionex Corporation, Sunnyvale, CA) anion exchange column at a flow rate of 0.4 mL/min. Before sample injection, the column was equilibrated with 5 mM NaOH for 5 min. The elution program involved two isocratic elution steps with 5 mM NaOH from 0 to 23 min to separate the neutral sugars followed by a ramp step to 450 mM NaOH from 23.1 to 41 min, which allowed separation of uronic acids and washing of the column. A monosaccharide standard of D-glucose was used for quantification (Sigma-Aldrich, catalog#47249). Sample quantitation was performed by linear regression. Experiments were performed in biological triplicates, e.g. three distinct samples of koji and noji were analyzed for free glucose levels. The HPAEC analysis was run once for each sample.

2.5 Alpha-amylase assays

To measure secreted alpha-amylase activity, we used the colorimetric Abcam alpha amylase assay kit (catalog#: ab102523). To prepare samples for analysis, 0.36 grams of rice (control) or rice fermented with *A. oryzae* or *N. intermedia* (koji and noji, respectively), was mixed with 1 mL sterile distilled water. The sample was vortexed briefly to resuspend and was then spun down at max speed for 10 minutes in a tabletop centrifuge. The supernatant was decanted and then 1 mL of alpha-amylase assay buffer was added (from the Abcam kit referenced above). The samples were vortexed to resuspend the particles, followed by sonication (Qsonica, model #Q500) to homogenize the sample (10 s ON, 25 s OFF, 2 min total, 25 % amplitude). The resulting slurry was spun down at max speed to clarify the supernatant, and 20 µL of the resulting supernatant was used as the enzyme source in the 150 µL enzyme reactions. We directly followed the protocol of the manufacturer for the enzyme assays. Absorbance was measured at 405 nm every minute using the BIOTEK Synergy H1 microplate reader, and the reaction rate was obtained from the linear reaction curve. Amylase activity *w*as calculated as (B/(ΔT*V))*D where B = Nitrophenol amount from the standard curve (in nmol), ΔT = reaction time (T2 – T1) (min), V = Pretreated sample volume added to the reaction well (in mL), D = sample dilution factor. Per the manufacturer's instructions, 1 Unit Amylase was defined as the amount of amylase that cleaves ethylidene-*p*NP-G7 to generate 1.0 µmol of nitrophenol per min at pH 7.20 at 25 °C. Experiments were performed in biological triplicates, e.g. three distinct samples of koji and noji were analyzed for the enzyme activity.

2.6 Physicochemical analysis

pH and °Brix were measured at 0 (rice), 48 h (noji) and 36 h (koji), and at the end of amazake incubation (10 h), all in triplicate. pH was analyzed on 2 g of samples and 10 mL of distilled water using a pH meter (Elite pH pocket testers, ThermoFisher Scientific). °Brix was measured in the same concentration using a digital hand-held refractometer (LLG-uniREFRACTO 1 and 2).

172 **2.7 Aroma compound analysis (HS-SPME-GC/MS)**

173 The analysis of aromas was carried out of the rice prior to fermentation by the fungi, and then
174 subsequently in samples fermented by *N. intermedia* at two different time points: after 48 h (the
175 final noji, prior to amazake process) and after 10 h of the amazake process. For *A. oryzae,* samples
176 were collected for aroma analysis after 36 h (the final koji, prior to amazake process) and after 10
177 h of the amazake process. All the samples were frozen in liquid nitrogen and kept at -80 °C until
178 analysis. Extraction and analysis were performed following the method by Feng et al. with minor
179 modifications (Feng et al., 2013). Briefly, 5 g of sample was homogenized with 15 g of sterile
180 water containing 0.9 % NaCl, vortexed and processed in a stomacher (Stomacher 400, Seward Co,
181 Worthing, West Sussex, UK) (max speed, 2 min) for each sample. 8 mL of samples were transferred
182 to 20 mL gas tight vials. To each vial 2.88 g NaCl was added and ten microlitres of 2-methyl-3-
183 heptanone (diluted to 27.2 mg/L in LC/MS-grade methanol). Steamed white rice was used as
184 negative control, following the extraction method. Distilled water was used as a blank sample. The
185 samples were stored at -80 °C until analysis. The volatile composition of the samples was
186 determined using headspace solid phase micro-extraction (HS-SPME).Solid-phase
187 microextraction (SPME) fiber, polydimethylsiloxane/divinylbenzene (PDMS/DVB), 65 μm was
188 used for the extraction of volatile compounds. After the extraction, the samples were incubated at
189 45 ºC for 15 min, after which the SPME fibre was inserted into the headspace at 45 ºC for 40 min,
190 before inserting into the GC injection port. The GC-MS analysis was performed on a Thermo
191 Scientific TRACE 1310 Gas Chromatograph equipped with a Thermo Scientific Q Exactive
192 Orbitrap mass spectrometry system with a Thermo fused-silica capillary column of cross-linked
193 TG-5SILMS (30 m x 0.25 mm x 0.25 μm) (ThermoFisher Scientific, Waltham, MA, USA). The
194 GC conditions: inlet and transfer line temperatures, 250˚C; oven temperature program, 40˚C for 2

min, 5˚C/min to 120˚C for 2 min, 7˚C/min to 220˚C for 5 min, 50˚C/min to 325˚C for 3 min; inlet helium carrier gas flow rate, 1 mL/min; split ratio, 20:1. The electron impact (EI)-MS conditions: ionization energy, 70 eV; ion source temperature, 250˚C; full scan m/z range, 30 - 350 Da; resolution, 60,000; AGC target, 1e6; maximum IT, 200ms. This volatile compound extraction method has been previously used to analyze different koji and soy sauce (Feng et al., 2013). Method of identification, by comparison of the MS spectra with the NIST library and by comparison of RI (Kovat indices). The areas were normalized to the Internal Standard (ISTD) 2-methyl-3-heptanone. Retention index based on Thermo TG-5SILMS column using C7-C27 as external references. Data were acquired and analyzed with Thermo TraceFinder 4.1 software package.

2.8 Sensory analysis: General protocol

The protection and processing of personal data was followed as prescribed by the Declaration of Helsinki and the 2016/679 EU Regulation. The experimental process was explained and written consent indicating voluntary participation in "Novel food sensory analysis" was obtained from each participant at the beginning of the study. Each study was conducted in a room with temperature and relative humidity (20 ± 2 ◦C; 75 ± 5% RH); the illumination was a combination of natural and nonnatural light. The order of evaluation was randomized, and samples were coded with 3-digit numbers. Both samples were served at the same time and consumers tasted them from right to left. It was mandatory for consumers to rinse their mouths with water between samples.

2.9 Sensory analysis: Amazake

The same general process as described above was followed, but with minor modifications. Amazake samples were processed in the Thermomix TM6 (full speed, 5 min) and roughly 5 mL of each amazake sample was served in a transparent plastic cup at 10 °C. A total of 60 consumers participated in the test by tasting each sample, and rated their liking using a 9-point hedonic scale (1= extremely dislike, 9= extremely like) for texture, appearance, color, taste, aroma, and overall liking of the product.

2.10 Sensory analysis: Oncom

Sensory analysis was conducted by a hedonic test with a total of 61 consumers. Peanut, pinenut, and cashew oncom were prepared to conduct the sensory analysis, following the traditional method. Approximately 10 g of each sample, was cut into a square, fried, and served. Consumers tasted each sample and rated liking on a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like) for flavor, texture, and appearance. Following this, a Check-All-That-Apply (CATA) was conducted, choosing among 21 attributes (Jaeger et al., 2015).

2.11 Data analysis

A student t-test was conducted to analyze each attribute (texture, appearance, taste, aroma, color and overall liking) for amazake sensory analysis. Data analysis was carried out with ANOVA test for volatile compounds and oncom samples, and post-hoc test was conducted using Tukey's HSD. Data from CATA was analyzed using Cochran's Q test with a pairwise comparisons approach based on the McNemar-Bonferroni to identify significant differences between attributes associated with each sample for the first part of the study (Meyners et al., 2013). All data analyses were

241 conducted using the statistical package XLSTAT Version 2009.6.03 (Addinsoft, USA). Results
242 were considered significant when $p < 0.05$. The heat map was carried out with RStudio Desktop
243 2022.07.1+554, with 95 % of confidence level and 0.001, 0.01 and 0.05 significance levels for the
244 correlation analysis.
245

2. Results

3.1 Production of oncom from alternative substrates

Oncom made from okara is used as a meat substitute, due to the ability of the fungus to remove off-flavors, release free amino acids and sugars, and alter the structure and improve texture during the solid-state fermentation (Maini Rekdal et al., 2023, Andayani et al., 2020). This led us to wonder whether similar effects could be achieved with other substrates. Thus, as a first exploration of the culinary potential, we evaluated the ability of *N. intermedia* to produce oncom from non-traditional substrates. We focused initially on nuts and legumes, including cashew, peanut, and pine nut, as these are related in composition to the traditional high-protein soy-based substrate. Employing our previously developed 48-hour solid-state fermentation protocol following the traditional process, we found that *N. intermedia* robustly grew on the cashew, peanut, and pine nut substrates, as indicated by the development of orange spores and mycelia (Fig. 1). As observed with okara, the fungal mycelium bound the individual particles together, creating a more cohesive and firm texture (Fig. 1). The fermented products browned nicely upon cooking, which may be explained by the ability of *N. intermedia* to release free amino acids and sugars that can participate in the Maillard reaction (Maini Rekdal et al., 2023).

Sensory analysis (N=61 Danish consumers) of the cooked oncom products indicated variable sensory attributes and liking scores across the different substrates. In Check-All-That-Apply (CATA) sensory trials, where participants were asked to rate the sensory attributes of the foods, cashew oncom was mainly described as nutty, sweet, mushroom, fermented, and while peanut had similar attributes, it was also frequently described as both dry and crumbly (Fig. 1). In contrast, pine nut oncom was uniquely associated with negative sensory attributes such as rancid, sour,

bitter, and astringent (Fig. 1). Principal Component Analysis (PCA) of CATA data supported these observations and showed a clear differentiation in sensory attributes between pine nut and the other two substrates, and consistent with the initial analysis, this appeared to be driven mainly by the negative attributes (Fig. 2). To evaluate the broader sensory appeal of these new products, we conducted a consumer rating study using a hedonic scale across flavor, appearance, and texture (1-extremely dislike to 9-extremely like). Liking scores from these experiments demonstrated that pine nut oncom also had significantly lower liking scores across all categories (<5.7 for flavor and texture), which is consistent with its negative sensory attributes (Fig. 2). Overall, cashew was the most liked of all substrates, scoring >6.5 across all categories, which is higher than the traditional okara oncom evaluated with the same consumer group (Maini Rekdal et al., 2023). Peanut was the second most liked substrate as it performed worse than cashew but scored >6.2 in all categories. Interestingly the liking of appearance did not significantly differ between the substrates (Fig. 2). Taken together, these results highlight that solid state fermentation with *N. intermedia* can convert substrates other than okara into foods that are positively perceived by Western consumers, outside the traditional cultural context where oncom is produced and consumed. The variability in sensory attributes and liking highlights the importance of considering the unique interactions between fungi and their substrates in the development of new fermented foods.

3.2 Production of a novel amazake using *N. intermedia* enzymes for starch-to-sugar conversion

Our promising results with oncom-type fermentations led us to further investigate the *N. intermedia* substrate range. In a small-scale growth screen with different grains, we noted that *N. intermedia* grew rapidly on rice in solid-state, and that the final fermented product was intensely

sweet (data not shown). The sweetness of the rice immediately recalled the sweetness that results from the traditional solid-state fermentation of rice with koji mold (*Aspergillus oryzae*). In the traditional production of rice koji, *A. oryzae*, which is inoculated onto steamed rice from spores, secretes high amounts of the enzyme alpha-amylase during its growth (Yamashita, 2021). The secreted alpha-amylase is the key enzyme responsible for the hydrolysis of starch into smaller oligosaccharides, and alongside the activity of enzymes such as glucoamylase, releases free glucose to create sweetness (Oguro et al., 2019, Gomi, 2019). The rice koji serves as a source of alpha-amylase in many traditional Japanese fermented foods. For example, when mixed with additional steamed rice and water, rice koji enzymes break down the starch to create a fruity, sweet slurry that can be served as is (amazake) or further fermented into alcohol (sake) (Yamashita, 2021, Redzepi and Zilber, 2018, Kitamoto, 2015, Feng et al., 2013, Watanabe et al., 1998, Oguro et al., 2019). Because of *N. intermedia*'s ability to similarly create sweetness from rice, we wondered whether we could harness the fungus in a similar starch-to-sugar conversion process.

We first analyzed the enzyme activity and sugar release by *N. intermedia* during rice fermentation. Consistent with the perceived sweetness, we found that *N. intermedia* released high levels of glucose from the rice (Fig. 3). Strikingly, the free glucose levels were as high as those found with traditional koji made with *A. oryzae*. Surprisingly, however, whereas alpha-amylase was highly secreted by *A. oryzae*, we could not detect any alpha-amylase activity during rice fermentation by *N. intermedia* (Fig. 3). We also found no evidence of secreted alpha-amylase when *N. intermedia* was grown in liquid cultures with rice or starch as the sole carbon source, a condition where *N. intermedia* must release enzymes to break down the starch to support its growth (data not shown).

These results strongly suggest that *N. intermedia* uses alternative, non-alpha-amylase enzymes for starch-to-sugar conversion in the fermentation of rice.

To explore whether the *N. intermedia* enzymes for starch-to-sugar conversion could be harnessed in similar ways to *A. oryzae* alpha-amylase, we explored the possibility of producing an amazake using *N. intermedia* fermentation of rice. Similar to traditional amazake production, we first grew *N. intermedia* on rice to generate the enzyme source. The corresponding procedure using *A. oryzae* produces 'koji' and we henceforth refer to this *N. intermedia* produced koji as 'noji'. Next, we mixed the noji (1 part) with additional steamed, uninoculated rice (2 parts) and water (1 part) and subsequently heated the mixture at 50°C to activate the starch-degrading enzymes. The resulting mixture had a liquified texture, similar to amazake, suggesting that *N. intermedia* starch-degrading enzymes were active in this protocol (Fig. 4). Consistent with the sugar analysis of the fermented rice, the final total dissolved sugar content, as assessed by Brix, was nearly indistinguishable from traditional amazake made with *A. oryzae* (Table 1). It is interesting to note that while the Brix was similar between both fermentations (Table 1), the free glucose level appeared higher with *N. intermedia* (Fig. 3). This is likely due to the fact that Brix measures total dissolved solids, rather than just detecting glucose, suggesting that other saccharides such as maltose could contribute to the total Brix value. Both fungi were exceptionally effective in sugar release upon heating, increasing the Brix more than ten-fold from noji/koji to each amazake. For both fungi, the pH decreased during the initial fermentation process (Table 1). Whereas *N. intermedia* is traditionally used in solid-state to make a meat substitute, these results highlight that the fungus is perfectly suitable for enzyme production to release sugars from starch.

337 To further characterize the novel amazake made with *N. intermedia* and to understand the
338 evolution of flavor during the process, we analyzed the volatile aroma compounds in rice, koji,
339 noji, and the corresponding amazake products. While there were many aromas and changes overall
340 across the volatile aroma dataset, overall, unfermented rice had by far the lowest concentration
341 and diversity of aromas, and koji and koji amazake had more volatile compounds than noji and the
342 corresponding noji amazake (Fig. 5). The difference could mainly be attributed to esters, which
343 were more abundant in koji-based products and are often responsible for fruity aromas in
344 fermented foods (Saerens et al., 2008) (Fig. 5). Many of these ester molecules were absent in the
345 noji product. The noji amazake was dominated by ethyl acetate, which has a fruity odor, octen-3-
346 one, a lipid-derived ketone that is regarded as the characteristic aroma of molds and mushrooms,
347 as well as butanol and butanal compounds, which may arise from amino acid degradation by fungal
348 enzymes during fermentation (Feng et al., 2013). The detected butanol and its derivatives are often
349 described as fruity, alcoholic, and malty (Cameleyre et al., 2015, Smit et al., 2009). Finally, some
350 of the compounds detected in the noji decreased to undetectable levels in the final noji amazake,
351 suggesting that the heating process changes the aroma composition. Taken together, these data
352 indicate that the novel amazake made with *N. intermedia* has a distinct volatile aroma profile from
353 traditional amazake made with koji.
354
355 Despite the apparent differences in aroma composition between noji and koji-based fermentations,
356 sensory evaluation with N=61 Danish consumers did not reveal any statistically significant
357 differences in consumer preference between the two products, across all analyzed attributes. For
358 example, the taste, appearance, and aroma received nearly identical liking scores (all >5.5, Fig. 5).
359 There was a trend towards a preference for the color of the noji amazake, potentially because of

its unique orange tint, but this was not statistically significant (5.75 for koji, 6.15 for noji, p=0.19) (Fig. 5). Overall, consumers rated the koji and noji amazake products close to 5.7, which indicates a slight liking. A limitation of these trials is that the consumer group may have been unfamiliar with amazake as a product, potentially making it difficult to distinguish between the *A. oryzae* and *N. intermedia* fermentations. Nonetheless, these data indicate a positive attitude towards a novel food produced with *N. intermedia*.

3.3 Culinary application of *N. intermedia* in a fine-dining restaurant dish

Based on the research findings and optimized protocols for solid state fermentation with *N. intermedia*, a dish was developed for restaurant Alchemist (Copenhagen, Denmark). The goal was to harness the starch-degrading *N. intermedia* enzymes to unlock sweetness during solid-state fermentation without the need for any downstream processing. In line with the successful development of the noji amazake, Japanese fermentation techniques and flavors served as a guiding philosophy. The final dish consists of a solidified rice custard set on top of a gel of umeshu (a plum liquor) and mirin (a sweet, fermented traditional Japanese rice "wine" that is similar to sake). The rice custard is inoculated with *N. intermedia,* which grows on the surface, similar to an agar plate. The fungal secretion of starch-degrading enzymes creates a natural sweetness, and the fermentation also produces fruity, fermented aromas that complement the solidified gel on the bottom. In addition to the secreted enzymes and flavor transformation, *N. intermedia* gives the dish a striking orange, fluffy appearance owing to its brightly colored spores and aerial mycelia (Fig. 6). The dish was prepared using the following components:

382 *Rice base:* 250 g of sushi rice (Hokkaido Yumepirika) was soaked for 10 h at 4ºC, strained, and
383 mixed with filtered water in a ratio of 1:4 w/w. It was then blended and cooked at 80ºC at maximum
384 speed in a food processor (Thermomix TM6). Finally, it was strained through a fine mesh and kept
385 in the fridge at 4ºC.
386
387 *Rice custard:* 150 g of the rice base was mixed with 200 g of filtered water, 18 g of sucrose and
388 jellified with 1.5 g of iota carrageenan (Texturas elBulli, Spain).
389 *Umeshu and mirin gel:* 225 g of umeshu (Choya, Extra Years 17% alc) was mixed with 150 g of
390 rice wine "seasoning" (Honteri, Mizkan) and jellified with 5 g of iota carrageenan (Texturas
391 elBulli) and 0.5 g of agar agar (Texturas elBulli).
392
393 *Plating of the bowl:* 10 g of umeshu and mirin gel was jellified below 15 g of the rice custard in a
394 bowl. The bowl was kept at 4ºC until inoculation with *N. intermedia*.
395
396 *Inoculation and fermentation:* The bowl was inoculated with *N. intermedia* inoculum and
397 fermented in a constant climate chamber (Memmert HPP110eco) at 30ºC for 60 h. Following
398 fermentation, the bowl was kept at 4ºC until served. Inoculum was prepared according to the
399 "Materials and methods" section.

3. Discussion

Expanding the biodiversity of microorganisms available in the kitchen to produce fermented foods and beverages holds promise to enable new gastronomic innovation. Here, we investigated novel culinary applications of *N. intermedia*, an edible, fast-growing fungus that is traditionally used in the production of the meat substitute oncom in Indonesia. We had previously sequenced its genome and discovered that this fungus not only had a promising safety profile, but also a broad substrate range in solid-state fermentation (Maini Rekdal et al., 2023). A separate study had also highlighted that *N. intermedia* can be used to ferment stale bread into burger-like patties with promising textures, suggesting potential use in new contexts (Gmoser et al., 2020).

In the current study, we demonstrated that *N. intermedia* has gastronomic potential across a range of applications. We found that *N. intermedia* can be used not only for producing oncom-like foods with new, alternative substrates, but also as an enzyme factory for starch-to-sugar conversion to produce an amazake-type ferment. Both the oncom and amazake fermentations produced foods that were liked by Western consumers, indicating that *N. intermedia* and the fermentation protocols and approaches presented here represent a welcome addition to the chef's toolbox. For example, we envision that the oncom ferments produced in this study may present attractive alternatives to Tempeh and other fungal products frequently used as vegetarian center-of-the plate alternatives in restaurants (Nout and Kiers, 2005). As a result, oncom provides an additional approach for catering to the growing consumer demand for non-meat alternatives and the awareness of the ethical and environmental issues with certain forms of animal meat production (Fonseca and Sanchez-Sabate, 2022, Sanchez-Sabate and Sabaté, 2019, Trinci, 1992, Perez-Cueto et al., 2022). *N. intermedia* was previously demonstrated to enhance the protein content and amino acid profile during solid

state fermentation of stale bread (Gmoser et al., 2020), suggesting that this fungus could lend both sensory appeal and nutritional value to novel foods.

To our knowledge, the use of *N. intermedia* to make amazake represents a novel technique for this fungus and suggests that in addition to its ability to lend structure and flavor to center-of-the plate meat substitutes such as oncom, *N. intermedia* represents a promising source of enzymes in solid state fermentation. The ability of the fungus to release free glucose to the same levels as the koji mold *A. oryzae* is notable because whereas *A. oryzae* was likely domesticated on rice and selected by humans for its starch-to-sugar conversion (Yamashita, 2021, Kitamoto, 2015, Machida et al., 2008), *N. intermedia* has no known evolutionary history with rice and has to our knowledge not frequently been grown on rice. Unlike *A. oryzae,* however, *N. intermedia* does not appear to produce an active alpha-amylase during the fermentation of rice, raising questions of what contributes to the efficient sugar release. Some fungi are known to use alternative enzymes, such as lytic polysaccharide monooxygenases, for starch degradation, which, together with glucoamylase, could explain the sugar release (Lo Leggio et al., 2015). Future efforts are needed to identify what enzymes are secreted by *N. intermedia* during growth on rice. Regardless of their precise identities remaining unknown at this point, the starch-degrading enzymes can be easily utilized in a culinary context, as demonstrated by the development of the amazake as well as the application in a final dish for the fine-dining restaurant Alchemist. The sugar release would also likely enable the downstream production of alcoholic beverages such as sake-type beverages. Overall, these results raise the possibility that other molds and mushrooms commonly used for food production may have unexplored enzymatic potential that is completely unrelated to their traditional use.

In addition to demonstrating new culinary applications and raising intriguing questions about the biochemical basis of *N. intermedia* starch degradation, this study sets the stage for further gastronomic innovation with *N. intermedia*. For example, the fungus can produce many other gastronomically relevant enzymes, such as pectinases and cellulases (Maini Rekdal et al., 2023), and it is worth exploring whether these enzymes could be harnessed in solid-state fermentation in similar ways to the amazake process described here, for example in the transformation of complex plant substrates into free sugars. Additionally, *N. intermedia* is known to grow on a range of byproducts (Maini Rekdal et al., 2023), and while we did not investigate the gastronomic potential of these byproduct fermentations in the current study, this fast-growing fungus might be ideally suited to upcycle waste into new flavors to promote food system circularity in restaurants and beyond. Finally, in addition to its capacity for texture and flavor transformation, *N. intermedia* has a unique property that it contributes a striking orange color through the development of spores and aerial mycelia. This orange color, which comes from carotenoid pigments similar to those found in salmon and carrots, is unique among edible fungi commonly used for fermented foods (Gmoser et al., 2017). *N. intermedia* therefore provides a unique biological approach to add intriguing color and visual dimensions to foods without the need for extensive processing or manipulation.

4. Conclusion

This study establishes that *N. intermedia* can be used for oncom-type foods from diverse substrates beyond the okara substrates traditionally used in red oncom production and presents an intriguing new use of *N. intermedia* for producing starch-degrading enzymes in solid-state fermentation. This study was made possible by basic scientific investigations of fungal biology (Maini Rekdal et al., 2023), and as such, it serves yet another example of the power of bridging fundamental science

and gastronomy to drive food innovation (Mestre et al., 2022). By building upon fundamental understanding of edible microorganisms developed by scientists, as well as their own knowledge of traditional fermentation practices, chefs are ideally positioned to unlock the full gastronomic potential of the microbial kingdom in kitchens and industry alike.

Author contributions

VMR: conceptualization, formal analysis, investigation, methodology, supervision, writing. NR: conceptualization, formal analysis, investigation, methodology, supervision, writing. MO: gastronomic applications, investigation. DP: gastronomic applications, investigation. PS: project administration, resources. RM: project administration, supervision, resources. JDK: conceptualization, resources, project administration. All authors reviewed and approved the final manuscript.

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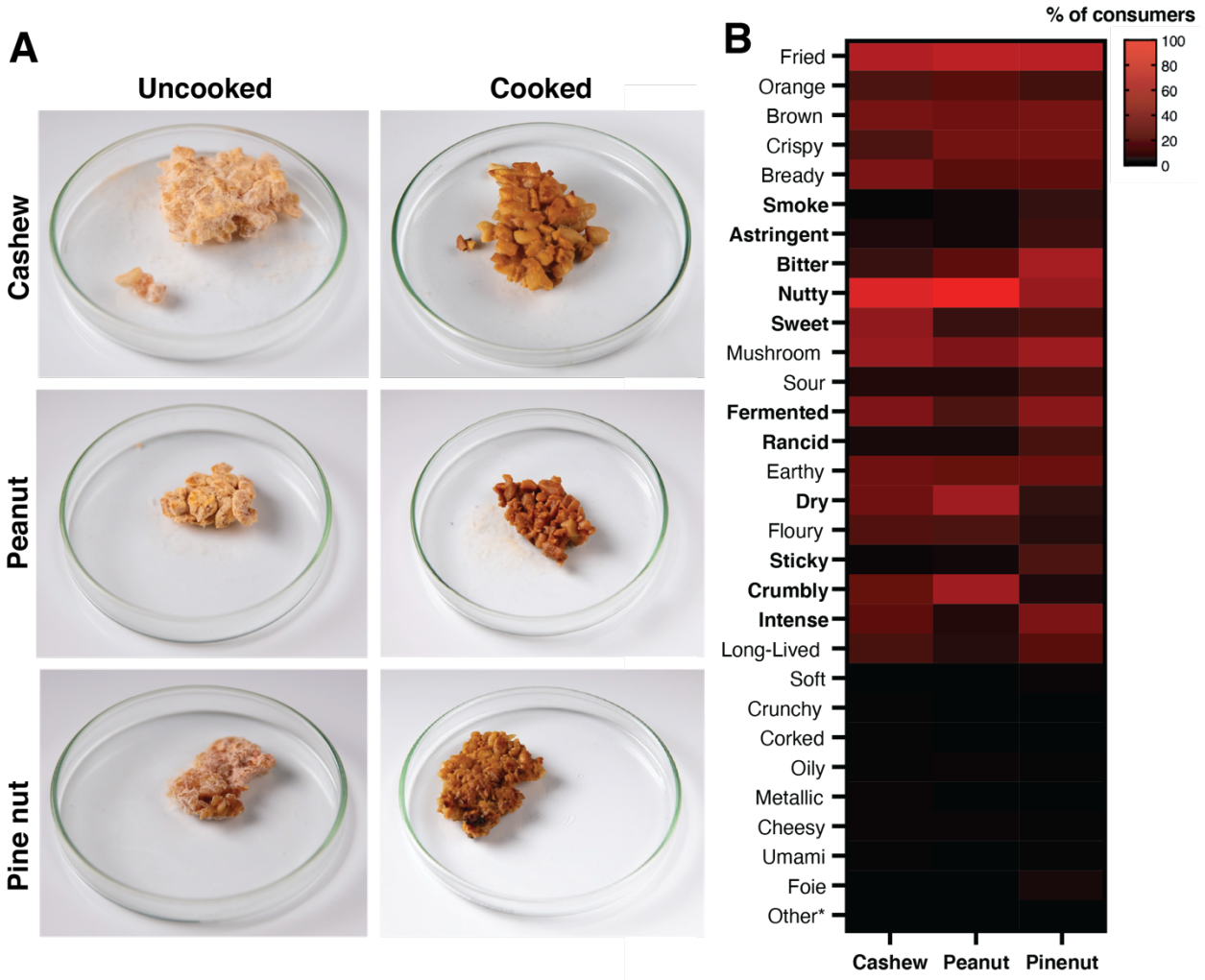
Competing interests

JDK. has financial interests in Amyris, Ansa Biotechnologies, Apertor Pharma, Berkeley Yeast, Cyklos Materials, Demetrix, Lygos, Napigen, ResVita Bio, and Zero Acre Farms. The other authors report no other conflicts of interest.

Data availability

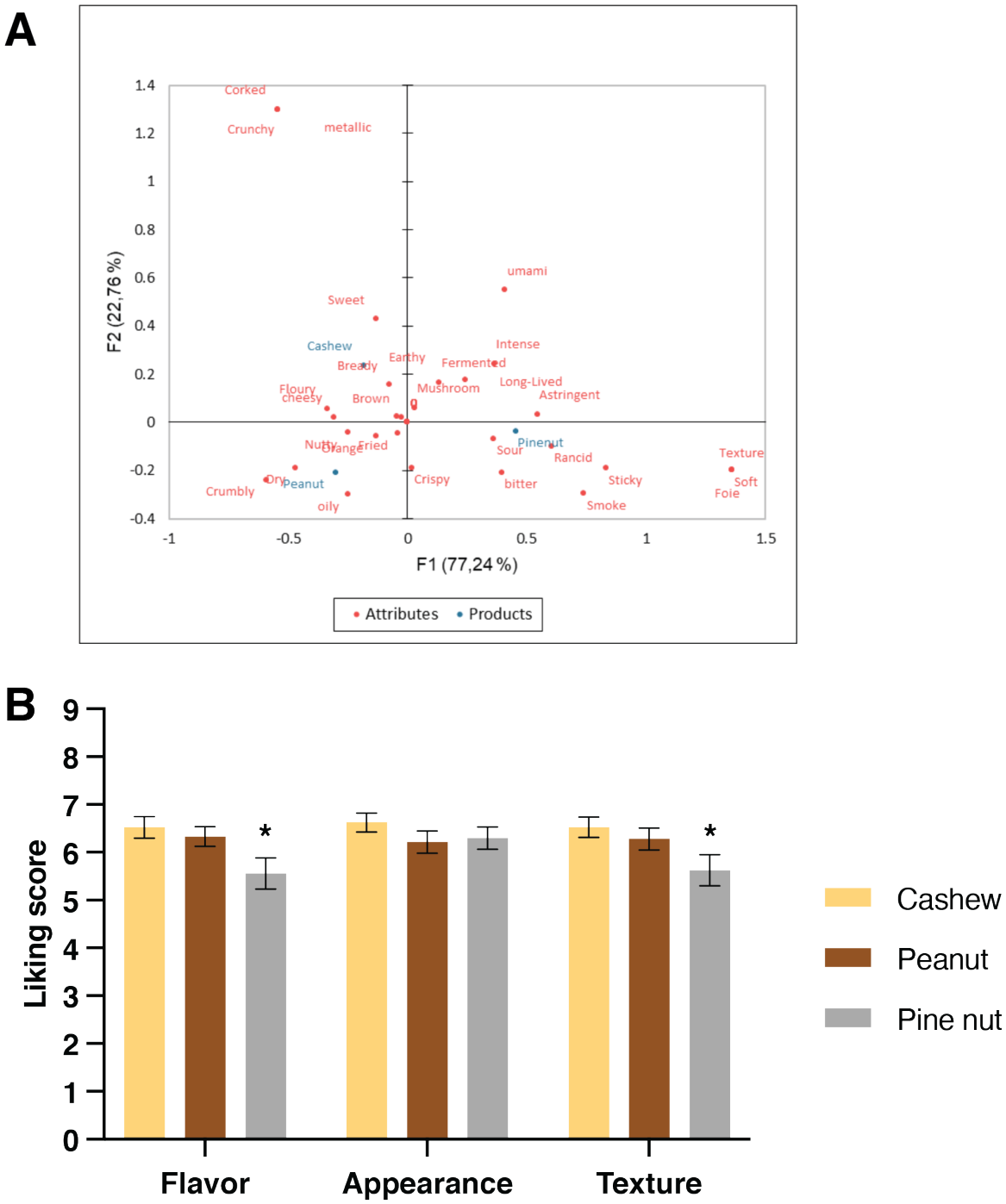
Data will be made available upon request.



501

502 **Figure 1. Appearance and sensory attributes of oncom produced from alternative substrates**
503 **in solid-state fermentation by *N. intermedia*.** A) Fermented products prior to and after cooking.
504 The fungus was grown on soaked, steamed substrates following the traditional protocol used with
505 okara. Samples were cooked by pan-frying. *N. intermedia* robustly grew on all tested substrates,
506 as indicated by the development of mycelia and the binding together of the individual nuts or
507 legumes.. B) Results from CATA sensory analysis of oncom produced with alternative substrates.
508 Heatmap colors correspond to the % of consumers (out of N=61 total) who named a particular
509 attribute in association with the food. Attributes in bold indicate a statistically significant
510 difference between the samples ($p<0.01$, Cochran's Q test with pairwise comparisons approach

based on the McNemar-Bonferroni test to identify significant differences between attributes associated with each sample). *Other indicates attributes that were not assigned to any food (value=0, includes Squishy, Grainy, Cereal, Hay, Mild, Yeasty, Fruity, Moldy) in the sensory trials.



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517 **Figure 2. Attribute association and liking scores of oncom produced from alternative**
518 **substrates in solid-state fermentation by *N. intermedia.*** A) Principal component analysis (PCA)
519 biplot illustrating the relationship among oncom samples and attributes. The data are derived from

the CATA sensory analysis presented in Figure 1. Blue indicates the substrate. Red indicates the attributes. The physical proximity of attributes and substrates highlights their relationship. Those far away from a substrate (for example, upper left or bottom right corner), did not have a clear association with a particular substrate. This graph clearly indicates that pine nut oncom is associated with distinct sensory attributes from the other two samples and is distinguished by negative attributes such as sour, rancid, bitter, and astringent. B) Sensory analysis of oncom samples using a hedonic scale for flavor, appearance, and texture (1=extremely dislike to 9=extremely like). Pine nut oncom, which was associated with negative sensory attributes, had a significantly lower rating for both flavor and texture (*=$p<0.05$, ANOVA analysis and Tukey HSD post-hoc correction). Cashew received the highest rating across all categories. Results are mean and standard error of the mean (SEM) of N=61 Danish consumers.

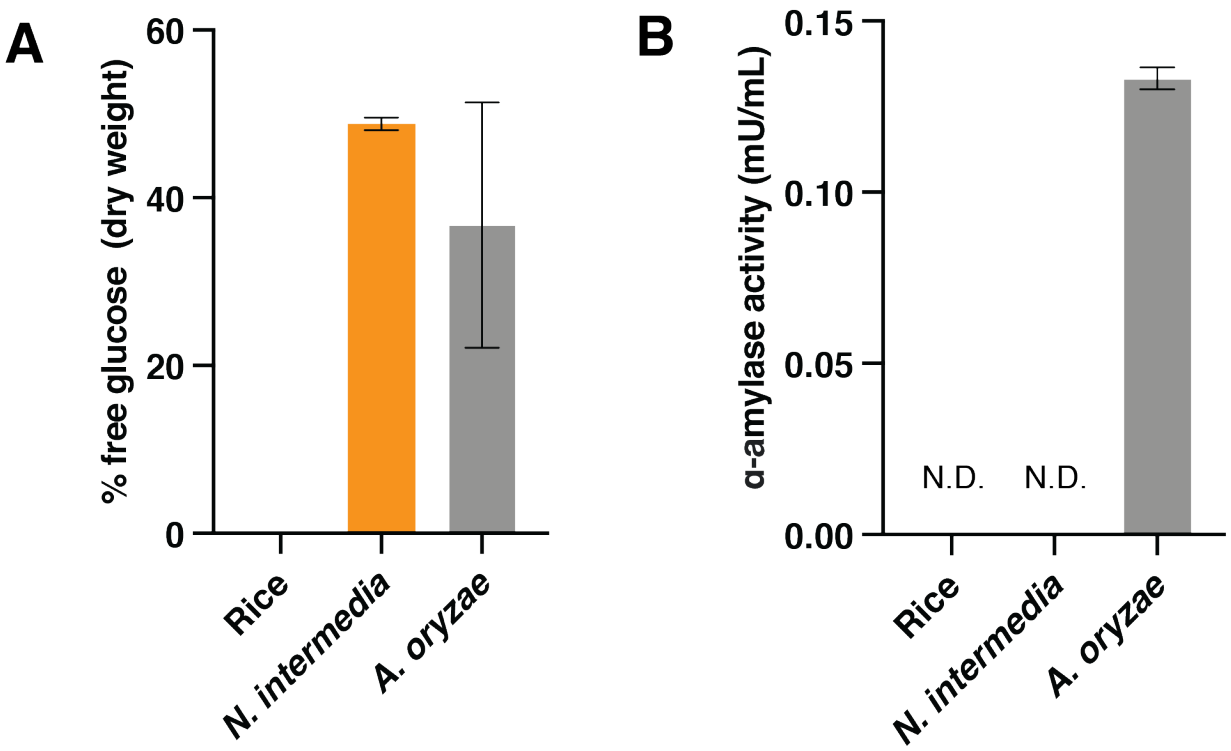

Figure 3. *N. intermedia* liberates high quantities of glucose but does not produce alpha-amylase during growth on rice. A) Levels of free glucose detected during fermentation of rice with *N. intermedia* and koji mold *A. oryzae* (control). This graph indicates high levels of glucose release during rice fermentation with *N. intermedia*, consistent with the perceived sweetness of the fermented rice. The sugar levels were analyzed at the end of the fermentation. Results are mean and SEM of three biological replicates. The results were normalized for potential dry biomass differences; thus, the variation in *A. oryzae* glucose levels likely originate from biological differences between batches. B) Alpha-amylase activity detected during fermentation of rice with *N. intermedia* and koji mold *A. oryzae* (control). Whereas *A. oryzae* uses secreted alpha-amylase for starch-to-sugar conversion, *N. intermedia* does not appear to secrete this enzyme. It was also not detected in liquid cultures when *N. intermedia* was grown with rice or starch as the sole carbon source (data not shown), strongly suggesting that alternative enzymes are involved. The enzymatic

545 activity was analyzed immediately at the end of the fermentation. Results are mean and SEM of
546 three biological replicates. N.D. = not detected.
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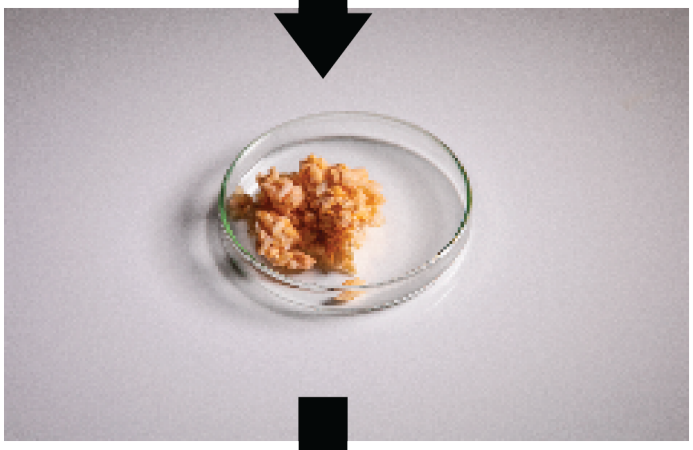
Steamed rice inoculated with *N. intermedia*

Solid state fermentation
48 hours, 29°C

Rice fermented with *N. intermedia* (Noji)



Add water, steamed rice
Heat for 10 hours, 50°C

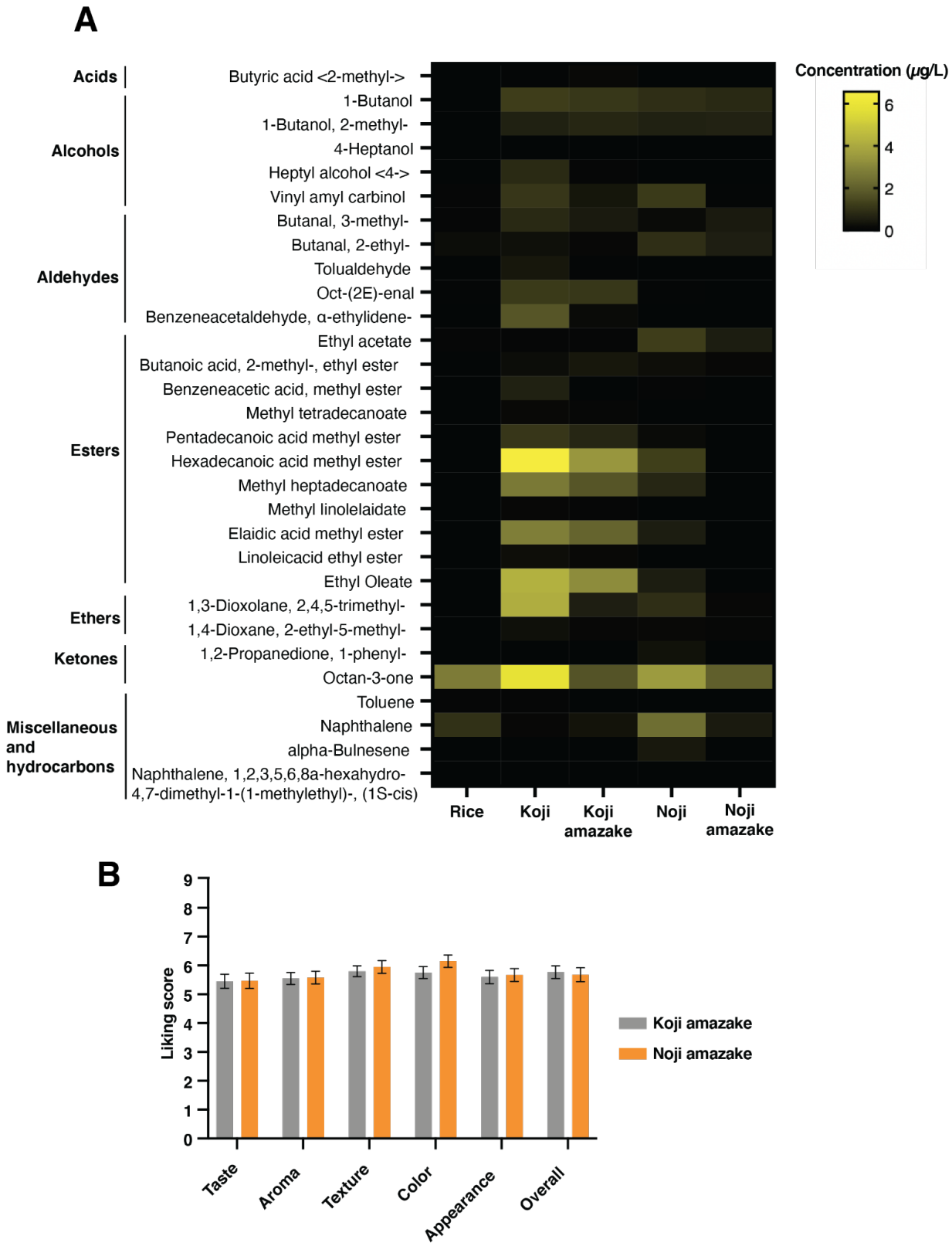
N. intermedia amazake (Noji amazake)

549

Figure 4. Production of a novel amazake using *N. intermedia.* General process for the use of *N. intermedia* starch-degrading enzymes to create a sweet "amazake". The protocol was adapted from the general process of producing traditional amazake with the koji mold *A. oryzae.* First, *N. intermedia* is grown on steamed rice, which produces enzymes. The fermented rice (called Noji, after *N. intermedia* koji) is mixed further with rice and water. Gentle heating for a short amount of time activates the enzymes and liquifies the rice (as can be seen in the picture) to generate a sweet product similar to amazake. We refer to this product as noji amazake.

Table 1. Physicochemical properties during *N. intermedia* and *A. oryzae* amazake production. pH and ºBrix were measured in triplicate samples at the end of each process. Steamed rice was measured at the start of the process to understand the evolution of the pH and ºBrix during the fermentation process. Koji = rice fermented with *A. oryzae.* Noji = rice fermented with *N. intermedia.* Results are mean ± standard deviation of three replicates.

Sample	pH	°Brix
Rice	6.47±0.17	0.10±0.00
Noji	5.23±0.09	1.50±0.08
Koji	5.10±0.03	1.60±0.02
Noji amazake	5.53±0.05	19.30±0.37
Koji amazake	5.87±0.05	21.40±0.36


A
Acids
Alcohols
Aldehydes
Esters
Ethers
Ketones
Miscellaneous and hydrocarbons
Butyric acid <2-methyl->
1-Butanol
1-Butanol, 2-methyl-
4-Heptanol
Heptyl alcohol <4->
Vinyl amyl carbinol
Butanal, 3-methyl-
Butanal, 2-ethyl-
Tolualdehyde
Oct-(2E)-enal
Benzeneacetaldehyde, α-ethylidene-
Ethyl acetate
Butanoic acid, 2-methyl-, ethyl ester
Benzeneacetic acid, methyl ester
Methyl tetradecanoate
Pentadecanoic acid methyl ester
Hexadecanoic acid methyl ester
Methyl heptadecanoate
Methyl linolelaidate
Elaidic acid methyl ester
Linoleicacid ethyl ester
Ethyl Oleate
1,3-Dioxolane, 2,4,5-trimethyl-
1,4-Dioxane, 2-ethyl-5-methyl-
1,2-Propanedione, 1-phenyl-
Octan-3-one
Toluene
Naphthalene
alpha-Bulnesene
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)
Rice
Koji
Koji amazake
Noji
Noji amazake
Concentration (μg/L)
6
4
2
0
B
Liking score
9
8
7
6
5
4
3
2
1
0
Taste
Aroma
Texture
Color
Appearance
Overall
Koji amazake
Noji amazake

564

Figure 5. Volatile aroma and sensory analysis of *A. oryzae* and *N. intermedia* amazake. A) Volatile aroma composition was determined using SPME-GC/MS. Results (reported in μg/L) are the average of three replicates. B) Sensory analysis of oncom samples using a hedonic scale (1=extremely dislike to 9=extremely like). There were no statistically significant differences in any of the attributes. However, there was a trend (p=0.19) towards higher liking of the appearance of the noji amazake. Results are mean and SEM of N=61 consumers.

Figure 6. *Neurospora* dish. A dish was developed for fine dining restaurant Alchemist (Copenhagen, Denmark) using the knowledge of *N. intermedia* solid state fermentation and starch degradation. Seen on top is the solidified rice custard inoculated with *N. intermedia,* which grows on the surface, similar to an agar plate. The rice custard is set on top of a gel of umeshu (fermented plum liquor) and mirin. The distinctly orange color comes from the fungal spores and aerial mycelia, which cover the surface as it grows on the solidified rice base that forms the basis of the dish.

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