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The Microbiota of a Lupine-based Moromi Fermentation

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Abstract

As an alternative to soy sauces, lupine-based seasoning sauce is likewise fermented in a two-step process. While in the first step a koji mould degrades macromolecules into substrates for bacteria and yeasts, in the moromi stage, aroma is formed over several months at a high salt content. In the present study, we investigated the microbiota of a lupine-based moromi in terms of dynamics, influencing factors and contribution to the process and flavour.

We were able to analyse the bacterial consortium without cultivation via 16S rRNA gene sequencing. Furthermore, we isolated bacteria and yeasts by cultivation under oxic and anoxic conditions and isolates were mainly identified by protein profiling using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. For the analysis of aroma-relevant substances, we determined volatile compounds via head-space analysis using gas chromatography–mass spectrometry.

We found out that the microbiota resembled the consortium of other wheat-free, legume-based seasoning sauces such as Korean *ganjang* and Indonesian *kecap* and no organism was recognized by us as spoilage organisms that were able to persist under the given conditions. Initially, *Candida guilliermondii* and *Weissella paramesenteroides* were the most abundant organisms, followed by *Tetragenococcus halophilus*, *Debaryomyces hansenii*, *Chromohalobacter moromii*, and finally *Staphylococcus equorum*. These organisms have already been described for other moromi or salty fermentation but we could also identify bacteria such as *Curtobacterium citreum* and *Oceanobacillus* sp. that have not been associated with food fermentation before. Of particular note is that *Chromohalobacter moromii* is a newly identified species that shares its closest genetic relationship with *Chromohalobacter beijerinckii* and possesses the capability to grow in environments with a salt concentration ranging between 5 and 25% NaCl. The lactic acid bacteria *Weissella paramesenteroides* and *Tetragenococcus halophilus* lowered the pH by forming acids like acetate facilitating the growth of yeasts like *Debaryomyces hansenii*. We assume, *Staphylococcus equorum* and the novel species *Chromohalobacter moromii* consumed acids or formed amines or ammonia which caused an increase of the pH. The aroma of matured moromi was mainly composed of acids and pyrazines which were also described for wheat-free soy moromi, and of the 4-hydroxyfuranones 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 4-hydroxy-5-methylfuran-3(2H)-one, important flavour components in all soy sauces.

We demonstrated that the microbiota is influenced by salt concentration, starter cultures or backslopping (using matured moromi), and additional carbohydrate sources such as wheat and buckwheat. At lower salt concentrations, the microbiota was more divers,

bacterial growth was enhanced and we did not detect spoilers. The use of backslopping accelerated the microbiota dynamics and yeast starters led to a very low diversity in both bacterial and yeast consortium. The addition of wheat or buckwheat both enabled *Zygosaccharomyces rouxii* to grow resulting in a delayed appearance and prevalence gain of *Tetragenococcus halophilus*. Here the aroma profile was dominated by esters, and pyrazines were partially degraded.

The current study provides new insights into the microbiota dynamics of a lupine-based seasoning sauce as well as examined methods to refine this consortium. With the demonstrated similarities to other legume-based seasoning sauces in the microbiota, these findings could also be transferable to other fermentation processes. Moreover, the isolated strains may benefit as novel starter cultures and the newly described species *Chromohalobacter moromii* can yield yet unknown metabolic abilities.

Zusammenfassung

Als Alternative zu Sojasauce wird auch Würzsauce auf Lupinenbasis in einem zweistufigen Verfahren fermentiert. Während im ersten Schritt ein Koji-Pilz Makromoleküle zu Substraten für Bakterien und Hefen degradiert, entwickelt sich während des Moromi-Schritts das Aroma über mehrere Monate bei hohem Salzgehalt. In der vorliegenden Arbeit haben wir die Mikrobiota von lupinebasiertem Moromi hinsichtlich Dynamik, Einflussfaktoren und Auswirkungen auf den Prozess und das Aroma untersucht.
Wir konnten das bakterielle Konsortium kultivierungsunabhängig mittels 16S rRNA-Gen-Sequenzierung analysieren. Darüberhinaus konnten Bakterien und Hefen durch Kultivierung unter oxischen und anoxischen Bedingungen isoliert und diese Isolate zum Großteil durch *protein profiling* mittels Matrix-assisted Laser Desorption-Ionization Time-of-Flight Massenspektrometrie identifiziert werden. Für die Analyse von aromarelevanten Stoffen wurden flüchtige Verbindungen über eine *Head-Space*-Analyse mittels Gaschromatographie-Massenspektrometrie bestimmt.
Wir konnten zeigen, dass die Mikrobiota dem Konsortium anderer weizenfreier, auf Hülsenfrüchten basierender Würzsaucen wie der koreanischen *Ganjang* und der indonesischen *Kecap* ähnelte und es wurden keine verderblichen Organismen nachgewiesen. Zunächst waren *Candida guilliermondii* und *Weissella paramesenteroides* die am häufigsten vorkommenden Organismen, gefolgt von *Tetragenococcus halophilus*, *Debaryomcyces hansenii*, *Chromohalobacter moromii*, und schließlich *Staphylococcus equorum*. Diese Organismen wurden bereits für andere Moromi bzw. salzige Fermentationen beschrieben, wir konnten zudem jedoch auch Bakterien wie *Curtobacterium citreum* und *Oceanobacillus* sp. identifizieren, die bisher nicht mit Lebensmittelfermentation in Verbindung gebracht wurden. Hierbei ist zu erwähnen, dass *Chromohalobacter moromii* als eine neue Art identifiziert wurde, genetisch eng verwandt zu *Chromohalobacter beijerinckii*, die die Fähigkeit besitzt, in Umgebungen mit einer Salzkonzentration zwischen 5 und 25 % NaCl zu wachsen. Die Milchsäurebakterien *Weissella paramesenteroides* und *Tetragenococcus halophilus* konnten den pH-Wert durch die Bildung von Säuren wie Acetat senken, was das Wachstum von Hefen wie *Debaryomyces hansenii* erleichterte. Es ist anzunehmen, dass der Anstieg des pH-Wertes verursacht wurde durch *Staphylococcus equorum* und die neue Spezies *Chromohalobacter moromii* indem diese Säuren abbauten oder Amine beziehungsweise Ammoniak bildeten. Das Aroma des gereiften Moromi bestand überwiegend aus Säuren und Pyrazinen, die auch für weizenfreie Soja-Moromi beschrieben sind, und auch aus 4-Hydroxyfuranonen, insbesondere 4-Hydroxy-2,5-dimethyl-3(2H)-furanon und 4-Hydroxy-5-methylfuran-3(2H)-on, wichtigen Geschmackskomponenten in allen Sojasaucen.

Wir konnten nachweisen, dass die Mikrobiota über die Salzkonzentration, Starterkulturen oder Backslopping (Verwendung gereiften Moromis) und zusätzliche Kohlenhydratquellen wie Weizen und Buchweizen beeinflusst werden kann. Bei niedrigeren Salzkonzentrationen war die Diversität der Mikrobiota höher, das Bakterienwachstum beschleunigt und auch hier konnten keine Verderber festgestellt werden. Die Anwendung von *backslopping* beschleunigte die Dynamik der Mikrobiota, und Hefe als Starterkulturen führten zu einer sehr geringen Diversität sowohl im Bakterien- als auch im Hefekonsortium. Die Zugabe von Weizen oder Buchweizen ermöglichte das Wachstum von *Zygosaccharomyces rouxii*, was zu einem verzögerten Auftreten und Wachstum von *Tetragenococcus halophilus* führte. Hier wurde das Aromaprofil von Estern dominiert, und zum Teil wurden Pyrazine abgebaut.

Die vorliegende Studie liefert neue Erkenntnisse über die Dynamik der Mikrobiota von Würzsauce auf Lupinenbasis und untersucht Methoden zur Beeinflussung dieses Konsortiums. Aufgrund der nachgewiesenen Übereinstimmungen in der Mikrobiota zu anderen leguminosenbasierten Würzsaucen könnte dies auch auf andere Fermentationsprozesse übertragbar sein. Darüber hinaus könnten die isolierten Stämme als neuartige Starterkulturen von Nutzen sein und die neu beschriebene Spezies *Chromohalobacter moromii* können noch unbekannte metabolische Fähigkeiten aufweisen.

List of Abbreviations

A.	*Aspergillus*
ANIb	Average Nucleotide Identity calculated using BLAST
API	Analytical Profile Index
BLAST	Basic Local Alignment Search Tool
C.	*Chromohalobacter*
Ca.	*Candida*
CFU	Colony forming units
D.	*Debaryomyces*
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen)
EPS	Extracellular polymeric substances
GC-MS	Gas chromatography–mass spectrometry
gDNA	Genomic DNA
GRAS	Generally recognized as safe
HDMF	4-Hydroxy-2,5-dimethyl-3(2H)-furanone
HEMF	4-Hydroxy-2-ethyl-5-methyl-3(2H)-furanone
HLFSS	High-salt liquid-state fermentation soy sauce
HMF	4-Hydroxy-5-methylfuran-3(2H)-one
HPLC	High performance liquid chromatography
IU	International Unit
LAB	Lactic acid bacteria
LSFSS	Low-salt solid-state fermentation soy sauce
MALDI-TOF-MS	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
ME	Malt extract
MH10	Moderate halophile
MRS	De Man-Rogosa-Sharpe
MS	Mass spectrometry
MSD	Mass selective detector
na	Not available
NIST	National Institute of Standards and Technology
PCR	Polymerase chain reaction
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
RAE	Retinol activity equivalent

RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
S.	*Staphylococcus*
s.	See
SPME	Solid-phase microextraction
T.	*Tetragenococcus*
T_m	Annealing temperature
TE	Tris-Ethylenediaminetetraacetic acid
TSB10	Tryptic soy broth
USDA	United States Department of Agriculture
v.	Version
W.	*Weissella*
Z.	*Zygosaccharomyces*

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1 Introduction

From time immemorial, fermentation is used to preserve food and beverages. While the term was previously used exclusively for anaerobic energy production from organic substances, in modern biotechnology it is also used to describe enzymatic reactions that take place under oxic conditions. Large molecules are degraded to free amino acids, mono-/disaccharides and nucleotides, which are sensorially detectable and contribute to taste and odour of the product.

To make food more flavourful, various fermented seasoning sauces are used all over the world. They are often based on protein-rich substrates and contain high amounts of salt to shape the required microbiota. About 2500 years ago, *garum* was made from salted fish and fish innards in ancient Rome and Greece, making it the oldest recorded seasoning sauce[128]. Fish and shellfish sauces are nowadays widely spread in Southeast Asia and are represented in a great variety. Also, the British Worcestershire sauce is made from anchovies and additional vinegar, molasses, and spices. On the other hand, there are also a lot of different plant-based sauces that vary in their substrates by given regional agricultural conditions. Maggi® seasoning is a wheat protein-based sauce, that was invented in Switzerland and is commonly used in many European and African countries. Although sauces based on peas and other legumes are now being tested, soy sauce is the most prominent and widely used.

1.1 Soy sauce production

The domestication of soybeans is assumed to have multiple origins in East Asia due to the divers genetic pool[1,196]. Nowadays, soybeans are used worldwide primarily as animal feed (about 76%) and only 20% of the production is used for human food, whereas the rest goes into industrial usage (from 2017 to 2019)[149]. In 2020, 353 million tons of soybeans were globally produced with Brazil having the largest market share with 121 million tons, followed by the US with 112 million tons. Since rich soils and large amounts of water are needed for cultivation of soy, it is criticized for environmental damage due to rainforest destruction and excessive use of resources[122]. Moreover, the acceptance of Roundup Ready transgenic soy plants is rather low in Europe, making the development of products based on alternative plants even more urgent[11].

Soy sauces are typically fermented in a two-step process. The sauces differ in their composition depending on regional conditions and preferences and there are no international standards for terms, quality or methodology. A draft codex standard for soy sauce

proposed at the joint food standards program of the Food and Agriculture Organization of the United Nations and the World Health Organization was not further elaborated in 2005[29]. Japanese *koikuchi shoyu* is widely spread in Japan and western countries and contains equal amounts of wheat and soybeans. After the first fermentation step, called koji, the mixture is immersed in a brine containing about 20% NaCl and fermented for months in a state called moromi[45]. Japanese alternative soy sauces are *usukuchi shoyu* (light-coloured due to short moromi fermentation), *saishikomi shoyu* (double fermented), and *shiro shoyu* (very light-coloured and low amino acid content due to high amounts of wheat and short incubation times)[52]. Moreover, for *tamari shoyu* only few or no wheat is used which leads to the deficiency in yeast growth and therefore aroma compounds but shows a higher amino acid content.

Chinese soy sauces can be divided into high-salt liquid-state fermentation soy sauce (HLFSS) and low-salt solid-state fermentation soy sauce (LSFSS) and some sauces are blended with additives like sugar, mushroom broth, starch, and spices[45]. For HLFSS, the ratio of wheat to soybeans is approximately 1:4, and brine at about 2 to 2.5 times the weight of the koji is added for moromi fermentation. The final salt concentration is at about 20% and the incubation is held at 15–30 °C for up to six months. The LSFSS is usually comprising of 3 parts wheat and 7 parts of soybeans. The moromi contains a comparably low amount of salt with about 13% NaCl and the added brine corresponds to the weight of the koji which leads to a rather solid mixture. Due to an elevated temperature of 45-55 °C and often the usage of microbial pure cultures as starters, the moromi fermentation period is reduced to 15-30 days.

The Korean soy sauce is traditionally made of *meju* and brine and is therefore called *(hansik) ganjang*[164]. For a *meju* brick, soybeans are cooked, pounded, and kneaded into a brick that is air-dried and fermented. After addition of brine at 20% NaCl, the fermentation is incubated for one to more that five years resulting in different variants of *hansik ganjang*. Korean soy sauces without the usage of *meju* are called *gaeryang ganjang* which includes the usage of rice, barley, wheat, or acid-hydrolyzed and enzyme-hydrolyzed sauces[26]. The meju brick is typically removed after a couple of months[28].

Indonesian *kecap manis* is based on black soybeans, and complemented with caramelized sugar and spices after fermentation[151]. This process is stopped after a few weeks and in some cases, no fermentation has taken place by then. There are several other soy sauces like *kya nyo* (Burmese), *toyò* (Philippinese), and *aloha shoyu* (Hawaiian) all of which have their unique composition and fermentation process, adding to the global diversity of soy sauces. Acid-hydrolyzed or sauces produced via enzyme-driven hydrolyzation will not be discussed in this work.

1.1.1 Koji

For most soy sauces, a koji fermentation is essential as the initial step. At first, soybeans are soaked in water. This loosens the hulls and moistens the beans, allowing spontaneous pre-fermentation and lowering the pH which enhances fungal growth even

further[153]. Afterwards, the soybeans are steamed or cooked and mixed with roasted and ground wheat grains[117]. For the koji fermentation, 0.05%–0.3% w/w of fungal spores of typically *Aspergillus* (*A.*) *oryzae* or *A. soyae* are added[39]. Traditionally, the fermenting koji is spread in wooden trays with a thickness of 3-5 mm height for three days[117]. The temperature is held consistent at approximately 25 °C, while stirring or automatic aeration allows metabolic produced heat to be released and oxygen to enter the koji.

The *Aspergillus* grows on these grains and beans, secreting enzymes that in addition to plant-derived enzymes degrade macromolecules into substrates for bacteria and yeasts[208]. Here, proteases for hydrolyzation of proteins to peptides and amino acids, as well as amylases for starch degradation appear to play a significant role. Several other organisms were detected in koji including yeasts like *Wickerhamomyces* and *Candida* species and bacteria like *Ochrobactrum*, *Staphylococcus*, and *Weissella* spp.[72,200].

1.1.2 Moromi

For the moromi fermentation, brine at 13 to 22% NaCl is added in a specific amount to the koji[39]. Matured moromi mash or pure cultures are added as starters to reduce the fermentation duration. The temperature is not controlled with incubation over summer or heated to about 35 °C for a shorter fermentation[208]. Traditionally, the mash is continuously stirred less frequently, from an initially daily aeration to weekly to more infrequently towards the end of the fermentation.
The high amounts of salt result in high osmotic pressure, DNA damage and effects the hydration shell of proteins, as well as the ion balance within cells[186]. This leads to the inhibition of the growth of pathogenic and spoilage microorganisms and the growth and enzymatic activity of the Koji mould is terminated[39]. It shapes a microbiota dynamic of halotolerant organisms that alters during the long term fermentation. In the early phase, the diversity is rather high and a lot of species persist within the first weeks. Due to a neutral pH, yeasts like *Pichia*, *Debaryomyces* and *Candida* were primarily found along with bacteria like *Weissella*, *Bacillus*, and lactobacilli, as well as *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, and *Staphylococcu*[66,170]. *Tetragenococcus* (*T.*) *halophilus*, the most crucial lactic acid bacterium in soy moromi, appears and contributes significantly to the production of lactic acid and acetic acid[176,177]. Due to the decreasing pH, acid-tolerant yeasts follow in succession and the koji mold disappears completely[170,208]. The most important yeast for flavour development, *Zygosaccharomyces* (*Z.*) *rouxii*, appears in this phase[66,176,191]. At the final stage, the moromi contains a consistent microbiota, often dominated by *Candida* species and *T. halophilus*[66,191].
The matured moromi is separated into sauce and solid residue via pressing in a vertical press and filtration[117]. The sauce is pasteurized at 70-80 °C for several minutes which terminates bacterial and yeast growth as well as enzyme activity[85].

Centrifugation or sedimentation of turbid materials clarify the soy sauce before bottling.

1.1.3 Aroma profile

The flavour and odour of soy sauce depends on the substrates, microbial growth, and process management and is therefore difficult to classify. Usually the evaluation is performed via sensory analysis, the components are furthermore identified via gas chromatography–mass spectrometry (GC–MS) or high performance liquid chromatography (HPLC) and the nitrogen content is typically quantified by Kjeldahl analysis [44,86,134,206]. Soy sauces all show a strong saltiness, umami taste and a roasted aroma with sweet and bitter notes. The saltiness is due to the NaCl concentration but also effected by other factors including KCl, fat, and spices [43]. The umami taste is mainly based on the amount of free amino acids resulting from protein degradation like glutamate or aspartate, although monosodium L-aspartate showed about 4 times lower umami taste intensity than monosodium L-glutamate [87]. Small amounts of several nucleotides including inosinate and guanylate are able to enhance the umami taste of these amino acids via a synergic effect [128]. Another taste interaction was observed with NaCl which showed a ternary synergism enhancing the umami flavour and, vice versa, monosodium L-glutamate boosting the perceived saltiness of sodium chloride [115].
The sweetness is based on mono- and disaccharides, hydrolyzed from starch and other large carbohydrate molecules. Nevertheless, as fermentation progresses, most of these are further metabolized into alcohols and acids or utilized for microbial growth [208]. Bitter notes are mainly due to polyphenols and free amino acids like valine, methionine, and isoleucine [42].

The aroma attributes are dependent on nearly 300 aroma compounds that could be detected in soy sauces [39]. Under aerobic conditions, alcohols in soy sauce are mainly formed from sugars and amino acids. These include ethanol (alcoholic flavour), 2/3-methyl butanol (malty, alcoholic), 2-phenylethanol (floral, sweet), as well as 2-methylpropanol (bitter), and 2,3-butanediol (fruity) [42,172]. Acids contribute to a sour flavour such as acetic acid and lactic acid but also add a cheese-like taste in case of e.g. butanoic acid, 3-methylbutanoic acid; Benzoic acid is known for a floral, fruity note, and long-chain acids like nonanoic acid and decanoic acid are more fatty in taste. As mentioned above, amino acids are the major compounds responsible for the umami taste of soy sauces. Aldehydes and ketones such as acetaldehyde (green, fruity), 1-hydroxy-2-propanone (caramel-like), benzene acetaldehyde (caramel-like) and, the most prevalent in soy sauces, 2/3-methylbutanal (malty, almond) are partly already present in the substrate. Esters are mainly fruity flavour compounds in soy sauces and occur in large quantities. They are typically formed via enzymatic esterification of alcohols with fatty acids. The most common in soy sauces are isopropenyl acetate, ethyl acetate, ethyl 2-methylpropanoate, and ethyl 3-methylbutanoate.

4-Hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) and 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (HEMF) are often referred to as the most important volatile aroma components and are frequently used to check the quality of soy sauces [104,161,172]. These and several other furanones and furans have a sweet and caramel-like flavour and their biosynthesis is still not fully understood. During the steaming of soybeans and the pasteurisation of the matured moromi, they typically result from Maillard reactions but they could also be synthesised by yeasts in the fermentation process. Furans are heterocyclic organic compounds consisting of a five-membered aromatic ring with one oxygen atom and furanones are derivatives of furan with a carbonyl group. HDMF and other furanones are found in fruits such as strawberries, but also other processed foods and beverages like wine, beer, ham, and cooked beef [158].
Another important group of flavouring substances in soy sauce are phenols. Especially 4-ethylguaiacol, guaiacol, and 4-vinylguaiacol contribute to the aroma with their smoky, burnt notes [42,86,166]. Other phenolic constituents found in soy sauces are vanillic acid, vanillin, ferulic acid and polyphenols like isoflavones. The soybeans contain these isoflavones which are responsible for bitter and stringent sensory characteristics. Phenols are typically synthesized by yeasts using precursors derived from roasted wheat [207].
Pyrazines are heterocyclic, aromatic compounds consisting of a benzene ring in which two methine groups are replaced by a nitrogen atom. They contribute to a pleasant aroma in different foods and beverages by means of roasted and nutty flavours [199]. Typically, these are formed through Maillard reaction of a carbohydrate with an amino compound at high temperatures during the roasting process [3]. In soy sauces, 2,6-dimethylpyrazine and 2,3,5-trimethylpyrazine are often detected in large quantities, but also 2-methylpyrazine, 2,5-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-methyl-6-vinylpyrazine, and other alkyl pyrazines were found in different products [3,172,199].
Several aroma relevant compounds contain sulfur which mainly originated from sulfur-containing amino acids like methionine and cysteine [104]. Here, methionol and methional are important ingredients for the soy sauce aroma, have a potato-like note, and are formed by microorganisms during fermentation or by heat degradation of sulfur-containing substrates [166,207]. Short-chain oligopeptides with a glutamyl group provide a kokumi flavour, also described as mouthfulness, complexity, and long-lasting taste [49,94,202].
There are several miscellaneous compounds that are known to contribute to the aroma and are not classified in the groups mentioned above. The terpene D-limonene gives a citrus fruit fragrance, styrene, in moderation, has a pleasant sweet odour, 3-hydroxy-2-methyl-4H-pyran-4-one (maltol) is found in most soy sauces and has a caramel-like flavour, nitrogen-containing compounds such as 2-pyrrolidinone and 2-acetylpyrrole have a roasty flavour and also alkanes like dodecane and hexadecane were detected in soy sauce [42,55,207].

In some cases, specific compounds are considered as unpleasant and should therefore be avoided or kept to a minimum. In particular, off-odours should be avoided, as the smell also affects the taste experience. These include borneol, butanoic acid (and the isomer 2-methylpropanoic acid), 3-methylbutanoic acid, biogenic amines,

dimethyl disulfide and dimethyl trisulfide[42,130,207]. To prevent these compounds to accumulate, responsible microorganisms like *Bacillus* species and biofilm-forming yeasts are prevented from growing by strict hygiene, sterilisation measures and the use of starter cultures[130].

1.1.4 Safety of the product

Microbial spoilage is defined as the undesirable change in taste, texture, and/or appearance of food caused by the growth and activity of microorganisms. Indicators for spoilage during the fermentation of seasoning sauces can be an increased pH (due to the conversion of lactic acid), a slimy or gritty consistency, visible mould growth, the formation of acetic, propionic, and butyric acids as well as the formation of biogenic amines[48,82]. Typical spoilage organisms in soy sauce are *Bacillus* species, as well as lactic acid bacteria like *Enterobacter aerogenes*, *Enterococcus faecium*, *Lactobacillus rennini*, and *Lactobacillus acidipiscis*[109,168,171]. Pathogens and spoiling organisms may contaminate the fermentation through raw materials or via the air. Nevertheless, it is unlikely that these could survive in a high-salt moromi fermentation and therefore, salt reduction should be tested carefully[39]. Moreover, microorganisms and enzyme activity are eliminated or deactivated by pasteurization after the fermentation[85].

Apart from spoilage, the high salt content and allergens of the substrates must be taken into account when enjoying seasoning sauces. Since high sodium intakes may cause hypertension or renal dysfunction, the use of high-salt soy sauce should be kept moderate[39]. The consumption of 15 mL (one tablespoon) of soy sauce can contribute to 38% of the recommended daily intake of sodium, defined by the World Health Organization. Although the gluten in the finished soy sauce should be completely hydrolysed, people who are allergic to wheat or have coeliac disease are advised to consume a wheat-free variant[107]. Moreover, about 0.27% of the population have a soy allergy and the prevalence of a sensitization after the consumption of soy-based formulas is 8.7 to 8.8%[88].
The potential carcinogen ethyl carbamate is most likely formed from the precursors ethanol and citrulline during the heat treatment at the end of the fermentation[212]. Therefore, fermented seasoning sauces may contain ethyl carbamate, if ethanol is produced during moromi fermentation. Currently, there is no regulated upper limit for this substance in fermented foods in the EU. Other possible carcinogenic compounds, 3-monochloropropane-1,2-diol and 1,3-dichloropropane-2-ol, are reported to be only detected in acid-hydrolyzed soy sauce due to the applied high temperatures[99,204].

Biogenic amines

Biogenic amines are organic bases that result from the decarboxylation of amino acids or from the amination of aldehydes and ketones. They contain one or more amine groups

and have a low molecular weight. As low amounts can influence the regulation of gastric acid secretion and body temperature, and high levels can cause intoxication symptoms like hypotension, nausea, palpitations, rashes, dizziness, headaches, tachycardia, hypertension, cardiac, and emesis, biogenic amines are potentially harmful [108]. Moreover, they can be precursors for carcinogens like N-nitrosamines when they react with nitrite [190]. Nevertheless, biogenic amines themselves do not usually pose a health risk to humans unless large quantities are ingested or the natural mechanism for degradation of amines is inhibited. Toxicological maximum limits vary between the compounds and if they are regulated, different countries established their own thresholds e.g. for histamine in fishery products or wine [136].
Throughout 40 different Chinese soy sauces, Yongmei *et al.* found tyramine, spermidine, histamine, cadaverine, and spermine in descending prevalence [209]. Later, Li *et al.* tested 53 Chinese soy sauce brands and found varying amounts of tyramine, 2-phenylethylamine, agmatine, histamine, serotonin, spermidine, putrescine, cadaverine, tryptamine, and spermine [108]. Depending on their concentrations in the product they may contribute to a bitter, sour or foul flavour. These findings show that due to the dynamic microbiota composition during the production process, soy sauces may contain biogenic amines to a certain amount and particularly high concentrations could be regarded as spoilage.
Biogenic amines are an important source of nitrogen for microorganisms and the production can be a defense mechanism of *Chromohalobacter* and other organisms against acidity [15,82,154]. Therefore, they can be detected indirectly by an increase of the pH during the fermentation.

There are several approaches to lower the level of biogenic amines in soy sauce by managing the consortium through the addition of coriander [121] or the use of starter cultures like *T. halophilus* to suppress the growth of amine-producing organisms [90]. It was also observed, that sauces with lower NaCl concentrations contained higher amounts of biogenic amines [60]. A starter culture of *Debaryomyces* (*D.*) *hansenii* might lead to the degradation of these compounds [21].

1.2 Lupine seeds as an alternative to soybeans

Due to the disadvantages such as environmental impact and allergic reaction triggered by soybeans explained above, alternative protein-rich plant seeds are tested to produce seasoning sauces with similar characteristics. Seeds of the blue, narrow-leafed lupine (*Lupinus angustifolius*) are a promising substrate and this work is based on the use of their seeds as the main substrate for the production of a novel seasoning sauce. They originate from Mediterranean Europe and North Africa and are spread to Central Europe as an ornamental plant [131]. They prefer sandy, well-drained soils and an acidic to neutral pH [194].
Therefore, Lupine seeds constitute a gluten-free, non-genetically modified alternative to soybeans and can be cultivated ecologically with short transportation routes within

Europe[56]. Compared to soybeans, lupine seeds contain similar protein levels, but a higher carbohydrate content (s. Tab. 1.1). The carbohydrates in the cell wall of the grains consist mainly of galactose, arabinose and galacturonic acid, and only traces of starch are detectable[19]. The proportion of lipids is lower in lupine seeds, especially polyunsaturated fatty acids. As a nitrogen reserve and as protection against herbivores, lupine seeds contain several quinolizidine alkaloids, which also cause a bitter taste[65]. The term 'sweet' lupine for species including *angustifolius* refers to a low alkaloid content from no to 500 mg/kg[140]. Health authorities suggest a limit of 200 mg/kg of total amount of alkaloids in lupine-derived products for safety[16,65].

Table 1.1: Nutritional values of lupine seeds and soybeans. The values of raw lupine seeds (*Lupinus albus*, NDB 16076) and raw soybeans (NDB 16108) were retrieved from the United States Department of Agriculture (USDA) FoodData Central (April 2023) [180].

Nutrient	Unit	Lupine seeds	Soybeans
Water	g	10.44	8.54
Energy	kcal	371	446
Protein	g	36.17	36.49
Total lipid	g	9.74	19.94
Carbohydrate, by difference	g	40.37	30.16
Fiber, total dietary	g	18.9	9.3
Minerals			
Calcium	mg	176	277
Iron	mg	4.36	15.70
Magnesium	mg	198	280
Phosphorus	mg	440	704
Potassium	mg	1013	1797
Sodium	mg	15	2
Zinc	mg	4.75	4.89
Vitamins			
Vitamin C, total ascorbic acid	mg	4.8	6.0
Thiamin	mg	0.640	0.874
Riboflavin	mg	0.220	0.87
Niacin	mg	2.19	1.623
Vitamin B-6	mg	0.357	0.377
Folate, dietary folate equivalents	μg	355	275
Vitamin B-12	μg	0	0
Vitamin A, RAE*	μg	0	1
Vitamin A, IU†	IU†	0	0.3
Vitamin D (D2 + D3)	μg	0	0
Vitamin K (phylloquinone)	μg	na	47.0
Lipids			
Fatty acids, total saturated	g	1.156	2.884
Fatty acids, total monounsaturated	g	3.940	4.404
Fatty acids, total polyunsaturated	g	2.439	11.255
Fatty acids, total trans	g	0	0
Cholesterol	mg	0	0

* 1 μg retinol activity equivalent (RAE) is equivalent to 1 μg of all-trans-retinol, 12 μg of all-trans-β-carotene, or 24 μg of other provitamin A carotenoids.
† 1 international unit (IU) is equivalent to 0.3 μg retinol, 0.6 μg β-carotene, or 1.2 μg other provitamin-A carotenoids.

1.3 Microorganisms involved in the moromi fermentation

Spontaneous fermentations contain a complex and divers microbiota and may be shaped by backslopping, pure starter cultures, and process management. Nevertheless, some organisms occur in many different moromi types and appear to play a major role in the process.

1.3.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore-forming, catalase-negative and typically aerotolerant anaerobes that produce lactic acid during fermentation [51]. Lactic acid fermentation are metabolic pathways in which glucose and other hexoses are converted into lactose (homofermentative) and additional end products such as CO_2, ethanol and acetate for energy production (heterofermentative). LAB are considered probiotic organisms and prevent the growth of spoilage organisms by lowering the pH [63]. Therefore, they are often used in the food industry in the fermentation of sourdough, dairy products, salami, beer and wine. However, they can also pose a risk for spoilage, such as the formation of glycerol, diacetyl and 1,2-propanediol in beer [141]. In the moromi fermentation, halotolerant LAB appear in the early phase and lower the pH of the mash [52]. Besides *T. halophilus*, *Lactobacillus* spp., *Weissella* (*W.*) *cibaria/confusa*, and *Leuconostoc mesenteriodes* were identified in soy moromi that were also present during the koji stage [176,177].

Tetragenococcus halophilus

T. halophilus belongs to the family of *Enterococcaceae* and is thus a Gram-positive, facultatively anaerobic LAB [31]. It is moderately halophilic, non-motile and usually forms tetrads of four coccoid cells. Growth requires 0.5 to 25% NaCl and is optimal at 5 to 10% [187]. The species can be divided into the two subspecies *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* and numerous strains occur simultaneously in one fermentation [83]. *T. halophilus* can adapt to osmotic stress using glutamate as the main organic solute and is able to actively uptake osmoprotectants like carnitine and glycine betaine [114,150].
In soy moromi, it is the most dominant bacterium and an important producer of lactic acid [181]. Moreover, *T. halophilus* is able to produce other short chain acids and several volatile aroma compounds such as 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, and benzaldehyde [182]. On the other hand, numerous strains are capable of producing biogenic amines, especially histamine and tyramine, which makes it advisable to select starter cultures without the decarboxylase activity [79,90].

Weissella

Weissella is a genus from the family of *Lactobacillaceae* that was previously classified as the *Leuconostoc paramesenteroides* group and, to date, comprises 19 valid species[30]. They are Gram-positive, non-spore-forming, catalase-negative like other LAB and produce lactic acid via the heterofermentative pathway. *Weissella* strains have been isolated from divers habitats like food fermentations (sourdough, sea food, Mexican *pozol*, cheese, tofu, kimchi, soy sauce) but also skin, milk, feces, saliva, and plants[53].
Especially at the beginning of the fermentation, the bacterial genus *Weissella* is predominant in the moromi with the most frequently mentioned species *W. confusa*, *W. cibaria*, and *W. paramesenteroides*[170,176]. In high-salt soy fermentation, it is replaced and vanishes after a few weeks whereas it persists under low-salt conditions[200,211]. Initially, *W. confusa* produces lactic acid to lower the pH, but on the other hand it is able to accumulate citrulline (see Section 1.1.4) due to salt stress via the arginine deiminase pathway[213]. Since some strains of *W. confusa* and *W. cibaria* have shown probiotic properties, like the formation of dextran and fructan, such salty fermentation could be a source of further probiotics[53,103].

1.3.2 Other halotolerant bacteria

Besides LAB, there are several other bacteria that contribute to the microbiota of the moromi and may have an influence on the flavour. These belong, inter alia, to the genera *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Kurthia*, *Klebsiella*, *Micrococcus*, and *Pantoea*[39].

Staphylococcus equorum

Staphylococcus (*S.*) *equorum* is a Gram-positive, non-sporulating, coagulase-negative bacterium[157]. It occurs on smear cheese, fermented sausages, and *jeotgal* (Korean salty fermented seafood) but was also isolated from horse skin, pregnant women vagina, and cat rhinitis[59,68,74,75,106,157]. There is no proven hemolytic activity nor methicillin resistance for *S. equorum* but different strains carry resistances against other antibiotics especially fusidic acid, and staphylococcal enterotoxin genes were found in some strains[46,80]. Due to potassium voltage-gated channels, they are able to grow at up to 25% NaCl[80].
S. equorum was detected in Korean *ganjang* and other *Staphylococcus* spp. could be found in Japanese soy sauce[121,176]. It can potentially contribute significantly to the flavour formation, for instance through the synthesis of diacetyl, 3-methyl-3-butenol, 2-butanone, methyl-branched ketone, acetoin, and traces of methyl-branched aldehydes[38,163].

Chromohalobacter

Chromohalobacter is a Gram-negative, oxidase and catalase-positive bacterial genus, that belongs to the family *Halomonadaceae*[185]. Currently, there are nine species included in this genus, one of which was described in the present work[119]. They are moderately halophilic and can grow at 5 to 30% NaCl, mainly by producing the compatible solutes ectoine and hydroxyectoine[184]. The majority of *Chromohalobacter* species were isolated from salterns and seas; in contrast, *Chromohalobacter* (*C.*) *beijerinckii*, *C. japonicus* and *C. moromii* were food associated as they were isolated from salted beans, Japanese salty food, and lupine moromi, respectively[119,139,174]. Additionally, one strain of *C. salexigens* was isolated from *ganjang* and this genus has also been detected in other works[28,82]. Their contribution to the flavour is yet unknown, but potentially they can add organic acids and undesired biogenic amines such as putrescine[82]. They might also synthesize extracellular polymeric substances (EPS), which may vary the rheologic properties[146].

1.3.3 Yeasts

In the fermentation, yeasts are particularly important for the flavour formation and typically appear after the pH decrease by LAB[161]. They produce alcohols including ethanol, higher alcohols, and also furanones and phenolic compounds[67]. Since the synthesis of these aroma compounds is rather slow, these processes determine the total fermentation time of the moromi, provided a rich flavour is desired[161]. The amount of degradable carbohydrates is crucial for yeast growth[62,151,164]. This leads to a lower influence of yeasts on fermentations without the addition of carbohydrate sources, such as Indonesian *kecap* or Korean *ganjang*, than on Japanese *koikuchi shoyu*.
Zygosaccharomyces rouxii can be isolated from the moromi at sufficiently low pH and subsequently *Candida etchellsii* and/or *C. versatilis*[161]. Furthermore, other yeasts including *Debaryomyces hansenii*, *Torulaspora delbrueckii*, *Candida guilliermondii*, and additionally *Cryptococcus*, *Microbotryum*, *Tetrapisispora*, and *Wickerhamomyces* can be detected[39,159,164,165]. Especially in low-salt fermentations, undesired bacterial growth can be suppressed by using yeast starters such as *Torulaspora delbrueckii* and *Candida guilliermondii*[164].

Zygosaccharomyces rouxii

Zygosaccharomyces is a genus placed within the family *Saccharomycetaceae* and was formerly assigned to the genus *Saccharomyces*[13]. Its high osmotolerance makes it an important yeast in food fermentation such as soy sauce, but also an undesirable spoilage agent in other foods and beverages including bread, fruit juice, wine[167]. *Z. rouxii* is able to grow at 0 to 70% sucrose, 0-20% NaCl and also has a tolerance for up to 13% ethanol[36,77,92,129,147]. Against osmotic stress, glycerol and/or D-arabitol are synthesized, imported and accumulated intracellularly[36]. To maintain membrane functions

at high salt concentrations, Na^+ cations are exported and the membrane fluidity is decreased by an increased degree of fatty acid saturation.
It prefers a pH of 3.5 - 5.5 and therefore appears in the moromi subsequently to the acid production by LAB[147,161]. Dependent on the NaCl concentration, it produces alcohols including about 2% ethanol, additionally 2-methyl-1-propanol, 3-methyl-1-butanol, and methionol and also highly desired 4-hydroxyfuranones[67,161]. The optimum NaCl concentration for alcohol and HEMF formation in moromi is at 17-18%, whereas the synthesis by a pure culture decreases with increasing amounts of salt[155]. The fermentation of sugar into alcohol is necessary for yeast growth under the low oxygen conditions in the moromi and due to maintenance of the proton gradient, only possible at low pH (3-4)[77,161]. *Z. rouxii* is limited in the use of different sugars and prefers to metabolise fructose rather than glucose[151,162,215]. It is sensitive to acetic acid, which is formed by *T. halophilus*, with the effect being intensified at a low pH[132,133].

Debaryomyces hansenii

The genus *Debaryomyces* belongs to the family *Saccharomycetaceae* and the species *D. hansenii* is also known as *D. tyrocola* var. *hansenii*, *Saccharomyces hansenii*, *Pichia hansenii*, *Torulaspora hansenii*, and *Candida* (*Ca.*) *famata*. It was initially isolated from marine water, and also from plants, cheese, wine, beer and soil[13,95]. It is weak-fermenting and is able to produce acids from a lot of different sugars including maltose, galactose, sucrose, and lactose[215]. Its osmotolerance enables *D. hansenii* to grow in media containing up to 23% NaCl or 60% sucrose[18,95]. *D. hansenii* responds to salt stress with similar mechanisms as *Z. rouxii*[36]. It is commonly used in the production of sausages, minced meat and cheese but can also be considered a spoiling agent in brine-preserved food[18,37,50,71].
It was also detected in fermented soy products such as *doenjang* (Korean fermented soybean paste), *ganjang*, and *kecap*, neither of which contains wheat[91,151,159,165]. As its potential aroma contribution, the yeast is able to produce methyl ketones which have a fruity, floral (rose), moldy, cheesy, or wine-like odor and alcohols like 2-phenylethanol, 2-methylpropanol, 2-methylbutanol, and 3-methylbutanol[47,98].

1.4 Aim and Hypotheses

The fermentation of soy sauce is a complex process and not yet fully understood. Depending on the substrate and the process management, different microorganisms participate in the fermentation and contribute to the characteristics of the resulting seasoning sauce. As an environmentally friendly option particularly for the European market, lupine-based seasoning sauce is a promising alternative. To create a soy-free, gluten-free, and salt-reduced seasoning sauce, the addition of wheat should be avoided and the salt content reduced without compromising the safety of the product. Due to the novel environment, the occurrence of yet unknown species and strains is possible.

Moreover, knowledge about isolated microorganisms can be beneficial in other salty fermentations and food processing for instance to understand spoilage effects and the benefits as a starter culture. Their metabolic pathways can be useful for the degradation of waste products or for the biosynthesis of metabolites that may be used for technical or medical applications.

In this thesis, the following hypotheses will be discussed:

1. **The microbiota of lupine-based moromi fermentation and the resulting flavour is equivalent to soy moromi due to the similar substrate composition and process.**
   A lupine-based moromi fermentation should be carried out on a laboratory scale under constant conditions. Bacteria and yeast should be isolated and cultivated to allow further analysis of these organisms. In addition, a culture-independent method should be used to verify the data and detect organisms that show no growth under the given conditions. GC-MS should be carried out to detect aroma compounds and pH will be measured to control acid production and altered growth conditions.

2. **A reduction in salt content alters the microbiota and, at a certain threshold, allows the growth of spoilage organisms or pathogens.**
   Simultaneously with the standard conditions at 20% NaCl, moromi batches should be sampled with altered NaCl concentration. The microbiota, pH value (as an indicator for spoilage) and aroma compounds should be compared at different times during fermentation.

3. **Lupine-based moromi is a promising source of new strains and species due to the novel habitat and growth conditions.**
   Isolated strains of promising organisms should be further investigated on genome level and regarding their physiological traits. Genomes of bacteria that could not be identified via the applied methods should be sequenced partially or fully and compared to valid species. If these cannot be assigned to the described taxa, they are to be characterised as new taxa.

4. **The microbiota and in particular yeast growth is dependent on the amount of carbohydrates and can therefore be modified by the addition of a carbohydrate source.**
   Lupine seeds contain a greater proportion of carbohydrates than soybeans, which implies that adding carbohydrate sources may not be necessary. To investigate the influence of additional carbohydrates, wheat and the gluten-free alternative buckwheat should be added to moromi batches. Especially the growth of *Z. rouxii*, the most dominant yeast in soy moromi, and *D. hansenii*, the major yeast in lupine-moromi, should be tested with the different substrates during fermentation.

2 Materials and Methods

Important materials and default methods used in this work are described in the following. Moreover, deviations and applied methods are shown along with the data.

2.1 Microbiological methods

2.1.1 Strains and isolates

In this work, a lot of microbial strains were isolated and identified from lupine- and buckwheat-based moromi. For further investigations, isolates of *Chromohalobacter spp.*, *S. equorum*, and *D. hansenii* were used in several experiments (s. Table 2.1). *D. hansenii* TMW 3.1188 was used as a starter for moromi and the whole genome was sequenced.

Table 2.1: Isolates from lupine moromi

Species	TMW No.	Moromi source	DSMZ* No.
C. canadensis	2.2310	10 % NaCl (week 1)	
C. canadensis	2.2316	20 % NaCl (week 6)	
C. canadensis	2.2317	20 % NaCl (week 6)	
C. moromii	2.2299	15 % NaCl (week 8)	
C. moromii	2.2304	20 % NaCl (week 8)	
C. moromii	2.2308	20 % NaCl (week 8)	113153
D. hansenii	3.1188	10 % NaCl (week 12)	
S. equorum	2.2497	10 % NaCl (week 6)	
S. equorum	2.2498	13.5 % NaCl (week 8)	
S. equorum	2.2499	13.5 % NaCl (week 12)	
S. equorum	2.2500	20 % NaCl (week 12)	

* Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures, DSMZ)

Moromi isolates of *S. equorum* was compared to isolates of other habitats. These are listed in Table 2.2.

Table 2.2: Staphylococcus isolates from other habitats

Species	TMW No.	Source
S. equorum	2.1355	bat feces
S. equorum	2.1763	Italian roe deer salami
S. equorum	2.1766	Italian salami
S. equorum	2.2343	domestic rabbit feces
S. equorum	2.2344	domestic rabbit feces
S. equorum	2.2347	domestic rabbit feces

In several cases, isolates were compared to closely related strains that were purchased from strain collections (s. Table 2.3). These were integrated to the in-house matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) database for a more reliable identification. *Z. rouxii* strain DSM 2531 and the isolate *D. hansenii* TMW 3.1188 were used as starter cultures for the moromi fermentation presented in Section 2.2.

Table 2.3: Type strains used in this study

Species	Strain	Source	Reference
C. beijerinckii	LMG 2148	Salted beans	[139]
C. canadensis	DSM 6769	Medium contaminant	[8,73]
C. israelensis	DSM 6768	Dead Sea	[8,73]
C. japonicus	CECT 7219	Japanese salty food	[174]
C. marismortui	DSM 6770	Dead sea and solar saltern	[185]
C. nigrandesensis	DSM 14323	Lake Tebenquiche	[144]
C. salexigens	DSM 3043	Saltern	[9]
C. sarecensis	DSM 15547	Saline in Andean region	[145]
Z. rouxii	DSM 2531	Japanese miso	

2.1.2 Cultivation conditions

For the cultivation of *Chromohalobacter spp.* and *S. equorum*, cultures were incubated at 37 °C under oxic conditions in tryptic soy broth (TSB10, pH 7, Table 2.5) by default. *D. hansenii* and *Z. rouxii* were incubated in malt extract media (ME, pH 5.6, Table 2.4) at 30 °C under oxic conditions. LAB like *T. halophilus* were inoculated in De Man-Rogosa-Sharpe medium (MRS, pH 5.7, Table 2.6) at 30 °C under anoxic conditions. The cultivation media for moromi samples were ME for yeasts, and MRS or TSB10 for bacteria. Glucose was soluted and autoclaved separately from the media to avoid Maillard reactions. NaCl were added to these media according to the compositions in this chapter; Adjusted amounts for several experiments are mentioned along with the results. The pH of all media was adjusted with HCl or NaOH (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) solution and autoclaved at 121 °C for 20 min. For agar plates, agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added to the adjusted medium at 15 g/L. To test growth under anaerobic and microaerobic conditions, agar inoculated plates were incubated in anaerobic jars with AnaeroGen sachet (Thermo Fisher Scientific Inc., Waltham, USA) or CampyGen Compact sachet

(Oxoid AGS,Basingstoke, United Kingdom).
Cultures in MRS medium were filled in falcon tubes and inoculated without shaking for anoxic cultivation. Plated cultures on MRS agar were grown under N_2 gas atmosphere or with Oxoid AnaeroGen sachets (Thermo Fisher Scientific Inc., Waltham, USA).

Table 2.4: Composition of ME medium

Compound	Concentration [g/L]	Supplier
Malt extract	20	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Soy peptone	2	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	50	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Table 2.5: Composition of TSB10 medium

Compound	Concentration [g/L]	Supplier
Casein peptone/tryptone	15	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Soy peptone	15	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	100	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Table 2.6: Composition of MRS medium

Compound	Concentration [g/L]	Supplier
Casein peptone/tryptone	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Meat extract	2	Merck KGaA, Darmstadt, Germany
Yeast extract	4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween80	1	Merck KGaA, Darmstadt, Germany
$K_2HPO_4 \times 3\ H_2O$	2.5	VWR International, Radnor, USA
Sodium acetate $\times 3\ H_2O$	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
di-Ammonium citrate	2	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MgSO_4 \times 7\ H_2O$	0.2	Sigma-Aldrich, St. Louis, USA
$MnSO_4 \times H_2O$	0.05	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	50	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-Glucose	20	Merck KGaA, Darmstadt, Germany

For type strains of *Chromohalobacter spp.* moderate halophile medium (MH10, pH 7.5) was prepared according to Table 2.7. All salts for Subov's salt solution were dissolved separately, sterile-filtered, and added to the medium after autoclaving.

Table 2.7: Composition of MH10 medium

Compound	Concentration [g/L]	Supplier
Proteose peptone	5	Thermo Fisher Scientific, Germany
Yeast extract	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-Glucose monohydrate	1	Merck KGaA, Darmstadt, Germany
Subov's salt solution		
NaCl	78	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MgCl_2 \times 6\ H_2O$	14	Sigma-Aldrich, St. Louis, USA
$MgSO_4 \times 7\ H_2O$	20	Sigma-Aldrich, St. Louis, USA
$CaCl_2 \times 2\ H_2O$	0.367	Sigma-Aldrich, St. Louis, USA
KCl	2	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$NaHCO_3$	0.067	Gerbu Biotechnik GmbH, Heidelberg, Germany

2.1.3 Conservation of organisms

Pure cultures were stored for long-term cryoconservation at -80 °C from fresh cultures with 40% (v/v) glycerol (Gerbu Biotechnik GmbH, Heidelberg, Germany). In preparation for experiments, strains were inoculated with cryoconserved cultures for 48 h and stored at 7 °C for up to two weeks. To avoid increased mutations, these were transferred a maximum of two times if necessary. For each experiment, precultures of strains were prepared in 5 mL of the same medium as used in the main culture.

2.1.4 Salt tolerance

To determine the salt tolerance of strains, TSB medium was prepared with different salt concentrations according to Table 2.5. We used NaCl concentrations of 0 to 30% in 2.5% steps. To avoid protein precipitation, all components were completely dissolved before NaCl was added and the volume was adjusted. Three pre-cultures were incubated and added to the prepared media in a 96 well plate (Sarstedt AG & Co., Nümbrecht, Germany) to an OD_{600nm}= 0.05 in triplicates. In the SPECTROstar Nano plate reader (BMG LABTECH GmbH, Ortenberg, Germany), the plates were measured every 10 min for 1.5 days after with 5 min double orbital shaking period at 500 rpm.

2.1.5 Temperature optimum

To investigate the temperature range and optimum of bacterial strains, triplicates of pre-cultures in TSB10 medium were incubated for 24 h at 30 °C. These were spread on TSB10 agar plates and incubated at 4, 15, 20, 25, 30, 37, 40, 42, and 45 °C. Plates at 4 °C and above 37 °C were wrapped in plastic bags to avoid drying. Plates were checked for growth after 8 h, 16 h, 24 h and then after each 24 h. First signs of growth as well as total cell counts were recorded.

2.1.6 pH optimum

For the determination of pH range and optimum of bacterial strains, buffered TSB10 was prepared. Therefore, citrate buffer (pH 4, 5, and 6), potassium phosphate (pH 7, and 8), Tris-HCl buffer (pH 9) and barbonate-bicarbonate buffer (pH 10) were prepared in 0.1 M solutions, sterile filtered and stored at room temperature (s. Table 2.8).

For the media, the buffers are diluted 1:10 and used instead of water in TSB10 medium preparation; Apart from this, it was prepared according to Table 2.5. For the experiment, the strains were cultivated in the different media in triplicates and growth was measured every 10 min for 1.5 days in a SPECTROstar Nano plate reader (BMG LABTECH GmbH, Ortenberg, Germany). In each case, three pre-cultures were used and the experiment

Table 2.8: Buffer components for pH range determination

pH	Component	concentration [M]	Supplier
4.0	tri-Sodium citrate dihydrate	0.0338	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
	Citric acid	0.0662	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
5.0	tri-Sodium citrate dihydrate	0.0581	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
	Citric acid	0.0419	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
6.0	tri-Sodium citrate dihydrate	0.0825	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
	Citric acid	0.0175	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
7.0	K_2HPO_4	0.0536	Merck KGaA, Darmstadt, Germany
	KH_2PO_4	0.0464	Merck KGaA, Darmstadt, Germany
8.0	K_2HPO_4	0.0935	Merck KGaA, Darmstadt, Germany
	KH_2PO_4	0.0065	Merck KGaA, Darmstadt, Germany
9.0	Tris	0.1000	Gerbu Biotechnik GmbH, Heidelberg, Germany
	HCl		Carl Roth GmbH & Co. KG, Karlsruhe, Germany
10	$NaHCO_3$	0.0461	Gerbu Biotechnik GmbH, Heidelberg, Germany
	Na_2CO_3	0.0539	Merck KGaA, Darmstadt, Germany

was carried out in a 96 well plates (Sarstedt AG & Co., Nümbrecht, Germany) with an initial OD_{600nm}= 0.05.

2.1.7 Biogenic amine formation

For every amine, a separate medium (s. Table 2.9) with 1% of an amino acid was prepared and, as a control, one medium without amino acid addition.

TSB10 medium (s. Table 2.5) with 0.1% of all precursor amino acids and 0.005% pyridoxal-5-phosphate (as cofactor) was prepared for pre-cultivation of bacterial strains. The precultures were incubated in daily fresh medium for four days to increase the enzyme activity of decarboxylases. For the experiment, the individual media were inoculated with an initial OD_{600nm}= 0.1 of the pre-cultured strain and incubated at 37 °C and 220 rpm for up to 4 days. This was carried out in a 48 well plate (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany), sealed with parafilm (Bemis Company Inc., Oshkosh, USA) to prevent drying. A positive reaction was

Table 2.9: Composition of biogenic amine medium

Compound	Concentration [g/L]	Supplier
Casein peptone/tryptone	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Meat extract	5	Merck KGaA, Darmstadt, Germany
NaCl	2.5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-Glucose monohydrate	1	Merck KGaA, Darmstadt, Germany
Tween80	1	Merck KGaA, Darmstadt, Germany
$MgSO_4 \times 7\ H_2O$	0.2	Sigma-Aldrich, St. Louis, USA
$MnSO_4 \times H_2O$	0.05	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$FeSO_4 \times 7\ H_2O$	0.04	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
K_2HPO_4	2	Merck KGaA, Darmstadt, Germany
$CaCO_3 \times 2\ H_2O$	0.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Pyridoxal-5-phosphate	0.05	Sigma-Aldrich, St. Louis, USA
L-Tyrosine disodium salt*	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Histidine monohydrochloride*	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Ornithine monohydrochloride*	10	Sigma-Aldrich, St. Louis, USA
L-Lysine monohydrochloride*	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromcresol purple	0.06	Merck KGaA, Darmstadt, Germany

* Each of these components was added to a separate medium.

visible by the purple coloration of the medium or by the disappearance of the tyrosine precipitate.

2.1.8 Antibiotic resistances

To identify if *S. equroum* isolates carry functional genes for antibiotic resistances, we performed a zone of inhibition assay. Therefore, over-night cultures (in TSB medium without NaCl at 37 °C and 200 rpm) of the strains were diluted to an OD_{600nm}= 0.5 and evenly plated on Müller-Hinton agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Sterile filter plates (BD Sensi-Disc, Becton Dickinson GmbH, Heidelberg,

Germany) were placed with maximum spacing and each was covered with 10 μL of antibiotic solution (s. Table 2.10, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The plates were incubated at 37 °C for 24 h and diameters of zones of inhibition were measured and compared to thresholds.

Table 2.10: Antibiotic solutions for sensitivity assay

Antibiotic	**Concentration [μg/10 μL]**	**Threshold diameter [mm]**	**Reference**
Ampicillin	2.0	18	[54]
Penicillin G	3.0	12-18	[70]
Oxacillin	1.0	20	[54]
Tetracycline	30.0	19	[54]
Erythromycin	15.0	18	[54]

2.2 Moromi fermentation

The koji was prepared using lupine seeds (*Lupinus angustifolius*) and spores of *A. oryzae* as starter. Previously, the lupine seeds were partially soaked, roasted, and crushed. The koji was incubated in an aerated tank for 48 h at a guided temperature dynamic of 25 to 35 °C.
The moromi was prepared in plastic beakers with an end volume of 600 mL, consisted of 40% (w/v) koji, 60% brine at usually 13.5% (w/v) NaCl and 0.07% (v/v) matured moromi as starter. The batches were incubated for six months by default at 25 °C at a humidity level of 80%. The evaporation was compensated with deionised water and the batches were mixed for ventilation once a week. Every moromi fermentation was done in duplicates from the same koji batch for each experiment.

To test the influence of additional carbohydrate sources, the koji for this experiment was supplemented with 30% (w/w) roasted wheat grains, buckwheat, or lupine seeds as a control. To start the moromi fermentation, 60% (w/v) brine (13.5% NaCl) and starter cultures of *D. hansenii* TMW 3.1188 and *Z. rouxii* DSM 2531 at 10^5 CFU/mL were added.

2.2.1 Moromi sampling

Each Moromi batch was regularly sampled to detect changes during the fermentation. 10 mL of moromi liquid were centrifuged at 7000 ×g for 6 min and the supernatant was diluted to a NaCl concentration of 5%. The pH was measured herefrom by means of 761 Calimatic pH meter (Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany). The pellet was washed in deionized water and dissolved in RNAlater (Invitrogen, Lithuania). This was stored at -20 °C and used for 16S rDNA amplicon sequencing after the end of the fermentation. 5 mL of liquid and solid moromi were stored in 20 mL glass vials with additional NaCl (end concentration 25%) at -80 °C until measurement for gas chromatography [198]. The samples were slowly thawed before the measurement to avoid the breakage of glass vials.
Moromi liquid was diluted in isotonic Ringer's solution (Merck KGaA, Darmstadt, Germany) supplemented with 5% NaCl and plated 5 to 6 times on ME, TSB10, and MRS agar (s. Section 2.1.2) using sterile glass beads (2.7 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). These were incubated for 4 days and CFU/mL were counted on all plates with 10 to 200 colonies larger than 0.5 mm. 100 colonies per condition were picked using sterile wooden toothpicks and streaked one to three times on fresh plates to isolate strains. These were identified via MALDI-TOF-MS or sequencing according to the protocols in Sections 2.3.1 and 2.4.3. 8 g of moromi liquid was taken and stored at 7 °C before the extraction of furanones and measurement via HPLC-MS. For sensory analysis, 50 mL moromi liquid was filtered and cooked at 80 °C before the samples were offered to the panel.

2.3 Biochemical analytics

2.3.1 MALDI-TOF-MS for species identification

To identify microorganisms at species level, we used MALDI-TOF-MS for a subproteome-profiling. Single colonies e.g. isolated from moromi-inoculated plates were smeared onto stainless steel targets (Bruker Daltonics GmbH, Bremen, Germany) [89]. The proteome was extracted on the target by applying 1 µL of a 70% (v/v) formic acid (Sigma-Aldrich, St. Louis, USA) and air-dried. These spots were coated with a matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid matrix (Merck KGaA, Darmstadt, Germany) solved in organic solvent solution (50% acetonitrile, 47.5% deionized water, 2.5% trifluoroacetic acid, from Bruker Daltonics, Bremen, Germany) and air-dried. The isolates were then measured using a Microflex LT Spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser ($\lambda = 337$ nm). For the identification, detected spectra were compared with the Bruker Biotyper database (Bruker Daltonics, Bremen, Germany) and an intern database, based on spectra of 16S rRNA gene sequenced strains and published type strains.

2.3.2 Gas chromatography–mass spectrometry

GC-MS was used to analyse volatile aroma compounds of moromi samples. As mentioned in Section 2.2.1, the samples were added up to a final concentration of 25% NaCl before measurement. The method was adapted from the measurement method described by Sun et al. [172]. The compounds were extracted from the sample at 50 °C for 40 min in a heat block. For the desorption, we used a polydimethylsiloxane/divinylbenzene (PDMS/DVB) solid-phase microextraction (SPME) fibre (Superlco, Bellfonte, USA) that was conditioned at 250 °C for 2 h before use. Therefore, the SPME device was inserted into the injection port and heated to 250 °C for 5 min. The injection into the GC 7890A (Agilent Technologies, Santa Clara,USA) was conducted in splitless mode with helium as carrier gas (flow rate 1 mL/min). We used a Zebron ZB-WAX column with a film thickness of 0.25 µm (Phenomenex, Aschaffenburg, Germany) and the temperature of the injector and detector was kept constant at 250 °C. The oven temperature for the measurement started at 40 °C for 2 min, was raised by 5 °C/min up to 230 °C and held for 15 min. Detection was performed in electron impact mode via a a 5975C mass selective detector (MSD) and a Triple-Axis Detector (Agilent Technologies, Santa Clara, USA) and masses were analyzed between 34 and 400 m/z. The temperature of the ion source was set at 230 °C and at 150 °C for the triple quadrupole. The resulting spectra was analyzed with the MassHunter Workstation Qualitative Analysis Software (Agilent Technologies, Santa Clara, USA) and identified by comparison with the National Institute of Standards and Technology (NIST) database [2].

2.3.3 High-performance liquid chromatography

Furanones could not be detected via GC-MS and were therefore extracted from the moromi and analyzed by HPLC-MS. 8 g of moromi were used for the extraction with 10 µL phenol (Merck KGaA, Darmstadt, Germany) and 25 mL distilled water. The solid residue after centrifugation at 5000 × g for 10 min was re-suspended in 25 mL water and centrifuged. The supernatant was subjected on an Amberlite XAD-2 polymeric adsorbent (20 – 60 mesh width; Merck KGaA, Darmstadt, Germany) for solid-phase extraction. 50 mL water were used to rinse the column and the compounds were eluted with 100 mL diethyl ether (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The extract was dried with sodium sulphate and further concentrated using a Laborota 4000 efficient rotary evaporator (Heidolph Instruments, Schwabach, Germany) constricted with a nitrogen stream for drying. The sample was transferred into 100 µL water and the aqueous phase was analyzed by an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, USA). It was composed of two isocratic pumps (G1310A), a micro well plate autosampler (G1377A), a column thermostat (G1316A), a diode array detector SL (G1315C), and an Agilent 6320 ion trap. The LUNA C18(2) column (150 mm × 2.0 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) was held at 28 °C during the measurement. Solvent A, containing water and 0.1% formic acid, and solvent B, containing methanol and 0.1% formic acid, were used for the binary gradient system. The program for HPLC analysis is shown in Table 2.11.

The injection volume was 5 µL and 10 mg/mL phenol was used as an internal standard. The compounds were identified due to their retention times and mass spectra compared to reference material and quantified through calibration curves and diode array detection (UV traces 272 nm for phenol, 290 nm for furaneol and 284 nm for norfuraneol).

Table 2.11: Gradient program for HPLC analysis. The gradual changes in the composition of the mobile phase are shown in percentages, with a constant flow rate of 0.2 mL/min.

Gradient time [min]	**Solvent A [%]**	**Solvent B [%]**
0	100	0
0-10	90	10
10-50	70	30
50-65	20	80
65-70*	0	100
70-80*	100	0

* These compositions were set directly without a gradient.

2.3.4 Physiological characterisation

For the characterisation of a novel species, several traits and metabolic capacities were tested. To detect a Gram-negative cell wall, a drop of 3% (w/v) KOH (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) solution was mixed on a glass slide with a fresh colony and the increase in viscosity was observed. For the determination of catalase activity, a drop of 1% (w/v) H_2O_2 solution (Merck KGaA, Darmstadt, Germany) was applied and foaming was observed. The hydrolysis of starch, Tween80 and casein was examined according to Cowan et al. [33]. To determine citrate utilization, the growth on Simmons' citrate agar (Merck KGaA, Darmstadt, Germany), with citrate as the sole carbon and nitrogen source, was observed. To determine their motility and the ability to produce H_2S, strains were pricked and incubated in sulphide indole motility agar (pH 7.3, s. Table 2.12).

For the determination of multiple other metabolic capabilities, the Analytical Profile Index (API) tests API 20NE and API ID 32E (both bioMérieux, Marcy-l´Étoile, France) were used according to the protocols. All media were supplemented with 10% (w/v) NaCl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for halophilic strains. and 10% (w/v) NaCl solution was used instead of isotonic salt solution provided by the kit. Pre-cultures were incubated for 24 h on TSB10 agar and a 10% (w/v) NaCl solution was inoculated with an initial OD_{600nm} between 0.08 and 0.13. Paraffin oil (Sigma-Aldrich, St. Louis, MO, USA) was coated over each tube where it was required. The inoculated strips were incubated under the indicated conditions and interpreted according to the protocols.

Table 2.12: Composition of sulphide indole motility agar

Compound	**Concentration [g/L]**	**Supplier**
Casein peptone	20	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Meat extract	6	Merck KGaA, Darmstadt, Germany
Sodium thiosulfate	0.3	Merck KGaA, Darmstadt, Germany
Ammonium ferric citrate	0.2	Sigma-Aldrich, St. Louis, USA
NaCl	100	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agar-agar	3.5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

2.3.5 Sensory Analysis

Various processed lupine sauces were tested by a sensory panel for the perceptible flavour components and their intensity. As mentioned in Section 2.2.1, moromi was heated before examination to ensure the safety of the product and to induce heat-dependent chemical reactions. In preparation for the analysis, panel members were selected based on their sensitivity to certain aroma substances. Therefore, a group of 15 people had to identify and assess the intensity of different smelling sticks. The chosen panel consisted of 3 women and 7 men, of which seven were fully trained in the food sector. The questionnaire for the analysis was based on typical soy sauce flavours[42] and results of the GC-MS analysis:

- fruity
- floral
- vinegar
- fermented
- wine
- mushroom
- mould
- earth
- grassy
- hay
- smoky
- burnt
- caramel
- chocolate
- nutty
- honey
- sweet
- bee wax
- cheese
- fatty
- umami
- Maggi®
- cooked vegetables
- soup
- bitter
- astringent
- salty
- sour
- rancid
- stinky
- alcoholic
- sweaty
- goat
- fish
- cooked meat

During the sensory analysis, each participant received a 25 mL beaker with 1 mL of the sample. The intensity of perceived attributes was assigned from 1 to 4 with 1 as very weak to 4 as strongly pronounced.

2.4 Molecular biological methods

2.4.1 Bacterial DNA extraction

The strain to be examined was incubated for 24 h at 30 °C in a suitable medium and 2-4 mL culture were harvested via centrifugation at 7000 × g. The cell pellet was washed in Tris-Ethylenediaminetetraacetic acid (TE) buffer (pH 8.0, 1 mM Ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris; ThermoFisher Scientific, Waltham, USA) before

use. The isolation of genomic DNA (gDNA) of most bacteria was conducted using the E.Z.N.A. Bacterial DNA Kit (VWR International, Radnor, PA, USA) following the manufacturer's instructions. For Staphylococci, the cell lysis was performed via beat beating 5 times for 30 s at 4.5 m/s using a FastPrep-24 (MP Biomedicals GmbH, Eschwege, Germany) and glass beads (0.5 mm). Extracted DNA was eluted in 2 × 20 µL pre-heated elution buffer and stored at -20 °C. The concentration and purity was controlled by a NanoDrop® ND-1000 spectrophotometer (Peqlab, VWR International GmbH, Darmstadt, Germany). For whole genome sequencing, the quality of gDNA was examined by agarose gel electrophoresis (s. Section 2.4.4).

2.4.2 Yeast DNA extraction

For the isolation of gDNA of *D. hansenii* TMW 3.1188, the strain was cultivated for 24 h at 30 °C in ME medium. Cells were harvested via centrifugation of 45 mL culture at 6000 × g for 5 min and DNA was isolated with the YeaStar Genomic DNA Kit (Zymo Research Europe, Freiburg im Breisgau, Germany). For cell disruption, the cell pellet was re-suspended in 2375 µL digestion buffer and 93.75 µL R-Zymolyase (5000 U/mL) and incubated at 37 °C for 1 h. 2375 µL lysis buffer was added and mixed gently. For DNA extraction, 4850 µL of phenol-chloroform (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were added and gently mixed. The mixture was centrifuged at 16,000 × g for 5 min and the upper, aqueous phase was transferred to a new falcon tube with a cut pipette tip to avoid shredding of the DNA. Ammonium acetate (7.5 M, Sigma-Aldrich, St. Louis, USA) was added to the sample in 0.5 parts and ethanol (VWR International, Radnor, USA) in 2.5 parts of this mixture and gently mixed. For the precipitation of DNA, the sample was kept at -80 °C for 2 h and centrifuged at 1 °C at 16,000 × g for 20 min to carefully remove the supernatant. The pellet was dried at room temperature for 10 min, re-suspended in 1000 µL TE buffer (pH 8.0, 1 mM EDTA and 10 mM Tris; ThermoFisher Scientific, Waltham, USA) with a cut pipette tip, and transferred to a new falcon tube. 40 µL Proteinase K (20 mg/mL; ThermoFisher Scientific, Waltham, USA) and 100 µL RNase (1 mg/mL; Zymo Research Europe, Freiburg im Breisgau, Germany) were added and the sample was incubated at 40 °C for 1 h. The DNA purification with phenol-chloroform was repeated and the aqueous phase was gently mixed with the same amount of chloroform (Sigma-Aldrich, St. Louis, USA) to remove residual phenol. After centrifugation, the DNA in the aqueous phase was precipitated again with ammonium acetate and ethanol at -80 °C for 2 h. The supernatant was carefully removed after centrifugation at 1 °C at 16,000 × g for 20 min and washed with ethanol (70%; VWR International, Radnor, USA) and dried by removing the supernatant after centrifugation 16,000 × g for 2 min twice. The genomic DNA was re-suspended in 300 µL TE buffer and stored at 4 °C.

2.4.3 Polymerase chain reaction

To amplify a specific DNA sequence, polymerase chain reaction (PCR) was performed using Taq DNA polymerase (MP Biomedicals Inc., Eschwege, Germany) and extracted gDNA (s. Section 2.4.1 and 2.4.2). The sequences were targeted using specific primers, provided by Eurofins Genomics (Ebersberg, Germany), listed in Table 2.13.

The mixture for PCR, composed of the Taq DNA CORE Kit 10 (MP Biomedicals, Irvine, USA), along with the thermal cycle program is provided in Table 2.14. Amplified 16S and 28S rDNA was purified using the QIAquick PCR Purification Kit (Qiagen N. V., Venlo, Netherlands).

For the intraspecific determination of strains, a PCR targeting randomly amplified polymorphic DNA (RAPD) was performed[32,193]. Therefore, one unspecific binding primer (M13V) was used in the usual concentration (1 μM) and $MgCl_2$ added at 5 mM using a $MgCl_2$-free incubation buffer. The cycle program was divided in three cycles with denaturation for 3 min at 95 °C, annealing for 5 min at 40 °C and elongation at 72 °C for 5 min followed by 30 cycles of denaturation for 1 min, annealing at 60 °C for 2 min and elongation for 3 min. The strain specific amplicons were compared via agarose gel electrophoresis.

To determine if the isolated and sequenced *D. hansenii* strain TMW 3.1188 was a diploid yeast or contaminated, it was streaked five times to ensure purity and four regions on the different chromosomes where amplified via PCR. The results of several colonies were compared by agarose gel electrophoresis.

Table 2.13: List of Primers. The indicated annealing temperature (T_m) is the applied temperature in the cycler program.

Name	**Application**	**Sequence [5'→ 3']**	**T_m**
616F	16S rDNA	AGA GTT TGA TYM TGG CTC AG	52 °C
609R	16S rDNA	ACT ACV VGG GTA GTA TCT AAK CC	52 °C
V9	28S rDNA	TTA CGT CCC TGC CCT TTG TA	60 °C
LR5	28S rDNA	TCC TGA GGG AAA CTT CG	60 °C
M13V	RAPD	GTT TTC CCA GTC ACG AC	
9050_F	Determination of hybrid yeast	GCA ATT GAA GGA AAA GAT G	55 °C
9050_R	Determination of hybrid yeast	AAT AGT CTT GGC GGC TCT A	55 °C
7190_F	Determination of hybrid yeast	ACA ATT AAA GGA AAA ATT C	55 °C
7190_R	Determination of hybrid yeast	GAT AGT CTT AGC AGC TCT G	55 °C
2020_F	Determination of hybrid yeast	CAG GAT ATC ATA TTG TTG	55 °C
2020_R	Determination of hybrid yeast	CGA AAG TTG TTT GTA TGC	50 °C
1670_F	Determination of hybrid yeast	TTC AAG ATA TTA TAT TAT TAA CCG	50 °C
1670_R	Determination of hybrid yeast	GAA AGT GGT TTG GAT CT	50 °C

Table 2.14: PCR for amplification of target DNA with Taq DNA polymerase.

PCR composition		**Cycler program**		
Component	Concentration [μL]	Temperature [°C]	Time [s]	
10× Incubation Mix +$MgCl_2$	2.50	94	120	
10 mM dNTP mix	0.50	94	45	30x
10 μM Forward Primer	0.25	T_m *	90	30x
10 μM Reverse Primer	0.25	72	120	30x
20 ng/μL gDNA	1.00	72	300	
Taq polymerase	0.25	12	∞	
HPLC grade water	ad 25.00			

* Annealing temperatures are provided in Table 2.13.

2.4.4 Agarose gel electrophoresis

Samples of extracted DNA and PCR products were tested via agarose gel electrophoresis. Therefore, gels with 1.4% (w/v) agarose dissolved in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) for RAPD PCR products or 1% (w/v) agarose in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.2) for every other sample were poured. Samples were mixed with 6× loading dye (ThermoFisher, Waltham, MA, USA) and a 1 kb DNA ladder (ThermoFisher Scientific, Waltham, MA, USA) was applied as reference. The electrophoresis was carried out at 100 to 130 V in a Peqlab electrophoresis chamber (Peqlab, VWR International GmbH, Darmstadt, Germany). Afterwards, the gel was bathed for 20 min in a saturated dimidium bromide solution (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), washed in demineralized water for 10 min and the stained DNA was visualized under UV illumination with a UVT-28M transilluminator (Herolab, Wiesloch, Germany).

2.5 Bioinformatic tools

Digital controlling, processing and storage of results were needed in all cases. Used software and databases are listed in Table 2.15.

Table 2.15: Software and databases

Tool	Application	Supplier/Reference
BioNumerics v.7.6	Clustering of RAPD-PCR band patterns	Applied Maths, Belgium
BLASTn/BLASTp *	Sequence similarity identification	[5]
ClustalW	Multiple sequence alignments	[97]
FoodData Central	Nutrient profile data	[180]
GGDC v.3.0	*in silico* DNA-DNA hybridization	[127]
JspeciesWSweb v.3.9.0	ANIb† calculation	[148]
MALDI Biotyper Automation FlexControl	Definition of measurement parameters and target layout	Bruker Daltonics, Germany
MALDI Biotyper Compass Explorer	Management of mass spectra databases	Bruker Daltonics, Germany
MALDI Biotyper FlexAnalysis	Analysis of single mass spectra profiles	Bruker Daltonics, Germany
MALDI Biotyper Realtime Classification	Mass spectra comparison	Bruker Daltonics, Germany [183]
MassHunter Acquisition Software	Control of GC and MSD for data acquisition	Agilent Technologies, USA
MassHunter Workstation Qualitative Analysis Software	GC compound analysis	Agilent Technologies, USA
Mega11 v.11.0.13	Phylogenetic tree calculation	[175]
NCBI Prokaryotic Genome Annotation Pipeline	Genome deposit & Annotation	[64,110,178]
NIST Chemistry WebBook	Reference for GC analyses	[2]
Office Professional Plus 2019	Data analysis and visualisation	Microsoft, USA
Perseus v.1.6.14.0	Genomic data comparison	[179]
SnapGene Viewer v.7.0	Genome viewer	Dotmatics, USA
Unicycler (Galaxy v.0.5.0 +galaxy1)	Genome assembly	[192]

* Basic Local Alignment Search Tool (BLAST)
† Average Nucleotide Identity calculated using BLAST (ANIb)

3 Results

3.1 Microbiota dynamics and volatile compounds in lupine based Moromi fermented at different salt concentrations

The full text of

Lülf, R. H., Vogel, R. F., Ehrmann, M. A. Microbiota dynamics and volatile compounds in lupine based Moromi fermented at different salt concentrations // International Journal of Food Microbiology. 9 2021, 354. 109316.

is provided in Appendix Section A.1 [120]. The supplementary data to this publication are shown in the Appendix Section A.2.

Summary

The microbiota and aroma compounds of different kinds of soy-based seasoning sauces were already investigated prior to this work. Moreover, alternative substrates for plant-based seasoning sauces were tested; however, there are no studies on lupine seeds as a substrate so far [128]. The salt concentration in the moromi defines the microbiota and its reduction requires an investigation to determine effects including the growth of potential spoilers.
The pH dropped within the first days of lupine moromi stage indicating the formation of organic acids, and it increased subsequently with up to 15% NaCl starting after 2 to 3 months. Unlike soy moromi, here no isolate of *Z. rouxii*, *Ca. versatilis*, nor *Ca. etchelsii* were identified using MALDI-TOF-MS [151,176]. Instead, *Ca. guilliermondii*, *Trichosporon loubieri*, and subsequently *D. hansenii* were detected and were dominating in the first few weeks with negligible differences depending on the salt concentration. Similar to soy moromi, LAB like *W. paramesenteroides* and *T. halophilus* dominated the bacterial consortium [176,200,210]. Other predominating bacteria were *C. japonicus* and in the late phase, *S. equorum* that were both also found in Korean *ganjang* [82,121]. The reduction of salt led to an early appearance of these species and additionally, the bacterial cell counts were particularly low at 20% NaCl compared to salt-reduced batches. Furthermore, 16S rRNA gene amplicon sequence analysis revealed the presence of *Oceanobacillus* sp. but no spoiling organisms like *Bacillus* or *Enterococcus* were found in any batch tested. The aroma was dominated by pyrazines and acids, but also alcohols,

esters and ketones were detected via GC-MS. No 4-hydroxyfuranones were found and only in a low-salt fermentation at 10% NaCl, some furanones were synthesized after months of incubation.

Investigating how to shape the yeast consortium, the addition of carbohydrate sources was tested and presented in Section 3.2. Isolates that are referred to as *C. japonicus* in this publication were further investigated and the results are shown in Section 3.4.

Author contributions

Rebekka H. Lülf was responsible for conceptualization, formal analysis, and wrote the original draft of the manuscript including visualization of the data. Rudi F. Vogel and Matthias A. Ehrmann developed the conceptual setup, and did the project administration, supervision and edited the manuscript.

3.2 Carbohydrate Sources Influence the Microbiota and Flavour Profile of a Lupine-Based Moromi Fermentation

The full text of

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is provided in Appendix Section A.3 [118]. The supplementary data to this publication are shown in the Appendix Section A.4.

Summary

As shown in Section 3.1, the microbiota of lupine-based moromi differs from soy moromi especially in yeast occurrence. The absence of *Z. rouxii* and other yeasts might be due to the lack of degradable carbohydrates. For *Z. rouxii* it was shown, that sugar fermentation is necessary for growth under anoxic conditions and the variety of usable saccharides is limited [77,151,161,162,215]. To investigate which yeast persists in a moromi with an additional carbohydrate source, the addition of wheat as the standard carbohydrate source in many soy sauces, and buckwheat as a gluten-free alternative was tested.
Therefore, 30% roasted wheat grains or buckwheat were mixed with the lupine seeds before the koji process and afterwards, starter cultures of *Z. rouxii* DSM 2531 and *D. hansenii* TMW 3.1188 were added to the moromi. In contrast to the standard conditions, the addition of wheat and buckwheat resulted in a temporary higher diversity of the bacterial consortium and in the appearance of *Z. rouxii*. Nevertheless, the final microbiota was similar under all conditions, namely dominated by *T. halophilus* and *D. hansenii*. Additional carbohydrate sources led to an aroma composition with more esters and less pyrazines, and HPLC analysis revealed that the concentration of 4-hydroxy-5-methylfuran-3(2H)-one (HMF) was increased. There were no significant differences in the taste and odour. Since the flavour formation takes a lot of time, we concluded that a longer persistence of *Z. rouxii* due to higher amounts of wheat or buckwheat might result in a distinct aroma [161].

Author contributions

Rebekka H. Lülf was responsible for conceptualization, formal analysis, and wrote the original draft of the manuscript including visualization of the data. Karl Selg-Mann conceptualised and conducted the sensory analysis. Thomas Hoffmann conceptualised and conducted the quantification of 4-hydroxyfuranones via HPLC-MS. Tingting Zheng did the statistical evaluation of the sensory analysis and data visualisation and also wrote parts of the original draft of this method. Melanie Schirmer was responsible for supervision and statistical evaluation. Matthias A. Ehrmann developed the conceptual

setup, and did the project administration and supervision. All co-authors reviewed and edited the manuscript.

3.3 Genome Sequence of the Diploid Yeast *Debaryomyces hansenii* TMW 3.1188

The full text of

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is provided in Appendix Section A.5 [119].

Summary

As described in Section 3.1 and 3.2, *D. hansenii* and *T. halophilus* are the predominant microorganisms in lupine-based moromi. For a further investigation of their co-existence, genomes of both organisms were necessary and for *D. hansenii* only one strain, TMW 3.1188, was identified.
Prior to this publication, the genome of other *D. hansenii* strains were already investigated and haploid as well as polyploid strains were published [138]. The majority of strains have six chromosomes with sizes of 9.4 to 12.6 Mb; However, the number of chromosomes varies from 5 to 10. The strain TMW 3.1188 was isolated from lupine-based moromi with 10% NaCl and the genomic DNA was isolated via phenol-chloroform extraction. The whole genome was analysed via PacBio sequencing and 26 contigs were assembled using HGAP4 and SMRT Link. The genome comprises two sets of 6 chromosomes and has a total size of 24.77 Mb. The GC content is at 36.23% and the data is available under accession number GCA_024256405.1. The 28S rDNA sequence is deposited under accession number OP179623.

Author contributions

The authors Link and Lülf share the first authorship due to equal contribution to this work. Tobias Link was responsible for data analysis, visualization, and wrote parts of the original draft of the manuscript. Rebekka H. Lülf did the investigation, formal analysis and wrote parts of the original draft. Marina Parr and Maik Hilgarth helped with the data analysis and reviewed the manuscript. Matthias A. Ehrmann developed the conceptual setup, and did the project administration, supervision and edited the manuscript.

3.4 *Chromohalobacter moromii* sp. nov., a moderately halophilic bacterium isolated from lupine-based moromi fermentation

The full text of

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is provided in Appendix Section A.6 [119]. The supplementary data to this publication is shown in the Appendix Section A.7.

Summary

Although *Chromohalobacter* has already been detected in soy moromi, its contribution to the microbiota dynamic or flavour formation is still unknown [28,82]. In lupine-based moromi, they appear to play a major role, even at different salt concentrations (s. Section 3.1). Since they might produce organic acids, biogenic amines or EPS, they should be further investigated [82,146].
Several lupine moromi isolates were identified as *C. japonicus* with barely enough similarity in their spectra via MALDI-TOF-MS. Strain TMW 2.2308^T showed the closest relation to *C. japonicus* CECT 7219^T with 99.67% identity in their 16S rDNA sequence and 93.12% as the highest ANIb value to *C. beijerinckii* LMG 2148^T. Therefore, it was proposed as a novel species named *Chromohalobacter moromii*. Optimal growth was observed at 37 °C (with a range of 4 - 45 °C), pH 7 (range 6 to 9) and 10 to 12.5% NaCl. The assembled genome consisted of 6 contigs with a total genome size of 3.47 Mb and showed a GC-content of 61.0%. Using an API test system, no amino acid decarboxylase activity could be detected.

The strains TMW 2.2299 and TMW 2.2304 were also isolated from lupine-based moromi and assigned to the new species *C. moromii*. TMW 2.2308^T was proposed as the type strain and deposited at the DSMZ (DSM 113153^T) and at the *Colección Española de Cultivos Tipo* (Spanish Type Culture Collection, CECT 30422^T). The genome is available under accession number JAHXDI000000000 and the 16S rDNA sequence has the accession number OM585595.

Author contributions

Rebekka H. Lülf was responsible for conceptualization, formal analysis, and wrote the original draft of the manuscript including visualization of the data. Maik Hilgarth supervised parts of the project and reviewed the manuscript. Matthias A. Ehrmann

supported the conceptual setup, and did the project administration, supervision and also edited the manuscript.

4 Discussion

In the following, we placed the results from the publications in Chapter 3 in a context and supplemented them with further data that were generated during the processing period but have not yet been published (s. Appendix Section A.8). In conclusion, the hypotheses formulated earlier were evaluated on the basis of the findings.

4.1 The microbiota of lupine-based moromi

The initial microbiota of lupine-based moromi is a rather divers consortium of mainly lactic acid bacteria and different yeast species. After several months, the microbiota is dominated by *T. halophilus* and *D. hansenii* under all tested conditions. By modifying the process, we were able to delay or accelerate the appearance and persistence of specific organisms. These findings might also be useful in other salty fermentations and for work in model systems with pure and co-cultures.

In this work, we could show the influence of different external factors on the moromi microbiota including the NaCl concentration, carbohydrate addition and inoculation of starter organisms. Due to the nutritional composition of lupine seeds as the sole substrate, a lack of glucose and other usable sugars might be the reason for the disappearance of most yeast species after a week. On the other hand, yeast growth was less effected by salt stress than bacterial growth. In Section 3.2 we demonstrated, that the microbiota was less divers than in previous experiments, independent of the addition of wheat or buckwheat. This is due to the use of yeast starters, that appear to suppress the growth of other yeast species and several bacteria. Unfortunately, the high impact of yeast starters on the bacterial consortium obscured the effects of wheat and buckwheat on bacterial growth.

It was also observed that in a spontaneous fermentation different bacterial species persist longer in the moromi (for at least two weeks) than with backslopping where *T. halophilus* already dominates the microbiota after two weeks (s. Appendix Section A.8 Figure A.1). Besides backslopping and starter cultures, the microorganisms could originate from the lupine seeds and should therefore be found in the koji state (s. Appendix Section A.2 Figure S1) or from the salt [208]. While early moromi yeasts and bacteria such as *Candida* and *Weissella* originate from the koji, the salt may contain extreme halophilic and spore-forming organisms such as *Bacillus*, *Oceanobacillus* and archaea. [156]. In our experiments, purified NaCl was used but for industrial manufacturing, sea salt is used. Through backslopping with moromi from exactly this facility, we could possibly

still find organisms in the experiments that originated from the marine salt. The use of purified salt is therefore not recommended for the production of soy sauce and similar fermentation processes[28,208]. To investigate the influence of NaCl or carbohydrates on the microbiota, we decided to use pure NaCl to eliminate other influencing factors.

4.1.1 Yeasts

Ca. guilliermondii is one of the yeast species detected at the beginning of the fermentation. It grew in all batches at 10-20% and had the largest share at 20% NaCl. It was missing in batches with additional yeast starters instead of backslopping and was also detected in the koji. It disappeared after 7 days (after 14 with 10% NaCl), which is not directly related to the pH drop or the appearance of another organism. Since the CFU decreased at the same time (except for 10%), it is likely that an essential resource like degradable carbohydrates was dried up. It is unlikely that the present strains do not withstand the osmotic pressure for a longer period of time because their highest abundance measured was at 20% NaCl. Furthermore, *Ca. guilliermondii* has already been described for soy moromi and grew at up to 18% NaCl[188,205]. As salt resistance mechanisms, they accumulate glycerol and export Na^+ ions via antiport with K^+[201]. Beneficial for the aroma, it is capable of producing phenolic compounds such as 4-ethylguaiacol and 4-ethylphenol and also produces glutaminase which converts L-glutamine in L-glutamate[10,41,168]. It is a an organism generally recognized as safe (GRAS) and might be a suitable candidate for a starter culture[160].
Another *Candida* species detected was *Ca. krusei*. It was present only irregularly and in small proportions at 10-20% NaCl but not with other yeasts as starter cultures. It had its largest share at 15% at day 4 and disappeared after 7 days. In preserved foods including wine, meat, and fruit juice, *Ca. krusei* is considered a spoilage agent due to its ability to grow at low pH, to form surface films and to produce CO_2 from sugars[22,142]. Since the moromi is not intended to be sterile and surface films are destroyed regularly, it should not be considered a spoilage risk for this seasoning sauce. In addition, growth of *Ca. krusei* can be prevented by using other yeasts as starter cultures as mentioned above.

Trichosporon asahii was only detected at 13.5% (day 0) and 20% (day 7), but was more abundant in a spontaneous fermentation and was originated from the koji (s. Appendix Section A.8 Figure A.1 and Section A.2 Figure S1). It was already described as part of the initial microbiota of soy moromi and typically disappeared early during the fermentation[176,191,200]. It is also considered a pathogen causing invasive trichosporonosis, a systemic disease that appears in immunosuppressed patients[126]. However, it is ubiquitous, occurs only in low numbers and there is no evidence of food spoilage. Since it was not present while yeast starters were used, its growth can be avoided if required.
Trichosporon loubieri occurred from day 0 to 7 at 10-20% NaCl (also detected at day 14 with 20%) with the highest amount at 10% after 4 days. Unlike *Trichosporon asahii*,

to our knowledge it has not yet been found in soy moromi or in other foods[39]. It is also pathogenic and its risk potential was assessed by us to be similar to *Trichosporon asahii* as mentioned above[125].

The predominant yeast in all lupine moromi batches at 10-20% and even with *Z. rouxii* as a competing starter culture was *D. hansenii*. It was present at 10-20% NaCl from day 7 until day 84 (and also after 168 days at 20% NaCl) with its highest cell count of 3.6×10^8 CFU/mL at 10% after 42 days. The decreasing CFU towards the end of fermentation may be explained by the decreasing availability of carbohydrates and other resources. This assumption is supported by the persistence after 6 months with consistently lower cell counts. Its ability to use a variety of different sugars might be the selection advantage for dominating the moromi[215]. It was also present in batches with additional wheat or buckwheat, but cell counts were decreased during the presence of *Z. rouxii*. On the other hand, growth of *Z. rouxii* was not influenced by *D. hansenii* showing that its dominance is at least not exclusively due to suppression of other yeasts. This species is able to produce a killer toxin that is lethal to certain yeasts excluding *Ca. versatilis*, *Ca. etchellsii*, and *Z. rouxii*[61]. The lethal activity of this toxin requires a pH below 5.1 and it increases with the presence of NaCl[123]. The suppression of other yeasts after the pH has decreased below 5 is therefore conceivable. But since the pH increased in most of our batches throughout the fermentation, this might not be the only explanation for its dominance. There is no sequence data for the formation of this toxin available and therefore, we were not able to screen the genome of the isolated strain TMW 3.1188[113]. Nevertheless, *D. hansenii* is a promising candidate for a starter culture for the suppression of disturbing yeasts and aroma contribution as mentioned in Section 1.3.3.

Wickerhamomyces anomalus (at 13.5% NaCl), *Lodderomyces elongisporus* (at 15% NaCl), and *Rhodatorula mucilaginosa* (at 20% NaCl), only occurred on day 7 in the different batches. This might be due to less competition as a result of the disappearance of most of the other yeast species. But since they were immediately overgrown by *D. hansenii* they may not have a significant impact on the product. *Wickerhamomyces anomalus* was found in several soy moromis[165,176,200] as well as *Rhodatorula mucilaginosa*[189], however, *Lodderomyces elongisporus* has not been detected in a moromi previously.

Z. rouxii could not be isolated from spontaneous or backslopped lupine moromi. Only with the addition of wheat or buckwheat, it appeared in week 2 and persisted for 2 - 6 weeks. This is due to the lack of fructose and glucose, since it is not able to use other monosaccharides including galactose[151,162,215]. The disappearance after a few weeks could be attributed to the depletion of available carbohydrates and the addition of a larger amount of wheat or buckwheat could prolong its persistence. Likewise, *Z. rouxii* was not detected in other wheat-free soy sauces (see Table 4.1) and was not able to grow when added as starter culture in wheat-free lupine moromi[151].
It was able to temporarily impede the growth of *D. hansenii* but no antagonistic

mechanisms against other yeast species could be found[188]. Furthermore, *D. hansenii* is not particularly sensitive to ethanol accumulation by *Z. rouxii*[18]. Therefore, the co-existence of *Z. rouxii* and *D. hansenii* need further investigation if *Z. rouxii* should be included in the lupine moromi microbiota.

4.1.2 Bacteria

W. paramesenteroides initially dominated the bacterial consortium at all tested salt concentrations and was detected at 10% NaCl up to day 21, at 13.5% and 20% until day 14 and at 15% until day 7. *W. cibaria* was present at 13.5-20% NaCl from the beginning of the fermentation until day 7. 16S rRNA gene amplicon sequence analysis revealed that the genus *Weissella* was still present after 12 weeks (84 days) even if it fell below the detection limit of our culture-dependent method (10^2 CFU/mL under total CFU/mL). Moreover, neither of them grew when yeast starters were used but the genus *Weissella* was detected via 16S rRNA gene amplicon sequence analysis after 4 weeks of incubation in batches with an additional carbohydrate source. At this point, *T. halophilus* was the only detected bacterium in a batch with yeast starters without an additional carbohydrate source. For the fermentation with wheat and buckwheat, no bacterial growth was observed via cultivation. This supports the assumption, that their cell counts were below the detection limit and they were not covered by our culture-dependent method. Both species were present in the koji and seem to be relevant LAB in the lupine moromi.

Leuconostoc mesenteroides was only detected on MRS agar at 10-15% NaCl on day 0. Via sequence analysis, it was also found at 20% NaCl and was still detectable after 12 weeks of incubation. It was originated from the koji and is commonly found in other fermentations and also in soy koji[81,173,176]. This indicates insufficient salt tolerance, which is supported by the fact that *Leuconostoc mesenteroides* showed no activity above 8% NaCl in pure culture[96]. In the moromi, it should therefore be rather unimportant and is more likely to influence the product during the koji phase.

Curtobacterium citreum was present at 10%, 13.5%, and 20% on day 0 or 2. This species was firstly isolated from a Chinese rice field soil and was not described in any other food related context[93]. It was also detected in the koji stage and might be originated from the lupine field soil[17].

One of the most dominant bacteria in all lupine moromi batches was *T. halophilus*. The isolated strains all belong to the subspecies *T. halophilus* subsp. *halophilus* and all cluster in one lineage[114]. It was present in every experiment and persistent until the end of the fermentation. Link and Ehrmann could show that this high adaptation to the lupine moromi is partly due to the degradation of galactose via strain-specific preferred pathways[112]. In a spontaneous fermentation or with yeast starters it appeared after 4 weeks but it showed high cell counts already after 2 weeks in all backslopped batches (with an exception at 20% NaCl were it was firstly detected after 6 weeks). This

demonstrates, that the use of starter cultures or backslopping enhances its growth and shortens the time needed to lower the pH for yeast growth. Furthermore, it indicates that *T. halophilus* originates from the koji since it appeared without backslopping or the use of sea salt.
T. halophilus was not detected with additional carbohydrate sources via cultivation whenever *Z. rouxii* was present. Devanthi *et al.* showed that growth of *T. halophilus* in soy moromi is enhanced by the co-inoculation with *Z. rouxii* but it has also been shown that it had an inhibitory effect at pH 6.0 under aerobic conditions[39,40]. Therefore, the addition of a *T. halophilus* starter prior to yeast starter cultures is recommended[40]. To our knowledge, the co-existence of *D. hansenii* and *T. halophilus* has not been investigated yet.

In Section 3.1 *C. moromii* was falsely identified as *C. japonicus*[120]. This is due to the fact, that it was identified via 16S rRNA gene sequencing that showed a 99.67% identity to *C. japonicus* CECT 7219^T [119]. This is also the case for *C. salexigens* strain KG13 (JABEVP01), isolated from *ganjang* since it shows ANIb identities below 84% to all *C. salexigens* strains but 96.43% identity to *C. moromii* TMW 2.2308^T (JAHXDI01)[28]. Through this adaptation, it becomes clear that *C. moromii* has also been identified in *ganjang* production (s. Table 4.1). Another candidate might be the putative *C. beijerinckii*, identified by Jung *et al.* since the partial 16S rRNA gene sequence (KP973975) shows 100% similarity not only to *C. beijerinckii* ATCC 19372^T (AB021386) but also to *C. moromii* TMW 2.2308^T (OM585595)[82].
In our work, *C. moromii* was detected in all batches at 10-20% NaCl via cultivation on ME agar[120]. It appeared after 14 days at 10-15% NaCl and was last found after 6 weeks and at 15% after 8 weeks. In moromi with 20% NaCl its appearance was delayed to week 6 to 8. It showed their highest cell counts at 15% NaCl after 28 days (1.9×10^9 CFU/mL) and optimal growth in pure culture at 10 to 12.5% NaCl[119,120]. The central osmoadaptation of *Chromohalobacter* appears to be the accumulation of compatible solutes by production or uptake of ectoin, hydroxyectoin, and glycine betaine[34,35,184]. Candidate genes for these systems could also be found in the genome of *C. moromii* TMW 2.2308^T. Therefore, it is possible for *C. moromii* to have a higher osmotolerance growing in a complex consortium than in pure culture.
C. canadensis was less abundant than *C. moromii* in all batches except for moromi with 20% NaCl where it appeared earlier and persisted longer, from week 4 to 24[120]. In pure culture, the type strain *C. canadensis* DSM 6769^T showed a weaker halotolerance, indicating that their transport systems for compatible solutes might be more efficient than those of *C. moromii*[119]. Both species were not able to grow on MRS agar and were therefore not detected while comparing spontaneous and backslopped fermentation. They do not originate from the koji stage (s. Appendix Section A.2 Figure S1) since they require at least 5% NaCl for growth[119]. This concludes why they were not detected in moromi batches with yeast starter cultures because neither an entry via the purified salt nor via the omitted backslopping was possible.
The annotation by NCBI revealed no genes encoding for amino acid decarboxylases, but all moromi isolates were tested positive for cadaverine, putrescine, and tyramine

synthesis using the biogenic amine media (s. Table 2.9). However, the lupine-based sauce was not rejected by testers or consumers due to off-flavors, indicating that these biogenic amines are not present in the product or only in small amounts. Therefore, the spoilage risk of biogenic amine synthesis by *Chromohalobacter* species does not seem to be crucial. Due to the high abundance of *C. moromii* in the lupine moromi and its presence in *ganjang*, the relevance in this fermentation including further spoilage potential and putative contribution to the flavour should be assessed in further investigations[28,82].

S. equorum was present in the moromi at 10% NaCl from the fourth week and two weeks later at 13.5% NaCl but was detected at 15% and 20% NaCl only after 6 months[120]. We could distinguish between at least three strains: TMW 2.2497 (isolated from moromi at 10% NaCl, genome accession number JAMBQA01), TMW 2.2498 (isolated from moromi at 13.5% NaCl, JAMBPZ01), and TMW 2.2499 (isolated from moromi at 13.5% NaCl, JAMBPY01). In pure culture, they grew weakly at up to 15% NaCl and at pH between 6.0 and 9.0 (data not shown). The delayed prevalence with increasing NaCl content might be related to the slower increase in pH, although, at 10% NaCl the pH was still below 5 while *S. equorum* was already present. Therefore, it is more likely that growth is decelerated due to the osmotic stress and cells need a longer time to adapt to higher salt concentrations. They were not found in the koji and were only detected in very small numbers with wheat and buckwheat addition at week 4 by 16S rRNA gene amplicon sequencing.
TMW 2.2497 and TMW 2.2498 were not able to produce biogenic amines in biogenic amine media (s. Table 2.9) while TMW 2.2499 produced cadaverine from lysine making it less suitable as a starter culture. Moreover, this strain showed resistance to oxacillin whereas the other two were sensitive to all tested antibiotic reagents (s. Appendix A.8 Table A.1). To select a starter culture, several other tests for its abilities including biofilm formation, protease and lipase activity and further safety hazards should be investigated[78]. Nevertheless, *S. equorum* TMW 2.2497 and TMW 2.2498 depict promising candidates for starter cultures in lupine moromi. These strains may also be applicable in other food fermentations since starter cultures of *S. equorum* are also used in the production of salami, *jeotgal* (sea food) and surface ripened semi-hard cheeses[101,124,143].

Brevibacterium sediminis was detected only at 10% NaCl from day 42 to day 168 and 13.5% at day 168 on ME agar and via 16S rDNA amplicon sequencing[120]. The physical parameters throughout moromi fermentation with higher salt concentrations should still be sufficient for growth since the type strain DSM 102229^T is described to grow at 0-20% NaCl and at pH 5–11[23]. It is therefore more likely to be in competition with *C. moromii* and *S. equorum* in the consortium and less well adapted to the conditions in the lupine moromi. We could not confirm its presence in a spontaneous fermentation or with yeast starter cultures nor was it part of the koji microbiota (s. Appendix Section A.8 Figure A.1 and Section A.2 Figure S1)[118]. Due to the late prevalence, its contribution in the moromi is probably rather indecisive and by optimising and shortening the process, its

presence may disappear completely.

Oceanobacillus sp. was not cultivable with the applied methods and therefore only detected via 16S rRNA gene sequence analysis. Most of the species assigned to this genus show moderate halotolerance, making this deficiency more likely due to the choice of agar formula with a too low pH[76]. However, *Oceanobacillus* was detected in relatively high abundances at 10% from week 4 and 13.5% NaCl (no data acquired at week 4) until week 12. To further investigate these organisms and their contribution to the product cultivation is essential and should be achieved by adjusting the cultivation conditions.

Lactobacillus helveticus (at 20% NaCl, day 0), *Klebisella oxytoca* (at 15% NaCl, day 0), *Klebsiella pneumoniae* (at 10% NaCl, day 0), *Enterococcus cloacae* (at day 0 with buckwheat and wheat), *Bacillus siamensis* (with yeast starter cultures but without additional carbohydrates, day 0), *Bacillus subtilis* (with wheat, week 2), and *Enterococcus faecium* (with buckwheat, week 2) were all detected at the beginning of their respective fermentation batch. Furthermore, there were several other genera including *Clostridium*, *Marinobacter*, and *Sphingobacterium* that were only detected via 16S rRNA gene sequence analysis. This indicates, that the microbiota of lupine moromi, especially at the beginning of the fermentation, is much more divers than we can display with culture-dependent methods. Due to their immediate disappearance and overall low prevalence, they may not be relevant for the development of a satisfactory seasoning sauce.

4.1.3 Archaea

Haloarchaea (also known as halobacteria) are a class of moderate to extremely halophilic euryarchaea, typically aerobic and chemoheterotrophic[137]. They were found in saline habitats such as solar salterns and hypersaline lakes and often dye their surrounding in a pinkish red colour due to β-carotinoide production[6,14]. Satari *et al.* showed that multiple genera remained in different samples of salt and were even cultivatable[156]. Therefore, haloarchaea could enter the moromi and might survive due to the high salt concentration and long fermentation period. Haloarchaea have already been found in fish and sea food fermentation as well as in kimchi but to our knowledge they were not yet detected in a moromi fermentation[100]. Unfortunately, in our experiments, no archaea were detected. This is due to the use of bacteria-optimised 16S rRNA gene primers and relatively short incubation periods for agar plates[12,203]. They may be spoilers producing off-flavours and disturbing growth of bacteria and yeast[58] or may improve the quality by inhibiting the growth of spoilers and improving the amino acid levels[4,7]. Therefore, growth of haloarchaea in lupine moromi need to be investigated. Potential isolates should be identified at species level to compare with published data and to find potential capabilities. In addition, a cultivation-dependent approach is necessary for a detailed investigation of their potential contribution to the fermentation.

4.2 The potential of lupine sauce as an alternative seasoning sauce

The seasoning sauce, based solely on lupine seeds as substrate, is a safe product with no persistent spoilage organisms even at a reduced salt content of 10% NaCl[120]. A direct comparison of different soy moromi-like fermentations with legume seeds as the sole substrate is challenging, as only a handful of studies exist, which moreover have different focuses and the methodology differs greatly (s. Table 4.1). In some cases, important information on the process is also missing, which is concealed for a competitive advantage or finished sauces were purchased and studied without the authors knowing the exact conditions[25,135,197]. There was no concrete data on flavour characteristics to any of these seasoning sauces (except for our study s. Section 3.2). To our knowledge, the microbiota of Japanese *tamari*, and also other legume-based soy sauce-like seasonings is not investigated and published yet[39,128,197]. The microbiota found in lupine moromi therefore resembles Korean *ganjang* and Indonesian *kecap* the most, due to no wheat addition resulting in low carbohydrate contents[25,151]. Yamana *et al.* were able to substantiate this by showing that grain-based seasoning sauces were high in saccharides such as glucose and trehalose, and bean-based sauces were characterised by amino acids such as glutamate[197]. Another example for an allergy-friendly alternative to soy sauce, is a sauce based solely on broad beans (*Vicia faba*, also known as fava bean, faba bead, and *Faba sativa*)[135]. It has a milder flavour than *koikuchi* soy sauce and contains two to three times more mannitol, erythritol, arabinose, and ornithine. These compounds were not detected in our experiments and might originate from the plant or be produced by yeasts[20,57,102,116].

The identified flavour compound composition of lupine sauce differs from those of other seasoning sauces (s. Table 4.1). This is partially due to the methods used for identification, as we were only able to detect volatile compounds with GC-MS. Highly polar and involatile substances including sugars, amines, and amino acids are very difficult to analyse by GC without derivatisation. For example, inosinate and glutamate have a synergetic effect for umami taste and studying these would therefore be particularly interesting[128]. In other works, these compounds were studied using nuclear magnetic resonance spectroscopy[28,82]. Moreover, without quantification, the amounts and their changes during the fermentation are missing which are needed in order to be able to draw conclusions about their relevance for the taste. Nevertheless, we could show which compounds persisted, occurred or fell below the detection limit due to metabolisation or evaporation. The number of substances with similar characteristics also give hints as to which compound groups may have an influence on the aroma. Our results can contribute to improving the data situation, as we show changes in flavour components and microbiota simultaneously over a period of 4-6 months (and a sensory analysis of the product).

The lupine moromi contained primarily acids, which form a sour taste (also fruity or cheesy) and pyrazines that have a nutty (or caramel-like, woody, smoky) flavour. Of

three furanones identified at 10% NaCl, only one (fatty aroma) was detected at 20% NaCl showing that the salt reduction influences the aroma. The only 4-hydroxyfuranones detected, which are often referred to as the most important flavour components in soy sauce, were HDMF and HMF. More obvious were the changes in the aroma profile with the addition of wheat and buckwheat where mainly esters (mostly fruity and floral) and acids were detected in the matured moromi, HMF was more and pyrazines were less abundant than without an additional carbohydrate-rich substrate. Pyrazines were often found in soy sauces and their quantity is sometimes even increased to optimise the taste[42,172,199]. Most of the other wheat-free sauces (s. Table 4.1) also contain a particularly high amount of organic acids[28,151,164,197]. Although the sensory analysis and GC-MS did not reveal any lupine-specific characteristics of the examined seasoning sauce, we could show matches with similarly processed products.

Table 4.1: Comparison of the moromi stage of wheat-free legume-based seasoning sauces. The pH values are shown for start/lowest/end of the fermentation.

Seasoning sauce	Pre-moromi treatment	NaCl [%]	Duration [months]	pH	Flavour compounds	Yeasts	Bacteria	Reference
Ganjang (soybeans)	*meju* brick (cooked, kneaded, air-dried)	8	0.5	na/na/5.2	alcohols (1-propanol, 2-methyl-1-propanol), ethyl acetate, acetic acid, aldehydes (benzaldehyde, 2/3-methylbutanal), ketones (1-phenyl-ethanone), phenol	no	*Bacillus* spp., *B. vallismortis*, *B. subtilis*, *B. amyloliquefaciens*, *B. methylotrophicus*, *Staphylococcus spp.*, *S. epidermidis*, *S. xylosus*, *Enterococcus* spp., *E. faecium*	[164]
		11 & 14	0.5	na/na/5.6	alcohols (2-phenylethanol, 2-methyl-1-propanol, n-butanol), ethyl acetate, acetic acid, aldehydes (butyraldehyde, 2/3- methylbutanal), ketones (2/3-octanone, 3-pentanone, ethyl propyl ketone)	no	*Bacillus spp.*, *B. vallismortis*, *B. subtilis*, *B. amyloliquefaciens*, *B. methylotrophicus*	
		20	12	6.9/5.6/6.7	organic acids (lactate, acetate, α-aminobutyric acid, γ-aminobutyric acid, butyrat), putrescine, amino acids (alanine, glycine, valine), betaine	na	*Cobetia crustatorum*, *B. licheniformis*, *C. beijerinckii*	[82]
		na	na	na	na	na	*Halanaerobium* sp., *Halanaerobium fermentans*, *T. halophilus*, *Staphylococcus* sp., *S. equorum*, *B. subtilis*	[25]

Table 4.1: Comparison of the moromi stage of wheat-free legume-based seasoning sauces. The pH values are shown for start/lowest/end of the fermentation.

Seasoning sauce	Pre-moromi treatment	NaCl [%]	Duration [months]	pH	Flavour compounds	Yeasts	Bacteria	Reference
		14.3	6	6.6/4.4/7.8	organic acids (lactate, acetate), sugars (fructose, galactose, glucose, mannose), amino acids, ethanol	*Debaryomyces* sp., *Wickerhamomyces* sp.	*T. halophilus, C. moromii, Idiomarina* sp., *Halomonas* sp., *Marinobacter* sp., *Corynebacterium* sp., *Bacillus* sp.,	[28]
Kecap manis (black soybeans)	*bungkil* (koji-like)	14.2	2	5.8/5.0/6.4	organic acids (lactate, acetate, oxalate, malate, formic acid), glycerol, ethanol, glutamic acid, 2-phenylethanol, isoamyl alcohol and isobutyl alcohol	*D. hansenii* var. *fabryi*, *C. guilliermondii* var. *membranaefaciens*	*T. halophilus*, *Staphylococcus* sp., coryneform bacteria	[151]
		20.8	2	6.4/4.8/5.0	organic acids (lactate, acetate, succinate, oxalate, malate, formic acid), glycerol, ethanol, glutamic acid, 2-phenylethanol, isoamyl alcohol and isobutyl alcohol	*C. parapsilosis*, *Sterigmatomyces*, *C. famata*	*T. halophilus*, *Staphylococcus* sp., coryneform	
Broad bean sauce (broad beans)	koji	17.0	na	na	mannitol, erythritol, arabinose, ornithine, lactic acid, phosphoric acid, glycine, arabitol, lysine	na	na	[135]
		na	na	na	amino acids (glutamic acid), urea, organic acids (γ-aminobutyric acid, butanoic acid), ornithine, putrescine	na	na	[197]

Table 4.1: Comparison of the moromi stage of wheat-free legume-based seasoning sauces. The pH values are shown for start/lowest/end of the fermentation.

Seasoning sauce	Pre-moromi treatment	NaCl [%]	Duration [months]	pH	Flavour compounds	Yeasts	Bacteria	Reference
Lupine sauce (lupine seeds)	koji	10	6	5.6/4.8/7.0	alcohols (3-methylbutanol, 2-phenylethanol), pyrazines, furanones	*C. guilliermondii*, *C. krusei*, *D. hansenii*, *Trichosporon loubieri*	*W. paramesenteroides*, *T. halophilus*, *C. moromii*, *C. canadensis*, *S. equorum*, *Brevibacterium sediminis*, *Klebsiella pneumoniae*	[120]
		13.5		5.6/4.9/6.3	alcohols (3-methylbutanol, 2-phenylethanol), pyrazines, furanones, organic acids (2-methylpropanoic acid, 3-methylbutanoic acid)	*C. guilliermondii*, *C. krusei*, *D. hansenii*, *Trichosporon loubieri*, *Trichosporon asahii*, *Wickerhamomyces anomalus*	*W. paramesenteroides*, *T. halophilus*, *C. moromii*, *C. canadensis*, *S. equorum*, *Brevibacterium sediminis*, *W. cibaria*	
		15		5.5/4.9/6.0	alcohols (3-methylbutanol, 2-phenylethanol), pyrazines, furanones, organic acids (2-methylpropanoic acid, acetic acid)	*C. guilliermondii*, *C. krusei*, *D. hansenii*, *Trichosporon loubieri*, *Lodderomyces elongisporus*	*W. paramesenteroides*, *T. halophilus*, *C. moromii*, *C. canadensis*, *S. equorum*, *W. cibaria*, *Klebsiella oxytoca*	
		20		5.5/5.0/5.3	alcohols (3-methylbutanol, 2-phenylethanol), pyrazines, organic acids (2-methylpropanoic acid, acetic acid)	*C. guilliermondii*, *C. krusei*, *D. hansenii*, *Trichosporon loubieri*, *Trichosporon asahii*, *Rhodotorula mucilaginosa*	*W. paramesenteroides*, *T. halophilus*, *C. moromii*, *C. canadensis*, *S. equorum, W. cibaria, Curtobacterium citreum, Lactobacillus helveticus*	

Table 4.1: Comparison of the moromi stage of wheat-free legume-based seasoning sauces. The pH values are shown for start/lowest/end of the fermentation.

Seasoning sauce	**Pre-moromi treatment**	**NaCl** [%]	**Duration** [months]	**pH**	**Flavour compounds**	**Yeasts**	**Bacteria**	**Reference**
Lupine sauce with 30% wheat/buckwheat	koji	13.5	4	6.2/4.7/4.7	organic acids (acetic acid, 2-methylpropanoic acid, 3-methylbutanoic acid, Benzene acetic acid), esters, pyrazines, HMF	*D. hansenii, Z. rouxii*	*T. halophilus*, *Enterococcus cloacae*, *Enterobacter* sp., *Lactococcus* sp., *Pseodomonas* sp., *Leuconostoc* sp., *Staphylococcus* sp., *Weissella* sp.	[118]

Also noticeable in the compared moromis is the slow increase in pH after the initial drop (s. Table 4.1), unlike other soy sauces where the pH remains low[117]. In lupine sauce with added yeast starters, the increase was not detected within the 4 months of incubation. The absence of several species like *C. moromii* and *S. eqourum* might be a reason for this constancy. Therefore, we assume that the pH increase is due to acid degradation as part of the aerobic respiration, and the production of amines and ammonia[151]. However, since all these sauces are not rejected by the consumer due to an unpleasant aroma, we may not consider this as spoilage.

Other than through salt and carbohydrate sources, the microbiota could be altered by managing the so far unregulated temperature and pH[152]. Röling *et al* showed that the stability and activity of proteases are elevated at a higher pH (5.4 - 6.2) which leads to higher glutamate contents. The growth rate of *T. halophilus* and other bacteria is increased at a higher pH, which was also shown in pure culture for *C. moromii* but this might also yield the risk of spoilage[119,152]. On the other hand, yeasts including *Z. rouxii* and *D. hansenii* prefer lower pH for optimal growth and their flavour production might be reduced[18,147]. In *kecap*, a higher temperature (37 - 45 °C) led to increased growth of microorganisms including *T. halophilus* and therefore, the pH was decreased faster than at lower temperatures[152,195]. This might enhance the ageing of the moromi without altering the total amount of nitrogen[69,195]. However, a slow, long-term maturation and lower temperature, especially during the first month, is suggested for a good quality soy sauce and maintaining a specific temperature would need further investigation[27,117,207,214]. Moreover, controlling the pH or temperature throughout the fermentation would be a major process engineering adjustment, making them more difficult to implement.

Another way to increase the availability of the carbohydrates of the lupine seeds would be germination. In the malting process during beer brewing, plant enzymes hydrolyze complex molecules including storage compounds[105,111]. Kaczmarska *et al.* showed that lupine seeds contain less starch and oligosaccharides but more sucrose and fructose after germination[84]. Therefore, germination of the lupine seeds might be, similar to malting, a promising optimization of the process with a microbiota potentially convergent to wheat-containing soy sauces. The amount of alkaloids is also decreased during the germination process resulting in a more easily digestible product[24].

We assume that the glutamate level might be sufficient due to the high protein content of lupine seeds, but it was not analysed in our work. Moreover, the allergic reaction to lupine alkaloids, histamine, and other biogenic amines is not yet addressed and should be investigated[169]. The alkaloid content could be lowered by germination or using less bitter lupines and the amount of biogenic amines might be controlled by using non-amine-producing starter cultures or even amine-degrading organisms[21,24,90,121]. On the other hand, the reduction of NaCl content showed no growth of spoilage organisms but a shortened time period until the formation of the final microbiota. The increase of available carbohydrates could be a useful tool for adjustments of the

preferred microbiota, especially for yeast growth. Furthermore, the use of starter cultures instead of backslopping allows the definition of the microbiota and accelerate its development.

4.3 Validation of hypotheses

The aim of this study was to understand how the microbiota of lupine-based moromi is composed, influenced and contributes to a delicious flavour. In the following, the hypotheses formulated at the beginning are validated in summary by means of the discussed results.

1. **The microbiota and flavour of lupine-based moromi fermentation and the resulting flavour is equivalent to soy moromi due to the similar substrate composition and process.**
   The microbiota was similar to other wheat-free, legume-based seasoning sauces including some of the most dominant organisms *T. halophilus* and *D. hansenii*, but also *W. paramesenteroides*, *C. moromii*, *S. equorum*, and *Ca. guilliermondii*. 16S rRNA gene sequence analysis confirmed that most of the more abundant bacteria were also culturable. Similar flavour compounds such as acetate and 2-phenylethanol were detected in lupine moromi but different methodological approaches complicate the comparison.

2. **A reduction in salt content alters the microbiota and, at a certain threshold, allows the growth of spoilage organisms or pathogens.**
   Although we could show that the microbiota of a salt-reduced moromi was more divers, no spoiling was determined in our experiments. This may be due to the still moderately high salt concentration at 10% NaCl. A further reduction of the salt content would be detrimental to the osmophilic organisms such as *T. halophilus* which is contradictory to the purpose of creating a soy sauce-like seasoning. Nevertheless, the reduction of salt led to a premature prevalence of *S. equorum* and *C. moromii* and higher total bacterial cell counts.

3. **Lupine-based moromi is a promising source of new strains and species due to the novel habitat and growth conditions.**
   Lodderomyces elongisporus, Curtobacterium citreum, Brevibacterium sediminis, and *Oceanobacillus* sp. have not been associated with moromi or similar food fermentations prior to our work. Since they appeared only in low cell counts or where not culturable, they were not further investigated. On the other hand, *C. moromii* is a novel species isolated from the lupine moromi. The nearest relatives to this species were also detected in salty fermentations and we postulate that one *C. salexigens* strain isolated from Korean *ganjang* needs to be re-classified to the species *C. moromii*. Nevertheless, to our knowledge there are no food fermentations known were *Chromohalobacter* plays a major role and therefore lupine-based moromi is a model system to investigate their coexistence with yeast

and LAB and their potential for flavour contribution. Isolates of *T. halophilus*, *S. equorum* and *D. hansenii* were further investigated and might be suitable candidates for starter cultures in this and other fermentation processes.

4. **The microbiota and in particular yeast growth is dependent on the amount of carbohydrates and can therefore be modified by the addition of a carbohydrate source.**
   We could show, that the microbiota of solely lupine-based moromi was more similar to wheat-free than to wheat-containing soy sauces. The use of wheat and buckwheat as additional carbohydrate sources enabled the growth of *Z. rouxii* while *D. hansenii* was still the dominating yeast in matured moromi. We assume that higher amounts of wheat or buckwheat result in an elongated presence of *Z. rouxii* throughout the fermentation. There were no differences detected between the moromi with wheat or buckwheat.

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A Appendix

A.1 Full publication: Microbiota dynamics and volatile compounds in lupine based Moromi fermented at different salt concentrations

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Microbiota dynamics and volatile compounds in lupine based Moromi fermented at different salt concentrations

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ABSTRACT

Fermented soy sauces are used as food seasonings in Eastern countries and all over the world. Depending on their cultural origins, their production differs in parameters such as wheat addition, temperature, and salt concentration. The fermentation of lupine seeds presents an alternative to the use of soybeans; however, the microbiota and influencing factors are currently unknown. In this study, we analyse the microbiota of lupine Moromi (mash) fermentations for a period of six months and determine the influence of different salt concentrations on the microbiota dynamics and the volatile compound composition. Cultured microorganisms were identified by protein profiling using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16S rRNA gene amplicon sequencing provided an overview of the microbiota including non-cultured bacteria. The volatile compounds were determined by gas chromatography–mass spectrometry (GC–MS). At all salt concentrations, we found that *Tetragenococcus halophilus* (up to 1.4×10^9 colony forming units (CFU)/mL on day 21) and *Chromohalobacter japonicus* (1.9×10^9 CFU/mL, day 28) were the dominating bacteria during Moromi fermentation. *Debaryomyces hansenii* (3.6×10^8 CFU/mL, day 42) and *Candida guilliermondii* (2.2×10^8 CFU/mL, day 2) were found to be the most prevalent yeast species. Interestingly, *Zygosaccharomyces rouxii* and other yeasts described as typical for soy Moromi were not found. With increasing salinity, we found lower diversity in the microbiota, the prevalence-gain of typical species was delayed, and ratios differed depending on their halo- or acid tolerance. GC–MS analysis revealed aroma-active compounds, such as pyrazines, acids, and some furanones, which were mostly different from the aroma compounds found in soy sauce. The absence of wheat may have caused a change in yeast microbiota, and the use of lupine seeds may have led to the differing aromatic composition. Salt reduction resulted in a more complex microbiome, higher cell counts, and did not show any spoiling organisms. With these findings, we show that seasoning sauce that uses lupine seeds as the sole substrate is a suitable gluten-free, soy-free and salt reduced alternative to common soy sauces with a unique flavour.

1. Introduction

The economic potential of plant-based fermented seasoning sauces is growing particularly in Western countries (Zhu and Tramper, 2013). Traditional oriental soy sauces are produced in a two-step fermentation and this manufacturing process differs depending on regional preferences (Devanthi and Gkatzionis, 2019). The most widely consumed soy sauce in Japan and Western countries is Koikuchi Shoyu. Therefore, equal amounts of boiled soybeans and roasted wheat are mixed with *Aspergillus oryzae* or *Aspergillus sojae*. For Chinese soy sauce smaller quantities of wheat (20 to 30%) are used, and Japanese Tamari Shoyu contains less or even no wheat. This initial fermentate is known as Koji and is incubated for two days. These moulds secrete enzymes, which, in addition to plant-derived enzymes, hydrolyse proteins and glycans to low molecular weight compounds. These compounds are precursors that can be subsequently metabolised in a second fermentation. A brine solution of 13% (for low-salt fermentations) up to 20% sodium chloride (for high-salt fermentations) is then added and the so-called Moromi is fermented for months, or even years. The high salt concentration inhibits the growth of spoiling or pathogenic microorganisms and also limits the growth and enzymatic activity of the Koji mould. Halotolerant yeasts and lactic acid bacteria (LAB) dominate Moromi fermentation.

The microbial composition changes during the fermentation process and comes to a fairly stable consortium in the late phase (Wei et al.,

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2013). In the early phase, many different species can be found. Yeasts such as *Pichia*, *Debaryomyces,* and *Candida* are predominant since the pH is high (Hanya and Nakadai, 2003). *Weissella*, *Bacillus,* and lactobacilli, are known bacterial genera detected at the beginning of Moromi fermentation, as well as *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas,* and *Staphylococcus* (Sulaiman et al., 2014). The LAB lower the pH by producing acids (Tanaka et al., 2012). One of the most important LAB, *Tetragenococcus halophilus*, appears in the first few weeks and forms lactic and acetic acid (Tanaka et al., 2012; Tanasupawat et al., 2002). With this continuous decrease in pH, acid-tolerant yeasts take a larger part in the microbiota (Sulaiman et al., 2014). The Koji mould *Aspergillus* disappears, and another crucial microbe for many soy sauce productions, *Zygosaccharomyces rouxii*, arises (Hanya and Nakadai, 2003; Tanaka et al., 2012; Wei et al., 2013). This yeast synthesises flavour components, such as alcohols and 4-hydroxyfuranones, which are necessary for the characteristic aroma of these soy sauces. In the matured Moromi, *Candida versatilis* and *Candida etchellsii* are predominant and continue the flavour development (Hanya and Nakadai, 2003).

In recent years, other protein-rich substrates have been used as an alternative to soy beans to create novel seasoning sauces (Mouritsen et al., 2017). Legumes such as lupine seeds, may be suitable for a similar fermentation process and a high-quality product. Lupine plants are cultivatable in European countries since they are adapted to the mid-European climate and can be produced ecologically with short transportation routes. Since lupine seeds contain a higher carbohydrate content than soybeans, the use of wheat may not be essential (see Supplementary Table S1, USDA Branded Food Products Database), resulting in a gluten-free product that people with celiac disease, soy intolerance or other dietary restrictions can consume. However, low carbohydrate contents may inhibit yeast growth . Furthermore, lupine seeds have a low lipid content, and lipids are typical precursors for aldehydes, esters, and alcohols (Diez-Simon et al., 2019). Therefore, a lower diversity of those compounds would be predicted when using lupine seeds instead of soybeans. Moreover, the high salt content of traditional seasoning sauces should be avoided due to health concerns, but it is also required to inhibit spoiling microorganisms (Devanthi and Gkatzionis, 2019). As proceeding of fermentation is still analogue, we hypothesise a comparable halotolerant microbiome especially for fermentations with higher amounts of salt.

In the present study, we investigate the influence of different salt concentrations on the microbiota dynamics in the Moromi fermentation of a lupine based seasoning sauce using culture-dependent and culture-independent methods. Additionally, we analyse the volatile compounds via GC–MS analysis to reveal potential flavour differences as compared to typical soy sauces.

2. Methods

2.1. Koji and mash fermentation

Lupine Koji was provided by Purvegan (Ramsen, Germany). For Koji fermentation, a mixture of untreated, roasted, and crushed lupine seeds (*Lupinus angustifolius*) were merged with spores of *Aspergillus oryzae* as the Koji starter. The Koji was incubated at 25 to 35 °C in an aerated tank for two days.

Moromi was prepared in batches with a total volume of 600 mL. Each batch consisted of 40% (*w/v*) fermented Koji, 0.07% (*v*/v) matured Moromi from a finished batch as the starter, and brine with 10, 13.5, 15 or 20% (*w/v*) NaCl. All batches were prepared in duplicate and incubated for six months at 25 °C at a humidity level of 80%. Batches were mixed once a week for ventilation and the volume was adjusted with deionised water if necessary.

2.2. pH measurement and cell counts

For pH measurement, samples were centrifuged at 7000 ×*g* for 6 min and supernatants were diluted to a NaCl concentration of 5% (w/v).

Fluid mash was diluted in Ringer's solution (Merck, Darmstadt, Germany) with 5% (*w/v*) NaCl and plated with sterile glass beads (2.7 mm, Carl Roth, Karlsruhe, Germany). CFU were counted in duplicate upon incubation at 30 °C for four days. We used Malt Extract peptone agar (ME) at pH 5.6 and De Man-Rogosa-Sharpe agar (MRS, composed of 10 g/L casein peptone, 2 g/L meat extract, 4 g/L yeast extract, 20 g/L glucose, 1 g/L Tween80, 2.5 g/L K_2HPO_4*$3H_2O$, 5 g/L Na-acetate*$3H_2O$, 2 g/L $(NH_4)_2$H-citrate, 0.2 g/L $MgSO_4$*$7H_2O$, 0.05 g/L $MnSO_4$*H_2O, 15 g/L agar) pH adjusted to 5.7, both with additional 5% (w/v) NaCl. ME plates were incubated under oxic conditions, and MRS plates under anoxic conditions. All colonies larger than 0.5 mm were recorded.

2.3. Identification of organisms

For identification of bacteria and yeasts at species level we used subproteome-profiling by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). 48 colonies per sample were streaked once and measured with a Microflex LT Spectrometer (Bruker Daltonics, Bremen, Germany). Detected spectra were compared with the Bruker Biotyper database (Bruker Daltonics) and an in-house database validated with 16S rRNA gene sequenced strains and published type strains.

For high-throughput 16S ribosomal RNA gene amplicon sequencing of bacteria samples were centrifuged at 7000 ×*g* for 6 min and washed in sterile Ringer's solution. Cell pellets were resuspended in RNAlater (Invitrogen, Vilnius, Lithuania) and stored at −20 °C until measurement. Samples were processed as described previously (Lagkouvardos et al., 2015). Cell lysis was achieved by bead-beating and heat treatment, and DNA was purified using gDNA columns (Macherey-Nagel, Düren, Germany). Concentrations and purity were tested using the NanoDrop® system (Thermo Scientific, Waltham, Massachusetts, USA). The V4 region of 16S rDNA was amplified (25 cycles) from DNA using primers 519F (5' CAG CMG CCG CGG TAA TWC) and 785R (5' GAC TAC HVG GGT ATC TAA TCC) (Klindworth et al., 2013). After purification using an AMPure XP system (Beckmann Coulter Biomedical GMBH, Munich, Germany) and pooling in an equimolar quantity, the 16S rRNA gene amplicon libraries were sequenced in paired-end modus (PE175) using a MiSeq system (Illumina, Inc., San Diego, California, USA) following the manufacturer's instructions. Processing of the samples and sequencing was conducted by the TUM Core Microbiome Facility (ZIEL - Institute for Food & Health, Freising, Germany). We analysed sequences using the IMNGS and RHEA default methods (Lagkouvardos et al., 2017; Lagkouvardos et al., 2016).

2.4. GC–MS

Aroma components were analysed via gas chromatography–mass spectrometry (GC–MS) with a measurement method described by Sun et al., 2010 (Sun et al., 2010). Therefore, 5 mL samples of mash with additional sodium chloride at a final concentration of 25% (*w/v*) were stored in glass vials (20 mL) at −80 °C until measurement (Yan et al., 2008). The Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) solid-phase microextraction (SPME) fibre was purchased from Supelco (Bellfonte, USA) and conditioned at 250 °C for 2 h before use. Samples were extracted at 50 °C for 40 min in a heat block and after insertion of the SPME device into the injection port, desorption was performed at 250 °C for 5 min.

A gas chromatograph (GC) 7890A from Agilent Technologies (Santa Clara, USA) with a 5975C mass selective detector (MSD) and Triple-Axis Detector (Agilent Technologies, Santa Clara, USA) was used for analysis; helium was used as the carrier gas (flow rate 1 mL/min). A Zebron ZB-WAX column (film thickness 0.25 μm; Phenomenex, Aschaffenburg, Germany) was used and the temperature of the injector and detector was kept constant at 250 °C. The injection was conducted in splitless mode.

The oven temperature during the measurement started at 40 °C for 2 min, raised by 5 °C/min up to 230 °C and held for 15 min. Masses were detected in electron impact mode (EI) and were accepted between 34 and 400 *m/z*. The ion source temperature was set at 230 °C, while the triple quadrupole temperature was 150 °C.

For detection of aromatic compounds, we used MassHunter Workstation Qualitative Analysis Software (Agilent Technologies, Santa Clara, USA). These compounds were identified by comparison with National Institute of Standards and Technology (NIST) database (William E. Acree and Chickos, 2020).

3. Results

3.1. Major characteristics of lupine based Moromi fermentation

To see the ratio of yeast and bacterial species present in lupine based Moromi we determined the CFU/mL and identified isolates via MALDI-TOF-MS. We also investigated whether the course of pH values was influenced by different salt concentrations and could affect the microbiota dynamics.

We found that yeasts only played a large part in the microbiota during the first few days (Fig. 1). At first, the bacterial cell counts were much higher than those of yeasts. After one week, they started to align, since bacteria are more strongly suppressed by salt stress. Salt stress also led to a stronger and longer-lasting decrease of bacterial growth along with increasing salt concentration in the Moromi. In all batches, bacterial growth recovered and yeast growth vanished completely in the mash with up to 15% of NaCl. Although cell counts were lower at 20% NaCl, even after six months, yeast species were still detected in this batch.

The pH decreased below 5 in all four batches within the first few days and increased again after six to eight weeks of fermentation. In the batch with 20% salt, the drop in pH took eight weeks, and showed only a limited increase over time. There were only slight differences in pH in the batches with 13.5% and 15% sodium chloride, indicating that this difference in salt concentration had low impact on the fermentation process.

3.2. Yeast composition via cultivation-dependent method

To see which yeast species are present in lupine based Moromi we determined CFU/mL and analysed isolates via MALDI-TOF-MS. At the beginning of Moromi fermentation, the dominating yeast species were *Candida guilliermondii* (2.2×10^8 CFU/mL, day 2 with 10% NaCl) and *Trichosporon loubieri* (5.4×10^7 CFU/mL, day 0 with 10% NaCl) (Fig. 2). These species are present at the end of Koji fermentation and appear to survive the osmotic shock from brine addition (see Supplementary Table 2). Within the first week, the species *Candida kruseii*, *Lodderomyces elongisporus*, *Trichosporon asahii*, *Rhodotorula mucilaginosa* and *Wickerhamomyces anomalus* were detected. *Debaryomyces hansenii* dominated after two weeks until the end of the fermentation (3.6×10^8 CFU/mL, day 42 with 10% NaCl) and no other yeast species were found in the matured Moromi. *D. hansenii* was the only yeast that grew in Moromi with 20% NaCl. There was almost no difference in the yeast microbiota, only an obvious decrease in the total abundance of yeast in the mash with 20% salt.

On MRS agar, almost identical results were observed (see Supplementary Fig. 2). These results indicate that *D. hansenii* is the predominant yeast in lupine based Moromi.

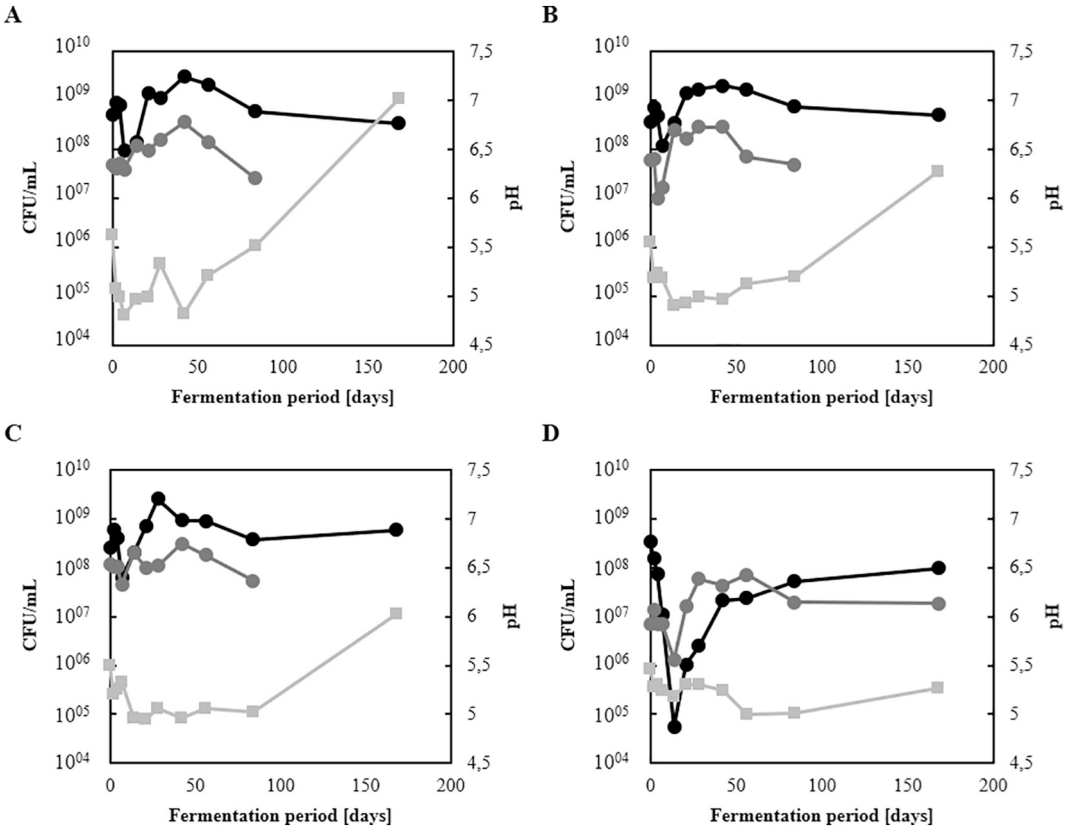

Fig. 1. Abundance of yeast and bacteria and pH values during Moromi fermentation. Moromi fermentation was prepared with 10% (A), 13.5% (B), 15% (C) and 20% (D) NaCl. pH values (squares, light grey) and absolute abundance (circles) of yeast (grey) and bacteria (black) are shown on ME agar. Relative abundance was calculated from MALDI-TOF-MS analysis and applied on total CFU/mL to determine absolute abundances of bacteria and yeast.

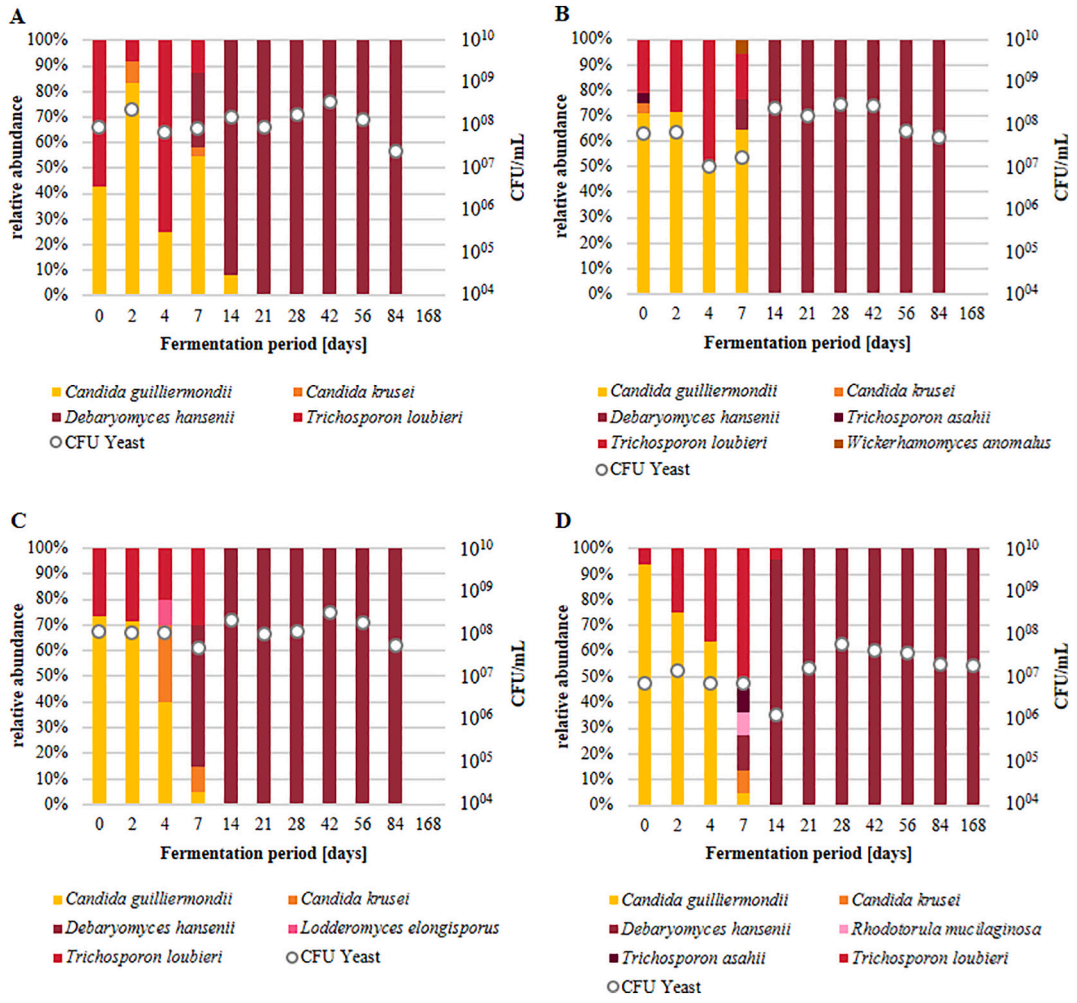

Fig. 2. Diversity of yeast during Moromi fermentation on ME agar via MALDI-TOF-MS. Species and total number of yeast CFU per mL in Moromi fermentation with 10% (A), 13.5% (B), 15% (C) and 20% (D) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of yeast.

3.3. Bacterial composition via cultivation-dependent method

Since LAB are predominant in soy-based Moromi we streaked colonies on MRS agar and incubated the plates under anoxic conditions (Devanthi and Gkatzionis, 2019). Yeasts were detected on ME agar, which was incubated under oxic conditions and we could also isolate bacteria from these plates. To identify which bacterial species play a role in lupine based Moromi fermentation, we used MALDI-TOF-MS analysis.

Figs. 3 and 4 show that the LAB *Weissella paramesenteroides* dominated the immature mash (1.1×10^9 CFU/mL, day 2 with 10% NaCl, on MRS agar). Additionally, we found *Curtobacterium citreum*, *Klebsiella pneumoniae, Klebsiella oxytoca*, *Lactobacillus helveticus,* and *Weissella cibaria* in the first few days of Moromi fermentation. On MRS agar, *Leuconostoc mesenteroides* was also detected. In the first two days, the CFU/mL of bacteria increased except the batch with 20% NaCl, but strongly decreased afterwards, which correlates with the presence of previously named species. Following species were *Chromohalobacter canadensis* (6×10^8 CFU/mL, day 28 with 15% NaCl, on ME agar) and *Chromohalobacter japonicus* (1.9×10^9 CFU/mL, day 28 with 15% NaCl, on ME agar) and *T. halophilus* (1.4×10^9 CFU/mL, day 21 with 10% NaCl, on MRS agar) and CFU increased again. Only in the moromi with 20% NaCl were bacteria almost absent between days 14 and 28 of fermentation. *Chromohalobacter* species only grew under aerobic conditions and after two months with 10 and 13.5% NaCl, *Brevibacterium sediminis* and *Staphylococcus equorum* grew on ME. With higher concentrations of sodium chloride, *S. equorum* only was detected after six months. On MRS agar (Fig. 4), it appears that *T. halophilus* was predominant in the lupine based Moromi after 14 days until the end of the fermentation.

3.4. Bacterial composition via cultivation-independent method

Bacterial composition was also investigated by 16S amplicon sequence analysis to detect any organisms missed by the cultivation-dependent method. Therefore, we selected samples from after the first species shift, 3 weeks into the fermentation process (and at 4 weeks with 10% NaCl) and from maturing Moromi (12 weeks). As before, we found *Chromohalobacter* and *Tetragenococcus* after 3 weeks and also *Weissella* and *Leuconostoc* (see Fig. 5 and Supplementary Fig. 7). A week later, with about 19% of the consortium *Oceanobacillus* was detected in fermentations containing 10% NaCl and these organisms remained in the batch until the end of fermentation, unseen by our cultivation-dependent method. At this late point, *Oceanobacillus* was also present in the fermentation with 13.5% NaCl. In the maturing Moromi, *Weissella* was still detectable in all batches via 16S amplicon sequence analysis but not by cultivation.

These results indicate that the microbiota in maturing Moromi was more diverse than observed with the cultivation-dependent method. *Chromohalobacter* had a high abundance after the first species shift, with lower salt concentrations and, in matured Moromi, with higher salinity. *Chromohalobacter* appears to play a major role in lupine based Moromi fermentation. We also identified significantly more genera that

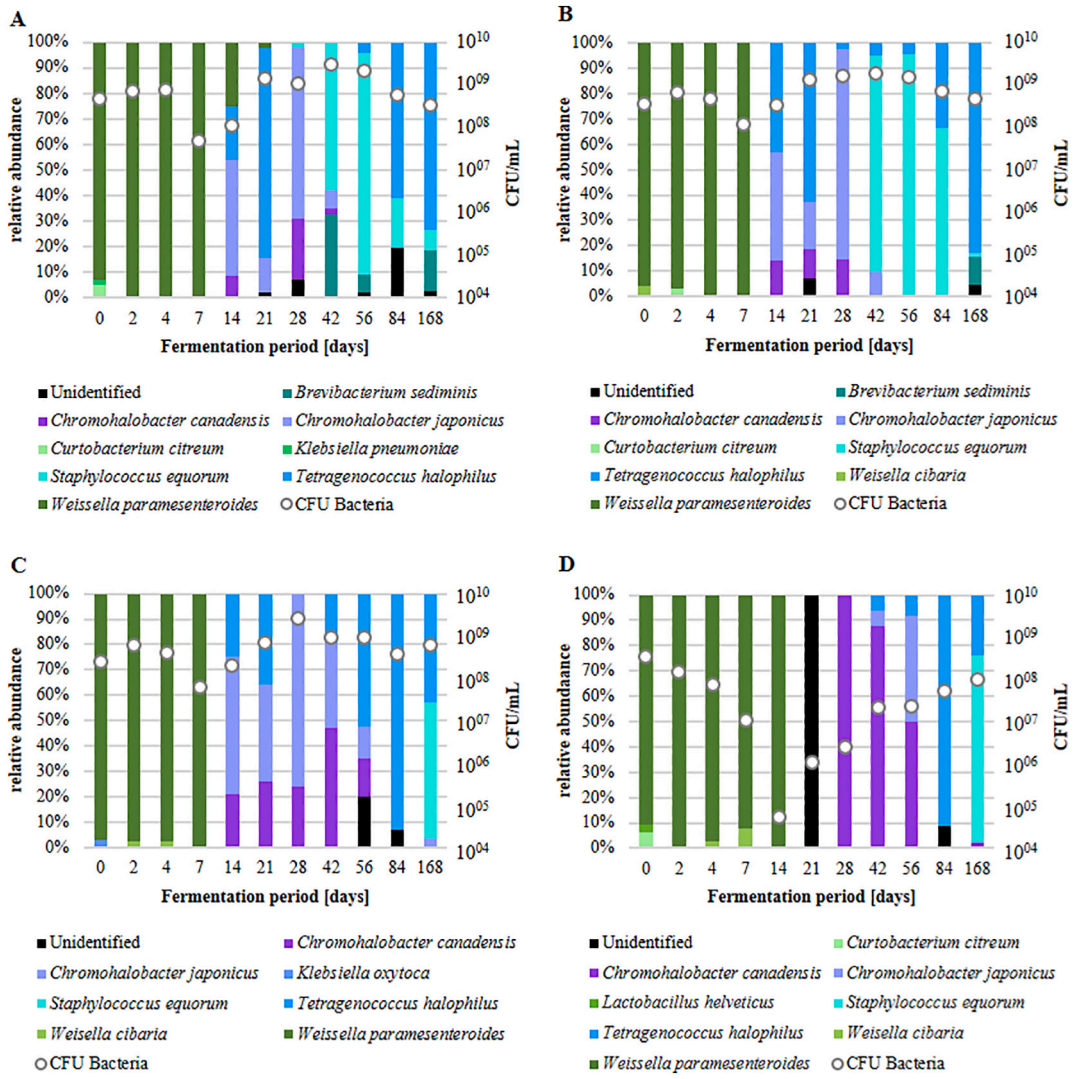

Fig. 3. Diversity of bacteria during Moromi fermentation on ME agar via MALDI-TOF-MS. Species and total number of bacterial CFU per mL in Moromi fermentation with 10% (A), 13.5% (B), 15% (C) and 20% (D) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of bacteria.

appeared within the Moromi fermentation. Still, species that are more abundant may have a greater impact on the quality of the final seasoning sauce.

3.5. Volatile dynamics during Moromi fermentation

Aromatic compounds and odour are important parameters in the evaluation of the quality of seasoning sauces. These parameters are based on the substrate composition (see Supplementary Table 1) and are directly defined by the microbiota and their metabolic actions. With GC–MS, we discovered that the volatile aroma compounds of lupine based Moromi fermentation differs from common soy sauces in some important compounds. In total, we found 94 volatile compounds, including 9 different alcohols, 11 esters, 13 acids, only 2 aldehydes, 12 ketones, 6 furan/furanones, 1 sulphur-containing component, 3 phenols, 24 pyrazines, and 12 miscellaneous substances. In Table 1, a representative selection of soy sauce-related compounds is shown for all four fermentation conditions during Moromi fermentation. The complete overview of all detected volatiles is provided in Supplementary Tables 2–3.

In matured Moromi only three alcohols: 3-methylbutanol, 2-phenylethanol, and (with 10% NaCl) 1-octen-3-ol, were present. These are the only alcoholic compounds that may influence the flavour of the finished product. In the fermentation with 13.5 and 15% NaCl, three esters remained in the matured Moromi: 2/3-methylbutanoic acid ethyl ester and (with 15% NaCl) ethyl 9-octadecenoate. In a repetition of the experiment, 3-methylbutanoic acid ethyl ester was also detected after six months in the Moromi with 10%, ethyl pentadecanoate with 13.5% and ethyl hexadecanoate with 20%. These findings show that esters are formed and metabolised or evaporated during the process. Of the 13 acids identified in all samples, 5 were still detectable in matured Moromi. Of these acids, 2-methylpropanoic acid was detected with all salt concentrations tested, while 3-methylbutanoic acid exclusively remained in the Moromi with 13.5% NaCl. Acetic acid, 2-methylhexanoic acid and benzeneacetic acid were also identified in the matured Moromi. In that late phase, both aldehydes, 3-methylbutanal and benzeneacetaldehyde, were only detected with 10% NaCl. 5 ketones were detected in that phase (two additional in the repetition) and the only one that was found with 13.5–20% was 2-Hydroxy-3-methyl-2-cyclopenten-1-one. The phenolic compounds included 2-methoxy-4-vinylphenol and 4-hydroxy-2/3-methylacetophenone, but neither was detectable at the end of the fermentation.

A the high number of different pyrazines were identified in the fermentation, indicating that pyrazines play a major role in lupine based Moromi fermentation. Even after six months, 20 of them were found and 8 of these were found with all salt concentrations. Between 12 and 13 of

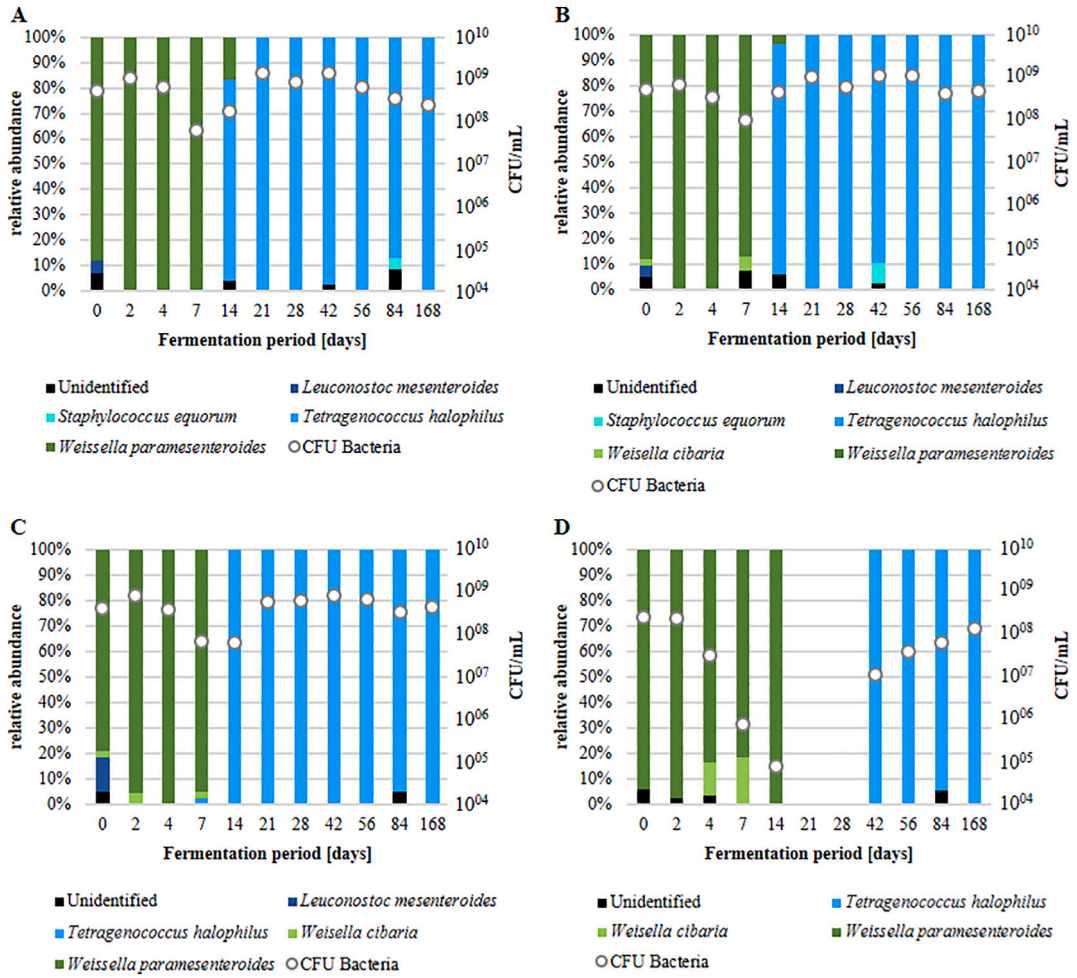

Fig. 4. Diversity of bacteria during Moromi fermentation on MRS agar via MALDI-TOF-MS. Species and total numbers of bacterial CFU per mL in Moromi fermentation with 10% (A), 13.5% (B), 15% (C) and 20% (D) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of bacteria.

these pyrazines were found at the start of fermentation and, therefore, were not formed during the fermentation process. In matured Moromi, three furanones but no 4-hydroxyfuranones were detected. Dihydro-5-((Z)-2-octenyl)-2(3H)-furanone was found with all salt concentrations, 5-heptyldihydro-2(3H)-furanone with up to 15% NaCl (in the repetition also with 20%) and dihydro-3,5-dimethyl-2(3H)-furanone solely in the Moromi with 10% NaCl.

4. Discussion

In this study, we investigated the microbiota dynamics and volatile compounds of lupine based Moromi fermentations that were supplemented with different quantities of NaCl. The results showed that yeasts only take a larger part in the microbiota during the first few weeks of fermentation. Yeast microbiota started with *C. guilliermondii* and *Trichosporon loubieri* and ended with *D. hansenii* in all batches. After three months, yeast species fell under the detection limit for the cultivation method (10^2 CFU/mL under total CFU/mL). Most of the yeast species we detected have already been described for soy mash, but to our knowledge, *Lodderomyces elongisporus*, *T. loubieri,* and *Candida krusei* have not been detected previously (Song et al., 2015; Tanaka et al., 2012; Wei et al., 2013). *D. hansenii*, one of the major yeast in lupine Moromi, has already been described for Korean soy sauce (ganjang), but only in early phase of fermentation (Song et al., 2015). Furthermore, we did not find yeast species that have been described as typical for soy mash, such as *Z. rouxii*, *C. versatilis* and *C. etchellsii* in lupine Moromi. This absence may have been be due to the lower amount of carbohydrates present in the mash, as postulated by Röling et al. for wheat-free Indonesian soy sauce (Röling et al., 1994).

Similar to the findings described by Wei et al. for soy mash, bacteria were found to be the numerically dominant microorganisms in lupine Moromi (Wei et al., 2013). *Weissella paramesenteroides* was the dominant species within the microbiota until at least the seventh sampling day and was not culturally detectable in the further course of the fermentation, although the presence of *Weissella* spp. was shown by the culture-independent approach until the end of fermentation.

Weissella is described for soy sauce, but Zhang et al. could only determine its persistence in low-salt fermentations (Yan et al., 2013; Zhang et al., 2016). Furthermore, species such as *Klebsiella pneumoniae*, *Weissella cibaria* and *Leuconostoc mesenteroides* and the genera *Curtobacterium* and *Lactobacillus* were found in soy sauce (Hui et al., 2017; Tanaka et al., 2012; Yang et al., 2017). One of the major bacterial genera in our Moromi fermentation was *Chromohalobacter,* with *C. japonicus* and *C. canadensis* dominating the fermentation for weeks; however, their presence was postponed with increasing salinity. Although they have also been shown to occur in Korean soy sauce and are therefore not unusual, these organisms deserve special attention as they have the potential to produce biogenic amines (e.g. putrescine) (Jung et al., 2015). Interestingly, Radchenkova et al. reported the presence of a strain of *C. canadensis* that produces extracellular polymeric substances under high saline conditions (Radchenkova et al., 2018), which may affect the rheological properties of the product.

One of the most important LAB in soy sauces, *T. halophilus*, has previously been identified as a dominating microorganism in lupine

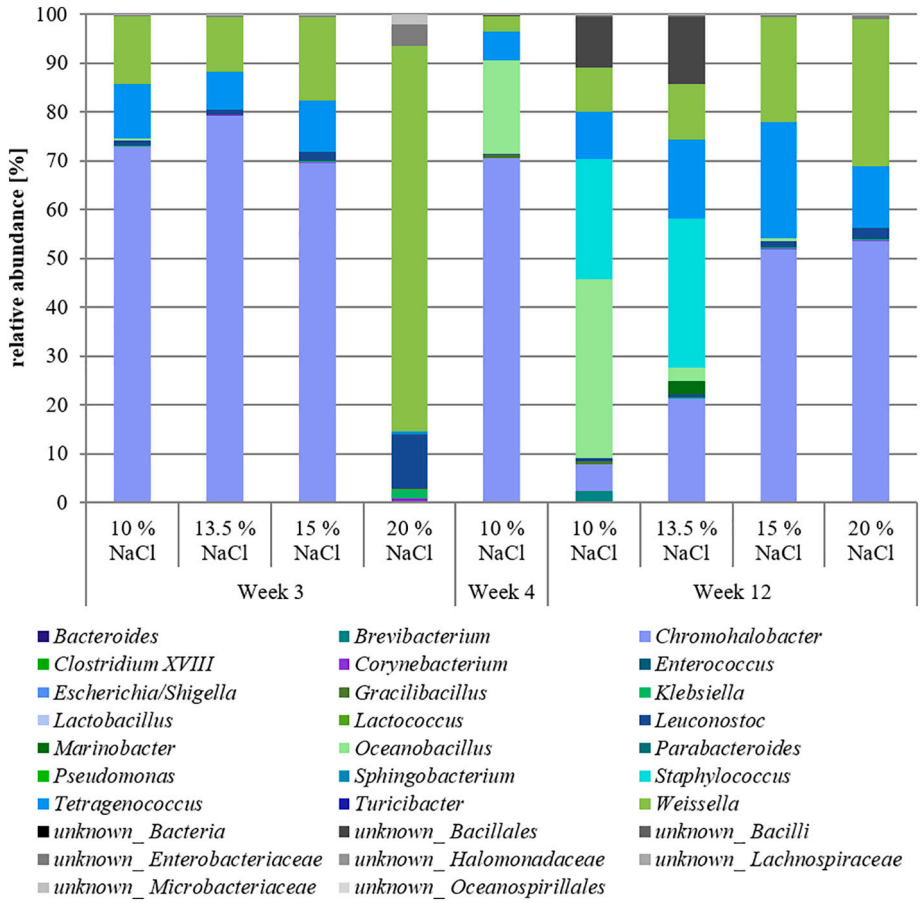

Fig. 5. Diversity of bacteria during Moromi fermentation via 16S amplicon sequence analysis. Evaluation was done using IMNGS and RHEA.

Moromi (Tanaka et al., 2012). This species could be responsible for volatile aroma compounds, such as acetic acid and 2-hydroxy-3-methyl-2-cyclopenten-1-one (Lee et al., 2013). To our knowledge, the subsequently upcoming *Brevibacterium sediminis* has not been described in soy sauce fermentation, but species of this genus were found in Chinese soy bean paste (Zhang et al., 2018). Despite its halotolerance up to 20% NaCl, *Brevibacterium sediminis* was not able to persist in Moromi batches with 15 or 20% salt, possibly be due to the slower increase in pH (Chen et al., 2016). *S. equorum* was strongly affected by salt concentration; therefore, its presence is adjustable with this parameter. This species has been detected in Korean soy sauce and has been described to convey certain benefits and threats (Mannaa et al., 2020), including resistance to antibiotics, haemolytic activity, biofilm formation, but also the production of protease and other enzymes that are important for aroma formation (Jeong et al., 2014). Via 16S rRNA amplicon sequencing, we detected the presence of *Oceanobacillus* in the matured Moromi with lower salt concentrations. This genus has not been mentioned in previous soy sauce fermentation studies, but Tominaga et al. isolated *Oceanobacillus sojae* from soy sauce fermentation equipment, and *Oceanobacillus gochujangensis* has been isolated by Jang et al. from another Korean fermented food, Gochujang (Jang et al., 2014; Tominaga et al., 2009). We were able to identify many more genera via amplicon sequencing than by cultivation, possibly due to the presence of remaining DNA from the Koji fermentate or early Moromi. Our sequencing detected all previously described genera with our cultivation method, therefore this method is suitable for the detection of bacterial consortium in lupine based Moromi. Fortunately, unwanted contaminants, such as *Bacillus* and *Micrococcus* were not detected in the lupine fermentations (Tanaka et al., 2012).

Due to different substrates and microbiota dynamics, the volatile aroma compounds differed from those found in soy mash. Compounds such as ethanol, isopropenyl acetate, ethyl laurate, acetaldehyde, furfural, 5-methylfurfural, 2-furanmethanol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDMF), 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (4-HEMF), and guaiacol were not detected with our technique in lupine based Moromi (Sun et al., 2010). This may have been due to the absence of *Z. rouxii*, C. *versatilis* and/or *C. etchellsii,* and/or the presence of *Chromohalobacter* species (Lee et al., 2013; van der Sluis et al., 2001). In addition, the absence of alcoholic compounds and 4-hydroxyfuranones may depend on the low level of carbohydrate present and the subsequent lack of Maillard reactions (Diez-Simon et al., 2019). As predicted due to the low lipid content in lupine seeds, fewer esters were detected. Nevertheless, typical predominant compounds for soy mash, such as 3-methylbutanol, 3-methylbutanal, acetic acid and 2-methoxy-4-vinylphenol were also detected in the lupine Moromi (Lee et al., 2006; Sun et al., 2010). Pyrazines that were detected at the beginning of the Moromi fermentation were likely formed via Maillard reactions during the roasting of lupine seeds (Koehler and Odell, 1970).

To our knowledge, this is the first published study to describe the microbiota of lupine based Moromi fermentation. This study reveals similarities and differences between soy sauces and lupine based seasoning sauce. In contrast to common (wheat-containing) soy mash, *T. halophilus*, *C. japonicus* and *C. canadensis,* and *D. hansenii* dominated the microbiota in the lupine based Moromi. The concentration of NaCl influenced the microbiota dynamics and volatile compound composition. The aromatic profile was composed of pyrazines, acids, and other compounds, and differed depending on the salt concentration. Furanones were also detected, but 4-hydroxyfuranones, which are typical in wheat-containing soy mashes, were not detected.

Table 1
Volatile compounds in lupine based Moromi fermentation.

Compounds[a]	10% NaCl											13.5% NaCl											15% NaCl											20% NaCl										
	Fermentation period in days											Fermentation period in days											Fermentation period in days											Fermentation period in days										
	0	2	4	7	14	21	28	42	56	84	164	0	2	4	7	14	21	28	42	56	84	164	0	2	4	7	14	21	28	42	56	84	164	0	2	4	7	14	21	28	42	56	84	164
3-Methylbutanol	+	+	+	+	+	+	+				+	+	+	+	+	+	+					+	+	+	+	+	+	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+
2-Phenylethanol		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethyl hexadecanoate		+	+	+	+	+	+	+					+	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+					+	+		+	+	+		
Acetic acid						+	+									+	+	+	+	+									+		+		+	+										+
3-Methylbutanoic acid	+	+	+	+	+	+			+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+			
2-Methylpropanoic acid			+			+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
Benzeneacetaldehyde[b]			+								+				+										+	+	+								+				+	+	+	+	+		
3-Methylbutanal[b]											+																																		
2-Methoxy-4-vinylphenol																		+																				+					+		
Methylpyrazine[b]	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2,5-Dimethylpyrazine[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2,6-Dimethylpyrazine[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dihydro-5-pentyl-2(3H)-furanone[b]	+	+		+								+	+	+	+	+							+	+	+	+	+	+						+		+	+	+		+	+	+	+	
2,4-Dimethylfuran[b]					+	+																																						
Dihydro-3,5-dimethyl-2(3H)-furanone[b]						+	+	+	+	+	+																																	
Dihydro-5-((Z)-2-octenyl)-2(3H)-furanone[b]								+	+	+	+					+	+			+	+	+					+	+				+	+										+
5-Heptyldihydro-2(3H)-furanone[b]										+	+											+											+											
2,3-Dihydrobenzofuran[b]																																											+		

+ Compound was detected qualitatively.

[a] Compounds were chosen from substances Sun et al. described as key aroma constituents for soy sauce and were most predominant in lupine-based Moromi (Sun et al., 2010).

[b] Compounds could potentially have been formed during roasting (Lee et al., 2006).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109316.

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A.2 Supplementary Data to Section A.1

Table S1 Nutritional values of raw, mature lupine seeds and raw, mature soy beans. Values are shown for 100 g published at USDA Branded Food Products Database on 1st April 2019.

	raw lupine seeds	raw soy beans
Water [g]	10.44	8.54
Energy [kcal]	371	446
Protein [g]	36.17	36.49
Total lipid (fat) [g]	9.74	19.94
Ash [g]	3.28	4.87
Carbohydrate, by difference [g]	40.37	30.16
Fiber, total dietary [g]	18.9	9.3

Table S2 Volatile compounds in lupine-based Moromi fermentation with 10 % and 13.5 % NaCl.

	10 % NaCl Fermentation period in days											13.5 % NaCl Fermentation period in days										
Compounds	0	2	4	7	14	21	28	42	56	84	164	0	2	4	7	14	21	28	42	56	84	164
1-Butanol, 3-methyl-	X	X	X	X	X	X	X				X	X	X	X	X	X	X	<				X
1-Hexanol, 2-ethyl-	X								>			X										
1-Octen-3-ol	X	X	<								>	>	X	X	X							
2-Phenylethanol	<	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3-Octanol	<																					
Heptaethylene glycol	<																					
3-Methyl-1-butyl acetate		<	X	>	>								>									
9-Octadecenoic acid, ethyl ester				X	X	X	X										X	>	X	>		
Benzeneacetic acid, ethyl ester									<										>	X	<	
Butanoic acid, 2-methyl-, ethyl ester																						
Butanoic acid, 3-methyl-, ethyl ester											<											X
Ethyl Acetate			>	X		>	>															
Hexadecanoic acid, ethyl ester	<	X	X	X	X	X	X	X					X	X	X	X	X	X	X	X		
Linoleic acid ethyl ester			X	X	X	X	X								>	X	X	X	X	X		
Pentadecanoic acid, ethyl ester										X												<
(R)-(-)-4-Methylhexanoic acid									<	<												
Acetic acid						X	X	<	<							>	X	X	>	>	X	
Benzeneacetic acid							X	X	<	<						X	X	>	X	X	X	
Butanoic acid, 3-methyl-	X	X	X	X	X	X	<	<	X	<		X	X	X	X	X	X	X	X	X	X	>
Butanoic acid, 4-hydroxy-		<		>		<									X							
Heptanoic acid		>	X																			
Hexanoic acid			X				>													<		
Hexanoic acid, 2-methyl-							>	>		>	X											<
n-Decanoic acid	<					<	X											>	<	<		
Nonanoic acid		X	X	>									X	<								
Octanoic acid	X	X	X	>	<	>	>	<				X	X	X				>	>	X		
Propanoic acid, 2-methyl-		<	X			X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X
Tiglic acid								X	<	X												
2-Butanone									<	<												
2-Cyclopenten-1-one, 2-hydroxy-3-methyl-						<	X									X	<	X	X	<		X
2-Hydroxy-5-methylacetophenone	<	<			<	<	X					X		<				<	X	<		
3-Hexen-2-one							>															
3-Octanone	X	X	X	X								X	X	<	X	<						
3-Pentanone, 2-methyl-											<											
4-Hydroxy-2-methylacetophenone													X	>	X	X	X					
4-Hydroxy-2-methylacetophenone		>	X	X	X	>																
4-Hydroxy-3-methylacetophenone	>																					
4-Octanone, 5-hydroxy-2,7-dimethyl-											<											
6-Methylenebicyclo[3.2.0]hept-3-en-2-one											X											
Acetoin	X	X	<	X		X	X					X	X	<	<	X	X	>				
Benzeneacetaldehyde			>		<						X				X							
Benzyl methyl ketone											X											
Butanal, 3-methyl-											X											
Butyrolactone		>	X	<	X								>			>						
Cyclohept-4-enone											<											>
2(3H)-Furanone, 5-heptyldihydro-										>	X											X
2(3H)-Furanone, dihydro-3,5-dimethyl-						>	X	>	>	>	>											
2(3H)-Furanone, dihydro-5-(2-octenyl)-, (Z)-							<	X	X	X	X					X	X			X	X	X
2(3H)-Furanone, dihydro-5-pentyl-	X	X		>								X	X	X	X	X	<					
2,4-Dimethylfuran					>	>																
2-Methoxy-4-vinylphenol																		>				
N-Morpholinomethyl-isopropyl-sulfide									>													
2,3,5-Trimethyl-6-ethylpyrazine	X	X	X	X	X	X	X				X	X	X	X	X	X	X	X	<	<		X
2,3-Dimethyl-5-ethylpyrazine	>	X	X	>								X	X	X	X							
2-Acetyl-6-methylpyrazine											X											
5H-5-Methyl-6,7-dihydrocyclopentapyrazine	X	X	X	X		X	X	<		>	X	X	X	X	X	X	X	X	X	X		X
5H-Cyclopentapyrazine, 6,7-dihydro-2,5-dimethyl-	X	X	X	X	X	X					<	X	X	X	X	>	<					>
Isopropenylpyrazine							>						X									>
Pyrazine, 2,3-dimetyhl-			<				X	>			X		>					X	X	<		X
Pyrazine, 2,5-dimethyl-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, 2,5-dimethyl-3-(1-propenyl)-,(Z)-											>					X	>	<				
Pyrazine, 2,6-diethyl-		X	X	>	X	>					X		X	X	X	X	<	X	<			>
Pyrazine, 2,6-dimethyl-	X	X	X	X	X	X	>	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, 2-ethyl-3-methyl-	X	X	X	X	X	X	X	X		>	X	X	X	X	X	X	X	X	X	<		X
Pyrazine, 2-ethyl-5-methyl-	X	X	X	X	X	X	X	>			X	X	X	X	X	X	X	X	>			X
Pyrazine, 2-ethyl-6-methyl-	>	X	X	X	X	X	X	X	X	X	X	<	X	X	X	X	X	X	X	X	X	X
Pyrazine, 2-methyl-5-(1-propenyl)-, (E)-						<					>		>						<			X
Pyrazine, 2-methyl-5-(1-propenyl)-, (Z)-		<	<																			
Pyrazine, 2-methyl-6-(1-propenyl)-, (E)-															<	<						
Pyrazine, 3,5-diethyl-2-methyl-		X									X	>	>				<	<				>
Pyrazine, 3-ethyl-2,5-dimethyl-	>			X	X	X	X	>		>	X	<				X	X	X	X	X	X	X
Pyrazine, ethyl-	X	X	X	X	X	X	>					X	X	X	X	X	X	X	X	<		
Pyrazine, methyl-	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, tetramethyl-		<									X											
Pyrazine, trimethyl-	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
1,5-Dimethyl-2-pyrrolecarbonitrile		<																				
1H-Benzimidazole, 5,6-dimethyl-											X											>
3-(4-Methyl-4H-[1,2,4]triazol-3-yl)-phenylamine	X	X	>			>					X		X				>	X		<		>
Benzimidazole		<								<						>						
Diethyl Phthalate	>								>													
Diisobutyl phthalate									>													
Dimethyl ether			X	>			>															
Indole											X											>
N-(3-Methylbutyl)acetamide																						<
Pyrimidine, 4,6-dimethyl-							<															
Pyrrolidine, 2-(cyanomethylene)-								>														
Styrene							X	>										X	<			
Sylvestrene									>													
Tridecane		>	X	X	>	X	>		>	X	X											

X Compound was detected in both replicates of the fermentation.
> Compound was detected in the first replicate.
< Compound was detected in the second replicate.

Table S3 Volatile compounds in lupine-based Moromi fermentation with 15 % and 20 % NaCl.

	15 % NaCl fermentation period in days											20 % NaCl fermentation period in days										
Compounds	**0**	**2**	**4**	**7**	**14**	**21**	**28**	**42**	**56**	**84**	**164**	**0**	**2**	**4**	**7**	**14**	**21**	**28**	**42**	**56**	**84**	**164**
1-Butanol, 3-methyl-	X	X	X	X	X	X	X		>		X	X	X	X	X	X	X	X	X	X	X	X
1-Hexanol, 2-ethyl-	X											X										
1-Octen-3-ol	>	X	X	X						>			X	X	X	X	X	X	X		>	
2-Phenylethanol	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
E-11,13-Tetradecadien-1-ol												<										
Ethanol, 2-(dodecyloxy)-												X										
Octaethylene glycol																	>					
3-Methyl-1-butyl acetate					>									>								
9-Octadecenoic acid, ethyl ester						>	X				>											
Benzeneacetic acid, ethyl ester									<	X												
Butanoic acid, 2-methyl-, ethyl ester											X											
Butanoic acid, 3-methyl-, ethyl ester											X											
Hexadecanoic acid, ethyl ester		X	X	X	X	X	X	X	X	>					>	>		X	X	X		<
Linoleic acid ethyl ester			>	>	X	X	X	X	X									X	<	>		
Acetic acid						X		X		X	X									<		X
Benzeneacetic acid						X	X	X	X	X	X											X
Butanoic acid, 3-methyl-	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	>		<	<	
Butanoic acid, 4-hydroxy-		<	<	<	X									<			>		>			
Heptanoic acid												X										
Hexanoic acid							>															
Hexanoic acid, 2-methyl-											X							<	X	>	>	X
n-Decanoic acid							X	X	>													
Nonanoic acid	>	X										X	X		>							
Octanoic acid	X	X					X	>	<	X		X	X	>	>							
Propanoic acid, 2-methyl-		X	X	X	X	X	X	X	X	X	X	<	X	X	X	X	X	X	X	X	X	X
2-Cyclopenten-1-one, 2-hydroxy-3-methyl-					X	X	X	X	X	>	X							<	<	X	X	X
2-Hydroxy-5-methylacetophenone	>				<	<	>	>	X			X	>			>			X		<	>
3-Octanone	X	<	<	X	>					>		X	<	X	<	<	X				>	
4-Hydroxy-2-methylacetophenone		X	X	>	>	X							<	X	<		X	X		X		
Acetoin	X		>		X	<	>					X	X	>	X	X	X	X	X	X	>	
Benzeneacetaldehyde		<	>	X	X								>		<	X	X	X	>	X		
2(3H)-Furanone, dihydro-5-pentyl-	X	X	>	>	X	X						X	<	X	>	>		>	>	>	>	
2(3H)-Furanone, 5-heptyldihydro-											X											<
2(3H)-Furanone, dihydro-5-(2-octenyl)-, (Z)-					X	X				X	X					<	<				<	X
Benzofuran, 2,3-dihydro-																				>		
2-Methoxy-4-vinylphenol	<			<			<	<							>						>	
2,3,5-Trimethyl-6-ethylpyrazine	X	X	X	X	>	X	X	X	X	<	X	X	X	X	X	X	X	X	X	X	X	
2,3-Dimethyl-5-ethylpyrazine	X	X	X	X								<	X	X	X							
5H-5-Methyl-6,7-dihydrocyclopentapyrazine	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X
5H-Cyclopentapyrazine, 6,7-dihydro-2,5-dimethyl-	X	X	X	X	X		X					X	X	X	X	X	X	>	>			
Isopropenylpyrazine								X	X	>				>							<	
Pyrazine, 2,3-dimetyhl-							X	X	X	>	<			>			>	X	X	X	X	<
Pyrazine, 2,5-diethyl-														>								
Pyrazine, 2,5-dimethyl-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, 2,5-dimethyl-3-(1-propenyl)-, (Z)-														>			>					
Pyrazine, 2,6-diethyl-					X	X	X	<	>				>	>		<	>	X	X	X	>	
Pyrazine, 2,6-dimethyl-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, 2-ethyl-3-methyl-	X	X	X	X	X	X	X	X	X	>	X	X	X	X	X	X	X	X	X	X	X	<
Pyrazine, 2-ethyl-5-methyl-	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X		X
Pyrazine, 2-ethyl-6-methyl-		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, 2-methyl-5-(1-propenyl)-, (E)-						>	<	<	X	>	>		>		>	>						
Pyrazine, 2-methyl-6-(1-propenyl)-, (E)-											<											
Pyrazine, 3,5-diethyl-2-methyl-					X									>			>	>				
Pyrazine, 3-ethyl-2,5-dimethyl-	X				X	X	X	X	X	X	X	>				X	X	X	X	X	X	X
Pyrazine, ethyl-	X	X	X	X	X	X	X	X	X			X	X	X	X	X	X	X	X	X	<	
Pyrazine, methyl-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Pyrazine, trimethyl-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
1,5-Dimethyl-2-pyrrolecarbonitrile														>	>					<		
3-(4-Methyl-4H-[1,2,4]triazol-3-yl)-phenylamine		X			<		<	X	<		<							<	>	<		
Benzimidazole						>												>				
Butyrolactone														>	<	>		<				
N-(3-Methylbutyl)acetamide											<										<	X
Pyrimidine, 4,6-dimethyl-										>											>	
Styrene							X	>														
Tridecane			<	X	X	<		<	X	>				>		X	X		X	X	X	

X Compound was detected in both replicates of the fermentation.
> Compound was detected in the first replicate.
< Compound was detected in the second replicate.

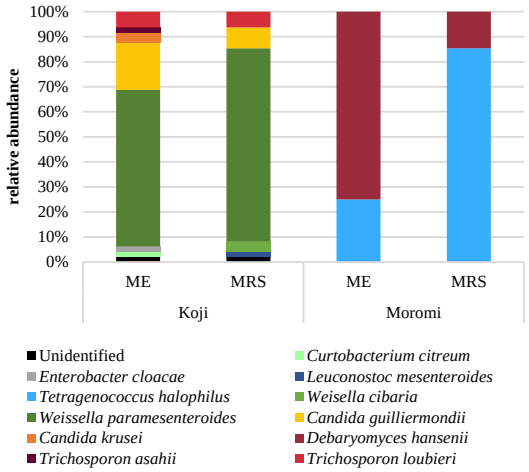

Figure S1 Microbiota in substrate material Koji and matured Moromi. Plated on ME and MRS agar and identified via MALDI-TOF-MS.

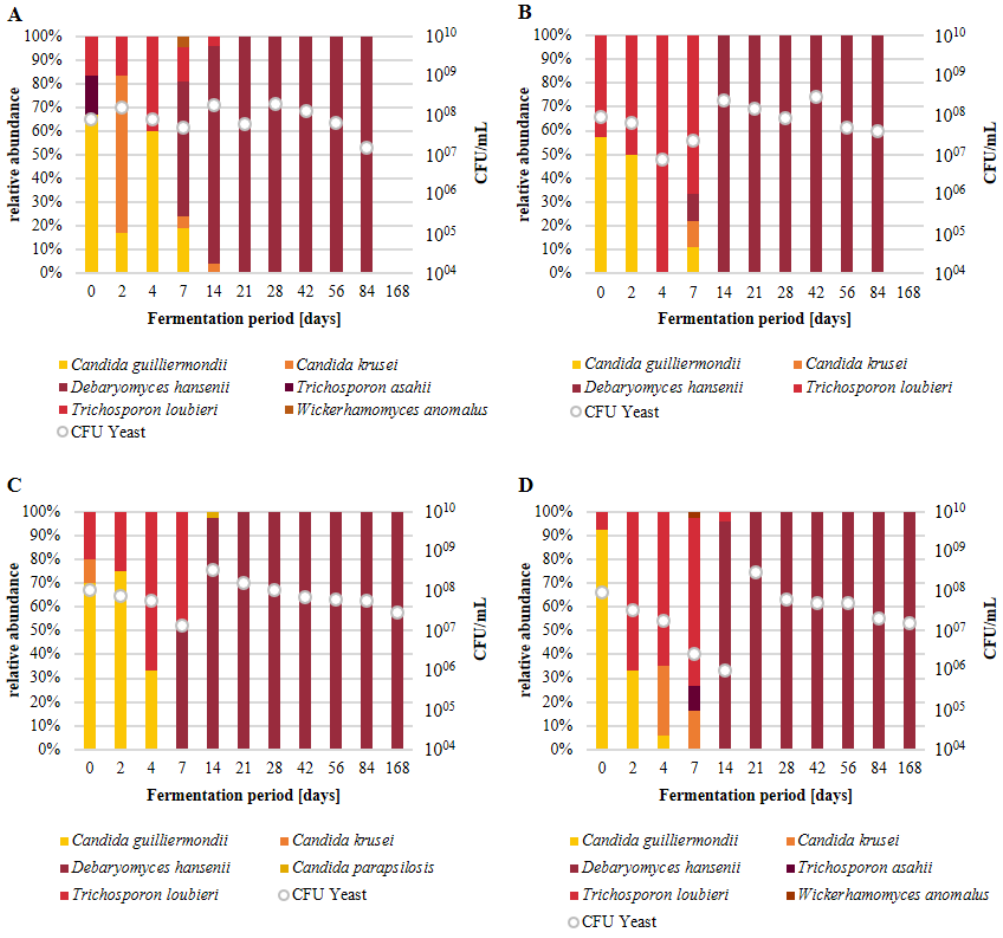

Figure S2 Diversity of yeast during Moromi fermentation on MRS agar via MALDI-TOF-MS. Species and total number of yeast CFU/mL in Moromi fermentation with 10 % (**A**), 13.5 % (**B**), 15 % (**C**) and 20 % (**D**) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of yeast.

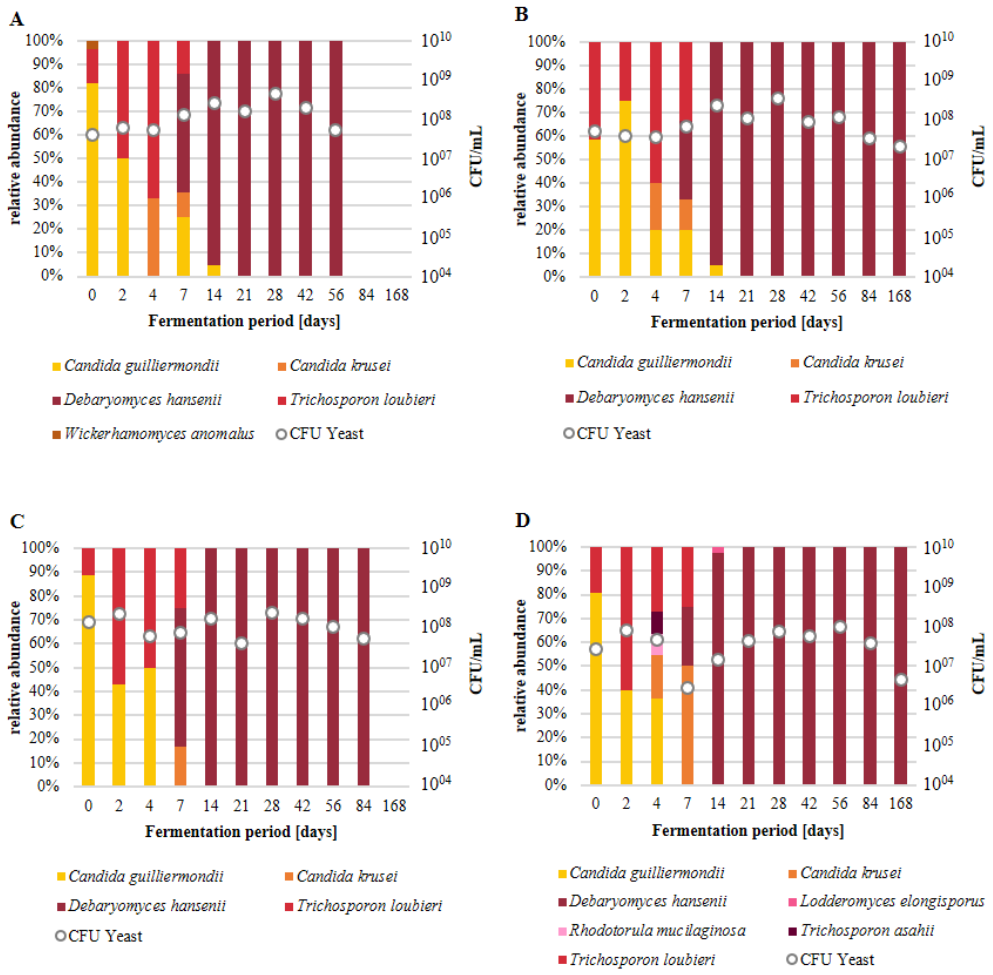

Figure S3 Repetition: Diversity of yeast during Moromi fermentation on ME agar via MALDI-TOF-MS. Species and total number of yeast CFU/mL in Moromi fermentation with 10 % (**A**), 13.5 % (**B**), 15 % (**C**) and 20 % (**D**) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of yeast.

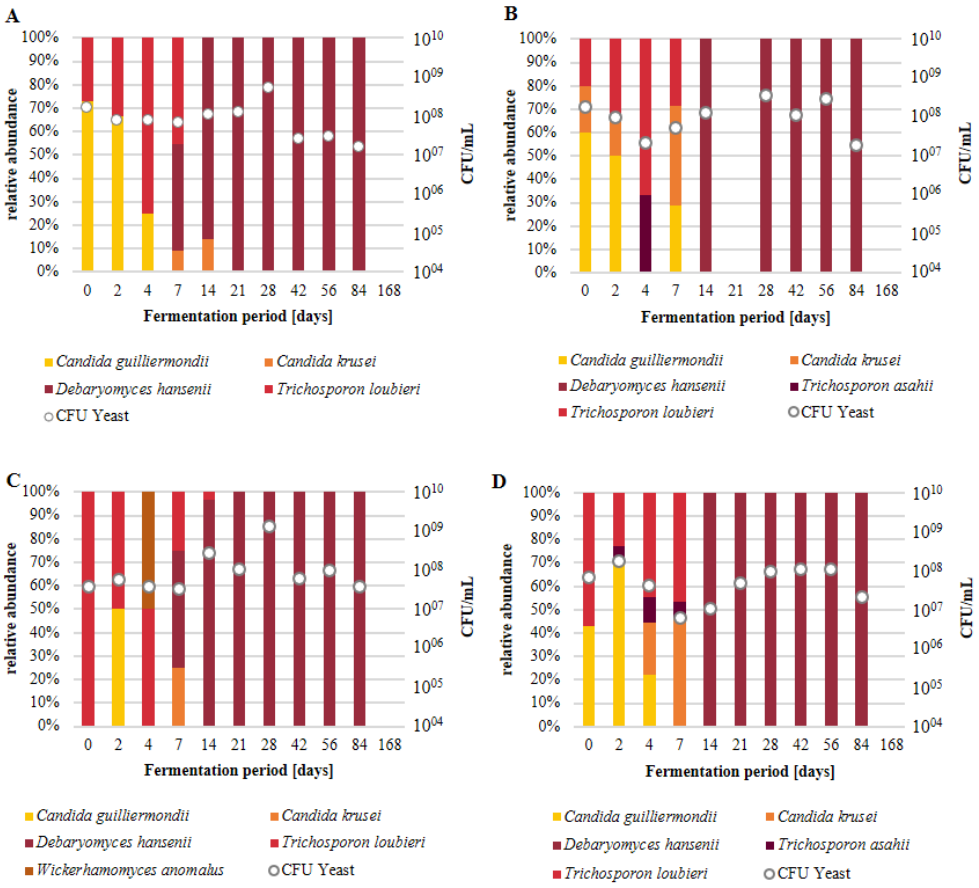

Figure S4 Repetition: Diversity of yeast during Moromi fermentation on MRS agar via MALDI-TOF-MS. Species and total number of yeast CFU/mL in Moromi fermentation with 10 % (**A**), 13.5 % (**B**), 15 % (**C**) and 20 % (**D**) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of yeast.

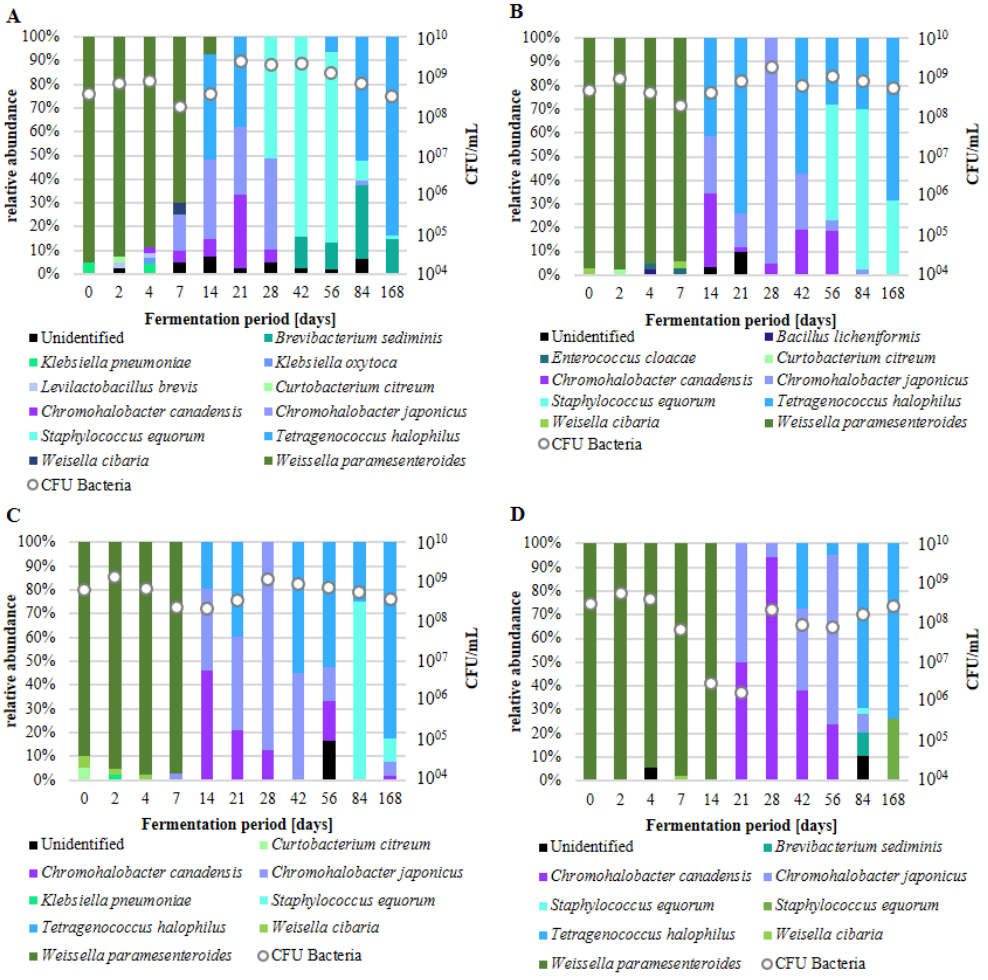

Figure S5 Repetition: Diversity of bacteria during Moromi fermentation on ME agar via MALDI-TOF-MS. Species and total number of bacterial CFU/mL in Moromi fermentation with 10 % (**A**), 13.5 % (**B**), 15 % (**C**) and 20 % (**D**) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of bacteria.

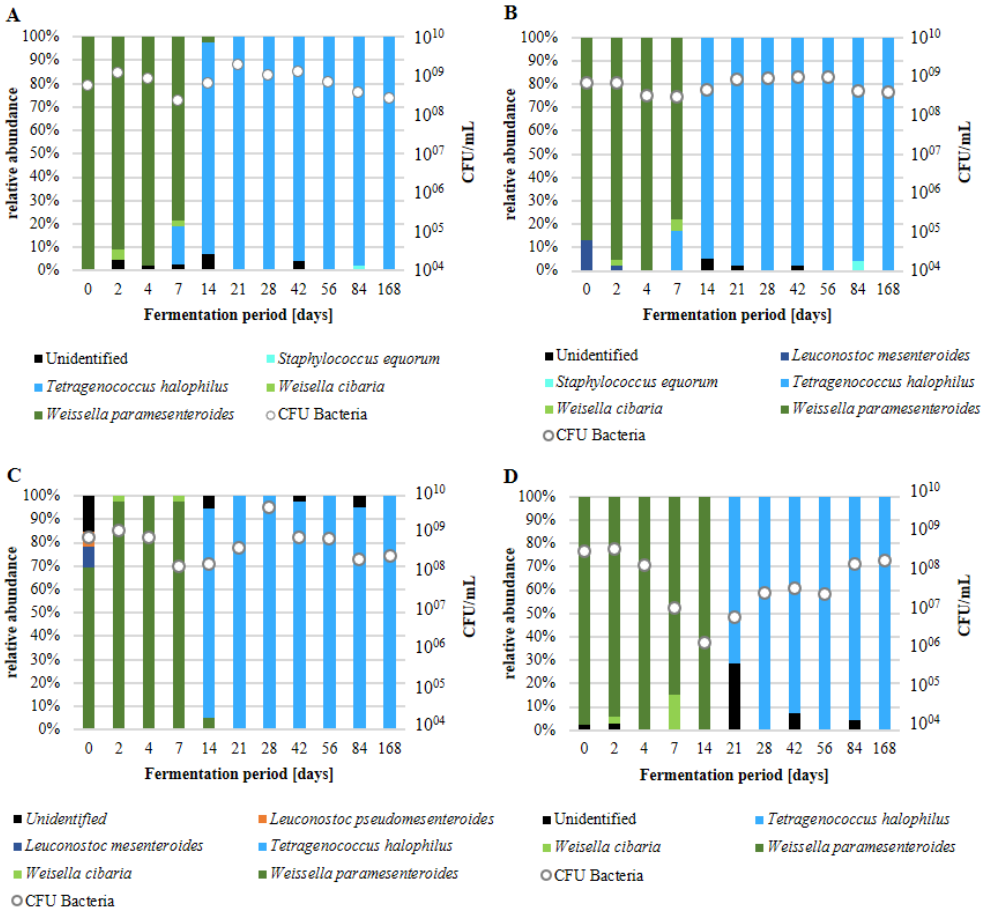

Figure S6 Repetition: Diversity of bacteria during Moromi fermentation on MRS agar via MALDI-TOF-MS. Species and total numbers of bacterial CFU/mL in Moromi fermentation with 10 % (**A**), 13.5 % (**B**), 15 % (**C**) and 20 % (**D**) NaCl. Relative Abundance was applied on total CFU/mL to determine absolute abundances of bacteria.

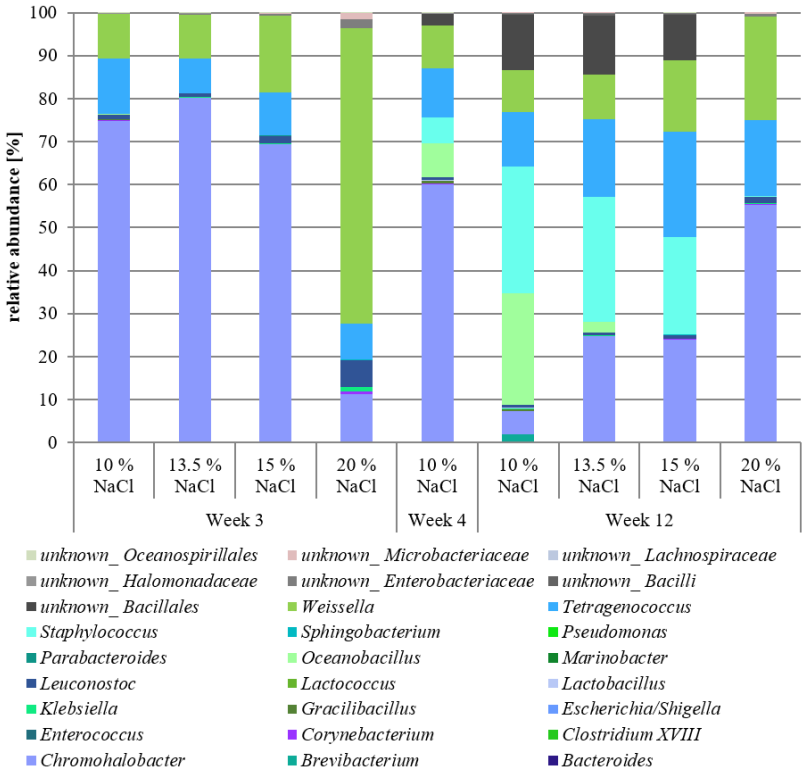

Figure S7 Diversity of bacteria during Moromi fermentation via 16S amplicon sequence analysis in a second batch. Evaluation was done using IMNGS and RHEA.

A.3 Full publication: Carbohydrate Sources Influence the Microbiota and Flavour Profile of a Lupine-Based Moromi Fermentation

The following full text of the research article was originally published in the Journal Foods.

Citation:

Lülf, R. H., Selg-Mann, K., Hoffmann, T., Zheng, T., Schirmer, M., Ehrmann, M. A. Carbohydrate Sources Influence the Microbiota and Flavour Profile of a Lupine-Based Moromi Fermentation // Foods. 2023. 12.

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Article

Carbohydrate Sources Influence the Microbiota and Flavour Profile of a Lupine-Based Moromi Fermentation

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Abstract: Lupine-based seasoning sauce is produced similarly to soy sauces and therefore generates a comparable microbiota and aroma profile. While the koji state is dominated by *Aspergillus oryzae*, the microbiome of the moromi differs to soy moromi, especially in yeast composition due to the absence of *Zygosaccharomyces rouxii* and *Debaryomyces hansenii* as the dominant yeast. In this study, we monitored the addition of a carbohydrate source on the microbiome and aroma profile of the resulting sauce. Compared to previous studies, the usage of a yeast starter culture resulted in a sparsely diverse microbiota that was dominated by *D. hansenii* and *T. halophilus*. This led to a pH below 5 even after four months of incubation and most of the measured aroma compounds were pyrazines and acids. The addition of wheat and buckwheat resulted in a temporary change in the yeast consortium with the appearance of *Z. rouxii* and additional bacterial genera. The aroma profile differs in the presence of pyrazines and esters. Since no significant differences in the taste and odour of wheat-added and buckwheat-added sauce was sensed, both substrates influence the lupine sauce in a similar way.

Keywords: lupine moromi; sensory analysis; GC-MS; *Zygosaccharomyces rouxii*; *Debaryomyces hansenii*

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1. Introduction

Fermented seasoning sauces such as soy and fish sauces are very popular to enhance the saltiness and umami taste of food all over the world [1]. Recently, plant-based alternatives are becoming more and more attractive for the industry. Compared to soy beans, lupine seeds contain a similar protein content but higher amounts of carbohydrates, which may make them a suitable alternative without the requirement of wheat addition [2]. Parallel to soy sauces, lupine-based seasoning sauce is produced in a two-step fermentation process.

For the initial step, the so-called koji, lupine seeds are roasted and crushed, and inoculated with *Aspergillus oryzae* for two days. With the addition of brine at 13.5% sodium chloride, the growth of the mould and potentially pathogenic microorganisms is inhibited, and this second fermentation step is called moromi (mash). Additionally, the microbiota can be modified using starter cultures as well as altered NaCl levels [3,4]. This is incubated for six months and lactic acid bacteria such as *Weissella paramesenteroides*, *Tetragenococcus halophilus*, *Chromohalobacter* species, and *Staphylococcus equorum*, and yeasts such as *Candida guilliermondii* and *Debaryomyces hansenii* are then dominating the moromi [2].

Previously, *D. hansenii* was isolated from the early phase of fermentation of *Ganjang*, a Korean soy sauce from a naturally fermented soybean block called *Meju* [5]. It also temporarily dominates the moromi-like *baceman* state of the Indonesian sauce *kecap manis* which is based on black soybeans [6]. Furthermore, *D. hansenii* is associated with other

foods such as cheese, raw sausage, wine, beer, and fruit [7]. They produce various flavour components, such as branched-chain aldehydes and alcohols [8].

In Japanese wheat-containing soy moromi, the most dominant yeast is *Zygosaccharomyces rouxii* [9]. It grows at a pH below 5 and uses glucose to produce alcohols such as ethanol and fusel alcohols [10,11]. Moreover, it is an important producer of flavour compounds such as 4-hydroxyfuranones and therefore, its absence in lupine moromi is further investigated in this study. Since the absence of *Z. rouxii* was often mentioned for moromi based solely on soy beans [9], the addition of wheat as the standard carbohydrate source in soy moromi, and buckwheat as a gluten-free alternative were tested in this study. As we wanted to compare the persistence of *D. hansenii* and *Z. rouxii*, a mixed starter culture was inoculated in the moromi.

2. Materials and Methods

2.1. Koji and Moromi Fermentation

For the koji, lupine seeds (*Lupinus angustifolius*), wheat grains, and buckwheat were roasted separately at 150 °C for 30 min. Each of these was mixed up with 70% soaked lupine seeds and merged with spores of *Aspergillus oryzae*. Incubation was executed at 25–35 °C for two days. Each moromi batch consisted of 40% koji, either with roasted lupine seeds, wheat, or buckwheat, and brine with 13.5% (*w*/*v*) NaCl. As starting cultures, 10^5 CFU/mL of *D. hansenii* strain TMW 3.1188 and *Z. rouxii* strain DSM 2531 were added. Each fermentation was prepared in duplicate and incubated at 25 °C at a humidity of 80%. A weekly mixing was executed for ventilation and the volume level was kept stable with deionised water.

2.2. pH Measurement, Cell Counts and Chromatography

The pH measurements, cell counts, and gas chromatography–mass spectrometry (GC-MS) were completed as in previous works [2]. We used malt extract peptone agar (ME, malt extract 20 g/L, soy peptone 2 g/L, NaCl 25 g/L, agar 15 g/L) at pH 5.6 and incubated under oxic conditions at 30 °C. Several dilutions of the samples were plated so that a standard deviation of cell counts could be calculated from quadruplicates. All colonies larger than 0.5 mm were recorded.

Furanones were detected via HPLC-MS from moromi supernatants in duplicates. For the extraction, an 8 g sample was mixed up with 25 mL H_2O and 10 µL phenol (10 mg/mL, internal standard) and centrifuged (5000× *g*, 10 min). The solid residue was re-extracted by 25 mL H_2O, centrifuged, and the combined supernatants were subjected to solid phase extraction on Amberlite XAD-2 polymeric adsorbent (20–60 mesh; Merck, Darmstadt, Germany). The column was rinsed with 50 mL H_2O and afterwards compounds were eluted with 100 mL diethyl ether. The diethyl ether extract was dried with sodium sulphate, concentrated by using a rotary evaporator, constricted to dryness with a stream of nitrogen and transferred into 100 µL of water. The aqueous phase was analysed by HPLC.

The HPLC system used was an Agilent 1200 HPLC system composed of two Isocratic Pumps (G1310A), a Micro Well-plate Autosampler (G1377A), a Column Thermostat (G1316A), a Diode Array Detector SL (G1315C), and an Agilent 6320 Ion Trap. The column was a LUNA C18(2) (150 mm × 2.0 mm, particle size 5 µm; Phenomenex; Aschaffenburg, Germany) held at 28 °C. The binary gradient system consisted of solvent A, water with 0.1% formic acid and solvent B, 100% methanol with 0.1% formic acid. The gradient program was as follows: 0–10 min, 100% A to 90% A/10% B; 10–50 min, 90% A/10% B to 70% A/30% B; 50–65 min, 70% A/30% B to 20% A/80% B; 65–70 min, 100% B; 70–80 min, 100% A. The flow rate was 0.2 mL/min and the injection volume was 5 µL.

Compounds were identified by their retention times and mass spectra in comparison with data determined for authentic reference materials. Quantification was achieved by means of calibration curves and DAD (UV traces 272 nm for Phenol, 290 nm for Furaneol and 284 nm for Norfuraneol).

2.3. Identification of Organisms

Identification of yeasts at species level were based on low molecular sub-proteome profiling using matrix-assisted laser desorption–ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). A total of 96 colonies per sample were streaked at least once and measured with Microflex LT Spectrometer (Bruker Daltonics, Bremen, Germany). Identification was achieved via comparison of detected spectra with the Bruker Biotyper database (Bruker Daltonics) and an in-house database validated with 18S rRNA gene sequenced strains and published type strains. Moulds were identified by microscopic analysis of hyphae and spores by Prof. Dr. Ludwig Niessen.

Bacterial samples were analysed along with yeasts by MALDI-TOF-MS and via 16S ribosomal RNA gene amplicon sequencing by Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany). Therefore, the V4 region was amplified from purified DNA using primers 519F (5′ CAG CMG CCG CGG TAA TWC) and 785R (5′ GAC TAC HVG GGT ATC TAA TCC) [12]. Sample preparation and analysis procedure were completed according to the method described previously [2].

2.4. Sensory Analysis

The different lupine sauce variants were tested by a panel for the perceptible flavour components and their intensity.

The panel members were selected from a group of 15 people who had to describe the aroma impression of eight different smelling sticks with defined aroma substances and classify their intensity using a scale. Only people who had sufficient sensitivity to the majority of these aromas were then included in the panel. The final panel then consisted of 10 people (3 female, 7 male). A total of seven of these people completed training in the food sector or were working in the sector for several years.

A list of typical soy sauce flavours was used as the template for a questionnaire [13]. The list was supplemented with a few more terms based on the results of GC analyses; similar and synonymous terms were removed as far as possible. A questionnaire with 35 aroma and flavour terms (taste and smell) remained. The participants received 1 mL samples of each variant in a 25 mL beaker to smell and taste. If a perception could be assigned to a concept, a number from 1 to 4 was assigned for the intensity (1 = very weak, hardly perceptible to 4 = very strong, dominating). Statistical tests on the pairwise comparisons of the sensory data of the biological replicates, as well as the aroma profiles of lupine sauces with additional carbohydrate sources, were performed using the Wilcoxon test.

3. Results

3.1. Major Characteristics of Lupine-Based Moromi Fermentation

The microbial cell counts and pH values changed during the fermentation and differed between the different batches (Figure 1). They all started with 1.7–2.4 $\times$ 10^7 CFU/mL and reached 1.4–8.0 $\times$ 10^9 CFU/mL after 16 weeks of incubation. Within the first four weeks in the fermentation with lupine seeds as sole substrate, the abundance of microorganisms increased with an average recorded growth rate of 7.4 per week for batch ML1 and of 8.2 per week for ML2. Subsequently, the stationary growth phase was reached. With additional carbohydrate sources, similar growth rates were detected within the first two weeks. However, after four weeks of incubation, cell counts were lower in these batches compared to the control group which correlates with the presence of *Z. rouxii* (Figure 2). Interestingly, they all reached cell counts of similar CFU/mL after 16 weeks.

In the same way as the cell counts, the pH values were similar at the beginning of the fermentation (approx. 6.2) and also after 16 weeks (approx. 4.7). During the first four weeks, the fermentation without wheat and buckwheat showed a fast decrease below a pH of 5. For the batches with wheat and buckwheat, these low pH values were firstly detected after eight to 12 weeks. The decrease in the pH matched with the appearance of *T. halophilus*, since it appeared in ML1 and ML2 after four weeks and was detected for the first time in batches with wheat and buckwheat after eight weeks (Figure 2).

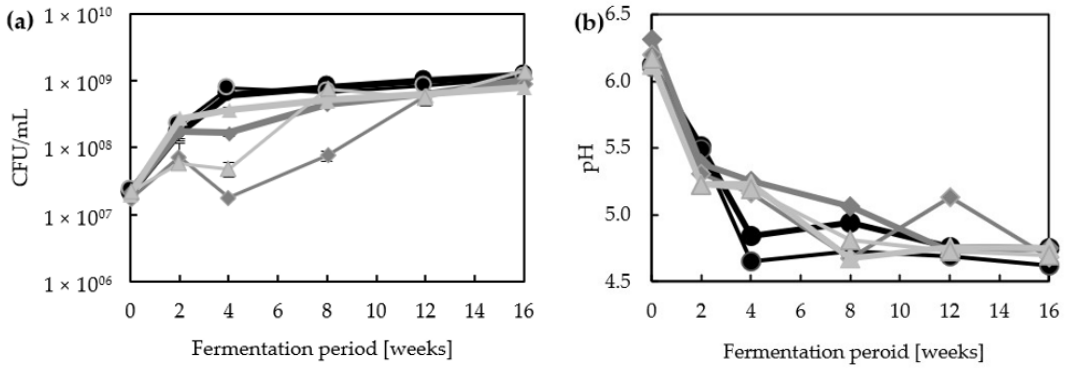

Figure 1. Abundance of microorganisms after four days of incubation on ME agar at 30 °C (**a**) and pH values (**b**) during moromi fermentation. Koji was prepared using lupine seeds with additional roasted lupine seeds (ML1 and ML2, circles, black), wheat (MW1 and MW2, squares, dark grey), or buckwheat (MB1 and MB2, triangles, light grey). The replicates could be distinguished by a grey border (ML2, MW2, MB2). Error bars indicate the standard deviation from quadruplicates.

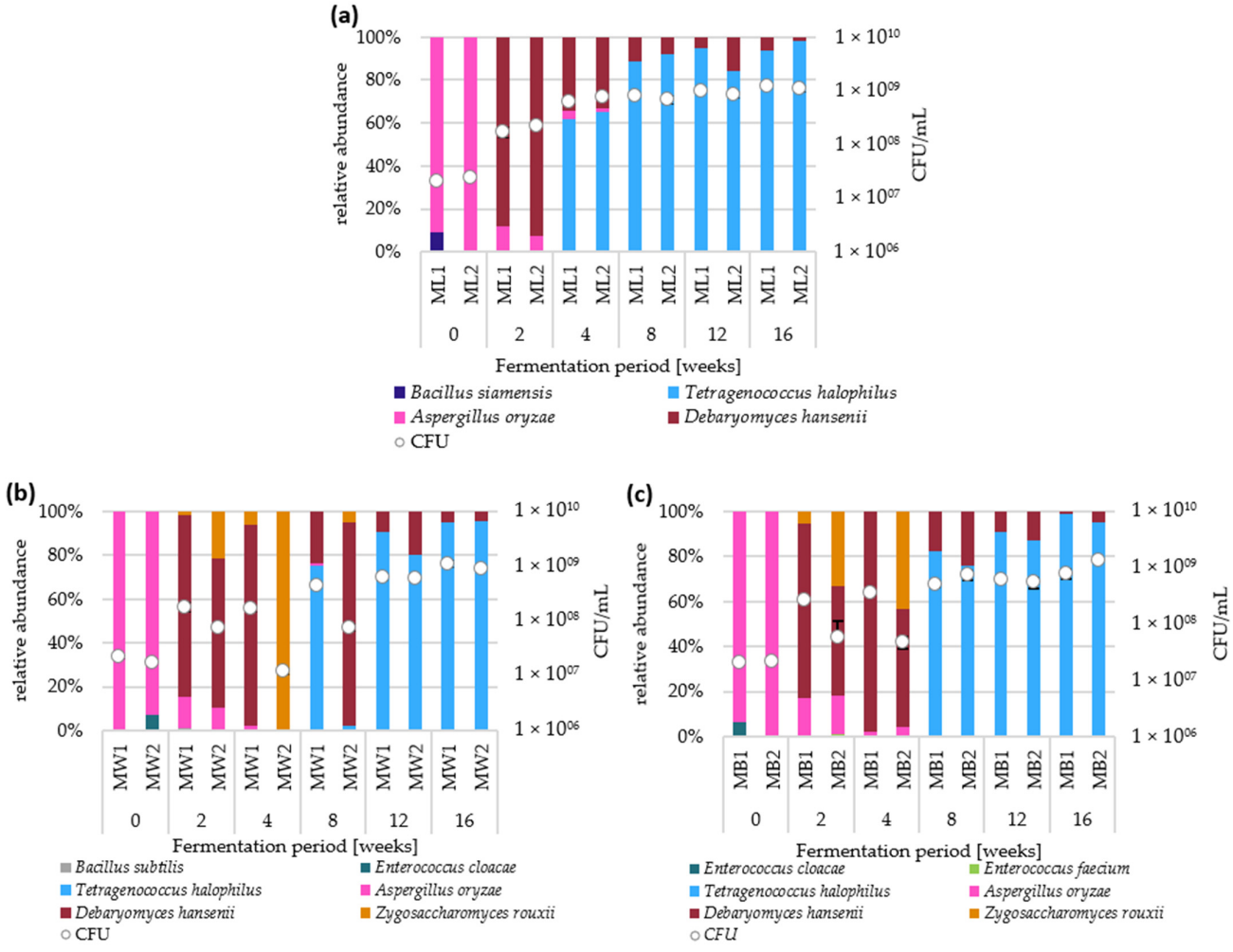

Figure 2. Microbiota dynamics during moromi fermentation, incubated for four days on ME agar at 30 °C via MALDI-TOF-MS. Species and CFU/mL (white dots) in moromi fermentation with (**a**) Lupine seeds as sole substrate, with (**b**) wheat and (**c**) buckwheat. Error bars indicate the standard deviation from quadruplicates.

3.2. Microbial Composition

For the identification of microorganisms, single colonies were analysed via MALDI-TOF-MS profiling. At the beginning of the fermentation, the plates were overgrown by *A. oryzae* which was the koji starter not yet inhibited by the salt addition. Therefore, it was barely possible to detect any other organisms.

After two weeks of moromi incubation with the lupine seeds as sole substrate, *D. hansenii* was detected (Figure 2a). In the following weeks, *T. halophilus* appeared and dominated the moromi until the end of the fermentation. Throughout the fermentation, no other organisms were identified.

In the wheat-containing batches, *Z. rouxii* was detected between week two and eight with its highest abundance (1.61×10^7 CFU/mL) after 2 weeks in batch ML2 (Figure 2b). The total amount of *Z. rouxii* is similar at any point, in both batches and after 2, 4, and 8 weeks (in ML2).

D. hansenii showed a decrease in cell counts in some samples which led to the drop of total cell counts and to a high relative abundance of *Z. rouxii*.

The addition of buckwheat led to a similar microbiota compared to wheat-added fermentations, but growth of *D. hansenii* was less severely suppressed (Figure 2c). The highest cell count of *Z. rouxii* (2.08×10^7 CFU/mL) was determined after four weeks of incubation in batch MB2. After eight weeks, this species was no longer detected in fermentations with buckwheat. Independent of the addition of a carbohydrate source, all batches were dominated by *T. halophilus* and *D. hansenii* after 16 weeks of fermentation.

The 16S rDNA amplicon sequencing revealed the presence of other bacterial genera in carbohydrate-supplemented moromi after four weeks of incubation (Figure 3). Besides *Tetragenococcus*, the most abundant organisms detected were *Enterobacter* (highest sequence similarity with *E. hormaechei*, and *E. cloacae*), *Lactococcus* (*lactis*, *taiwanensis*), *Leuconostoc* (*holzapfelii*, *falkenbergense*), *Pseudomonas* (*juntendi/putida*), *Staphylococcus* (*warneri*, *aureus*, *caprae*), and *Weissella* (*confusa*, *paramesenterioudes*). It is probable that these were not detected via MALDI-TOF-MS due to the detection limit for the cultivation method (<10^2 CFU/mL) or due to poor growth in media used in this study. In the same way as the culture-dependent method, 16S rDNA amplicon sequence analysis revealed *Tetragenococcus* as the only bacterium present at the end of the fermentation in all batches.

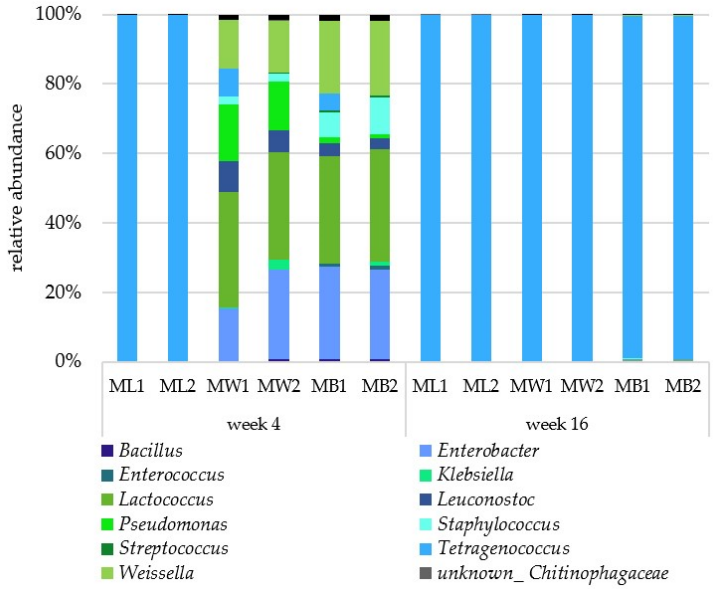

Figure 3. Diversity of bacteria during moromi fermentation determined via 16S rDNA amplicon sequence analysis. Data were evaluated using IMNGS and RHEA [14,15].

3.3. Aromatic Compounds

The aroma profile is one of the most important parameters in the evaluation of the quality of a seasoning sauce. The effect of fermentation on the aroma compound composition was determined by GC-MS analysis. In total, 108 volatile compounds were detected and a representative selection of acids, esters, ether, pyrazines, and furan(on)es are shown in Table 1. During the fermentation, acids were formed in all batches. Esters and dimethyl ether were not detected at the beginning of the fermentation but appeared, especially in fermentations with wheat and buckwheat, during the four months of incubation.

Table 1. Excerpt of volatile aromatic compounds measured via GC-MS *headspace* analysis after 0, 12, and 16 weeks of fermentation.

	Moromi with Lupine Seeds			**Moromi with Wheat**			**Moromi with Buckwheat**		
Compound	**0**	**12**	**16**	**0**	**12**	**16**	**0**	**12**	**16**
2-Methylpropanoic acid		X	X		X	X		X	X
3-Methylbutanoic acid		X	X		>	>		X	X
Acetic acid		X	X		X	>		X	X
Benzene acetic acid		<	X		X	X		X	X
n-Decanoic acid		X	>						
Nonanoic acid	<	X			<			>	
Octanoic acid	X	X	>	X	<	<		>	
2-Methylbutanoic acid ethyl ester					X	X		X	X
2-Methylpropanoic acid ethyl ester					X	X		X	X
2-Phenylethyl acetate					X	X		X	<
3-Methyl-1-butyl acetate					X				<
3-Methylbutanoic acid ethyl ester					>	X		X	X
3-Methylbutyl 2-methylbutanoate					<			>	X
3-Methylbutyl 3-methylbutanoate					<	X		X	X
3-Methylbutyl butanoate					>	<		X	
Ethyl 9-octadecenoate			>		X	X		X	X
Ethyl Acetate					X			X	
Ethyl benzene acetate					X	>		<	X
Ethyl hexadecanoate		X	>		X	X		X	X
Isopentyl 2-methylpropanoate					>	>		<	X
Linoleic acid ethyl ester		X	X		X	X		X	X
Octanoic acid ethyl ester					<			X	
Phenylethyl 3-methyl-butanoate					>	>		<	X
β-Phenylethyl butyrate					X	X		X	X
Dimethyl ether					X	X		X	<
2,3,5-Trimethyl-6-ethylpyrazine	X	X	X	X					
2,3-Dimethyl-5-ethylpyrazine	>	<		>					
2,3-Dimetyhlpyrazine	X	X	X	X		>	X		
2,5-Diethylpyrazine	X	X	<						
2,5-Dimethylpyrazine	X	X	X	X	X	X	X	X	X
2,6-Diethylpyrazine	>	X	X						
2,6-Dimethylpyrazine	X	X	X	X		>			
2-Ethyl-3-methylpyrazine	X	X	X	X	>	>	X		
2-Ethyl-5-methylpyrazine	X	X	X	X	>	>	X		
2-Ethyl-6-methylpyrazine	X	X	X	<	X	X		X	X
2-Methyl-5-((E)-1-propenyl)-pyrazine	>	<	<						
2-Methyl-5-((Z)-1-propenyl)-pyrazine	X	<							
3,5-Diethyl-2-methylpyrazine	X	X	X						
3-Ethyl-2,5-dimethylpyrazine	X	X	X	X					
5H-5-Methyl-6,7-dihydrocyclopentapyrazine	X	X	X	X	>	>	X		<
6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine	X	<	>						

Table 1. *Cont.*

	Moromi with Lupine Seeds			Moromi with Wheat			Moromi with Buckwheat		
Compound	**0**	**12**	**16**	**0**	**12**	**16**	**0**	**12**	**16**
Ethyl pyrazine	X	X	X	X	>		X		
Methylpyrazine	X	X	X	X			X		
Trimethyl pyrazine	X	X	X	X	X	X		X	X
2,3-Dihydrobenzofuran		>	X				X		
5-Isopropyl-3,3-dimethyl-2-methylene-2,3-dihydrofuran		>							
Dihydro-5-((Z)-2-octenyl)-2(3H)-furanone		X	>		X	X	<	>	X

X Compound was detected in both replicates of the fermentation. > Compound was detected in the first replicate. < Compound was detected in the second replicate.

At the beginning, a number of different pyrazines were determined. However, they were metabolised during the fermentation with additional carbohydrate sources. The 2-Phenylethanol, 3-methyl-1-butanol, and benzene acetaldehyde (not shown) were measured in all batches during the whole fermentation. At the end of the fermentation, moromi with lupine seeds as sole substrate contained primarily pyrazines and acids and the volatile compounds of moromi with additional wheat and buckwheat were mainly esters and acids.

Since 4-hydroxyfuranones were not detected via GC-MS, we analysed 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 4-hydroxy-5-methylfuran-3(2*H*)-one (HMF) via HPLC in matured moromi (Table 2). The 4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF) quantities were below the detection limit.

Table 2. Furanone analysis via HPLC, measured in technical duplicates. The moromi was sampled after four months of incubation with lupine seeds as sole substrate (ML), wheat (MW), and buckwheat (MB).

	HDMF (µg/g)	**HMF (µg/g)**
ML1	1.47	1.74
ML2	0.92	1.16
MW1	0.76	2.67
MW2	0.60	3.62
MB1	0.83	2.68
MB2	1.87	4.52

HDMF was present in all moromi batches in similar amounts. However, the concentration of HMF was increased in fermentations with wheat and buckwheat compared to sole lupine seeds.

3.4. Sensory Analysis

For a description of the flavour, a sensory analysis of the resulting seasoning sauces was performed. Since the sensory data of the biological replicates differ from each other (Figure S1 (Supplementary Materials)), they will be described separately. The taste was described using the six attributes of sweet, umami, bitter, astringent, salty, and sour (Figure 4) with intensities of 0 (not perceptible) to 4 (very strong).

All products were described with similar strong saltiness (2.1–2.8) and umami taste (1.3–2.0). None of the sauces was described as more than faintly astringent or bitter. The taste attributes of these products were not significantly different.

However, the seasoning sauce without additional carbohydrate sources showed a quite intense aroma of beeswax and chocolate but alcoholic, sweaty, goat-like, or wine-like flavours were not perceptible (Figure 5). The sauces with wheat showed a fermented, cheesy and nutty aroma and no floral, mouldy or hay-like flavour. For sauces with buckwheat the most dominant flavours were cheesy and fermented. Except for alcoholic, all aroma attributes were perceived in low amounts in at least one of the replicates with buckwheat.

Therefore, it appears to have a complex, hard to describe aroma profile using these attributes. Overall, the lupine sauces with additional carbohydrate sources did not show significant differences (Figure S2).

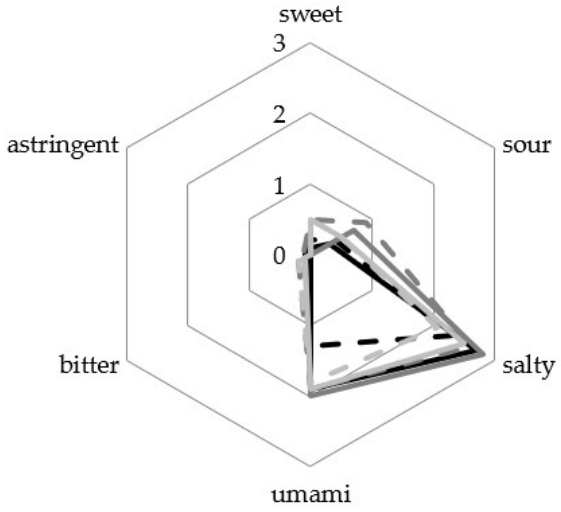

Figure 4. Sensory analysis of the taste of resulting seasoning sauces. Values of moromi with lupine seeds is shown as black lines (ML1) and dashed lines (ML2), with wheat as dark grey lines (MW1) and dashed lines (MW2), and with buckwheat as light grey lines (MB1) and dashed lines (MB2). Data are presented in a 6-point hedonic scale.

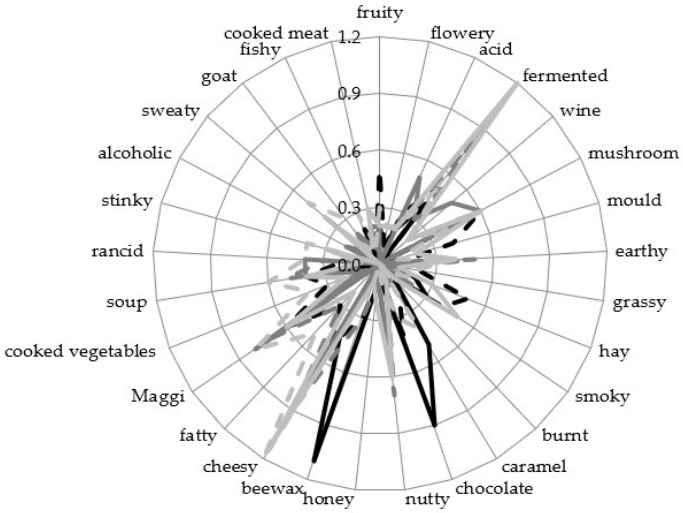

Figure 5. Sensory analysis of the aroma attributes of resulting seasoning sauces. Values of moromi with lupine seeds is shown as black, continuous lines (ML1) and dashed lines (ML2), with wheat as dark grey, continuous lines (MW1) and dashed lines (MW2), and with buckwheat as light grey, continuous lines (MB1) and dashed lines (MB2). Data are presented in a 29-point hedonic scale.

4. Discussion

There are several options how to influence the microbiota and flavour of a fermentation. In a lupine-based moromi, higher salt content leads to a less diverse microbiota and a delay in the appearance of several organisms [2]. Here, we could show that the usage of yeasts as starter cultures resulted in an early dominance of *D. hansenii* and *T. halophilus*. In moromi solely from lupine seeds, scarcely no other organisms were detectable. Similar effects of the inhibition of bacterial growth by yeast starters in moromi were observed by Song et al. [3] in Korean soy moromi. The absence of several species might also be the reason for the lack of pH increase that was measured at the end of the fermentation in backslopped lupine

moromi [2]. In several soy moromi analyses, the pH remained constantly low; Therefore, we assume that a pH increase is not beneficial for a high-quality product [4,16,17].

The addition of wheat and buckwheat led to a temporary growth of *Z. rouxii* along with a decrease in CFUs of *D. hansenii*. We assumed that higher amounts of wheat or buckwheat would elongate the presence of *Z. rouxii* in the fermentation. The lack of specific sugars might explain the absence of *Z. rouxii* in sole lupine moromi [18]. Röling et al. [6] held the presence of galactose as the sole sugar in wheat-free *kecap* fermentation was responsible for the absence of *Z. rouxii*. Despite the higher tolerance of *D. hansenii* to several stresses [19], *Z. rouxii* appears to suppress its growth temporarily in some cases. The inhibition could not be explained by the bacterial consortium, since it was similar in all carbohydrate-added batches.

The aroma profile of wheat- and buckwheat-added moromi showed a lot of different esters and acids, whereas pure lupine moromi contained predominantly pyrazines and acids. Since *T. halophilus* dominated the microbiota in all batches we assumed that these acids and the resulting decreased pH were mainly caused by *T. halophilus* [20]. Yeasts, probably *Z. rouxii*, formed esters from alcohols and acids that are usually known to enhance the fruity character in foods [21,22]. Pyrazines may contribute to a variety of flavours such as potato, popcorn, fermented soybeans, and cocoa [23]. Unfortunately, these differences were not detectable in the sensory analysis. The microbial degradation of pyrazines is still not fully investigated; therefore, to our knowledge, the appearing organisms were not yet described for pyrazine degradation [23]. Since higher concentrations of HMF were measured in samples with additional carbohydrate source, *Z. rouxii* might be responsible for HMF formation [24].

In the present study, we could show that the addition of carbohydrate sources to a moromi fermentation could lead to a temporary change in the microbiota and therefore a shifted aroma profile that was not sensorially detectable. The addition of wheat and buckwheat led to similar results and therefore appeared to be both equally suitable carbohydrate sources. We assume that higher levels of carbohydrates may promote the persistence and flavour formation of *Z. rouxii* in the moromi.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12010197/s1, Figure S1: Comparison of biological duplicates of Sensory Data; Figure S2: Comparison of sensory data of lupine moromi with wheat versus buckwheat.

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Data Availability Statement: The data related to this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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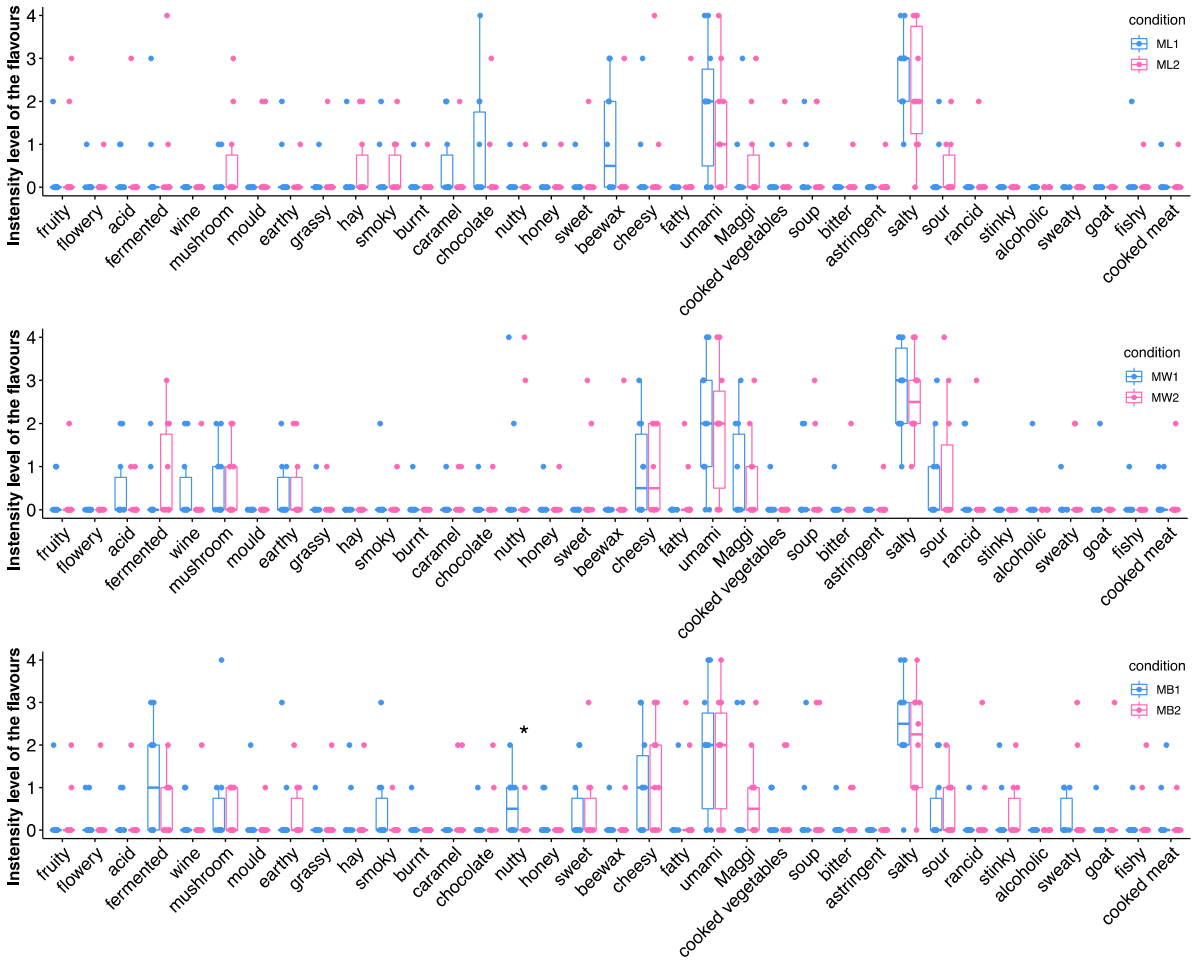
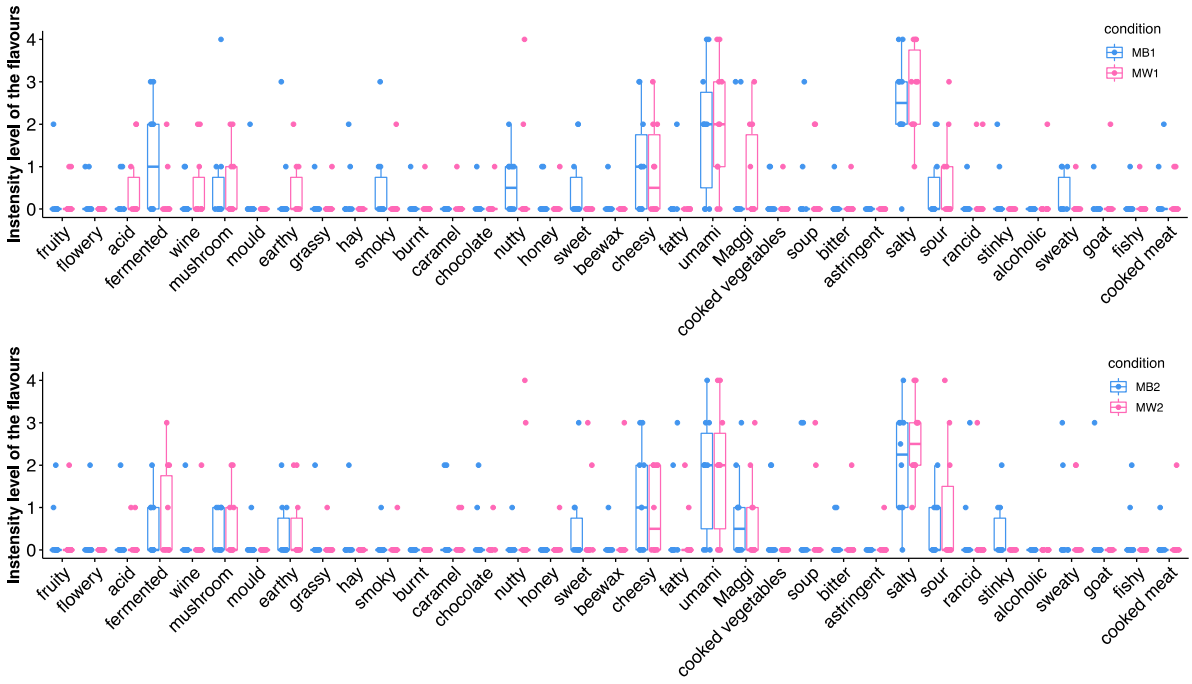
A.4 Supplementary Data to Section A.3

Supplementary Data



Supplementary Figure S1. Comparison of biological duplicates of Sensory Data. The intensity of aroma and flavour attributes of the replicates of lupine moromi (ML1 and ML2), moromi with wheat (MW1 and MW2), and moromi with buckwheat (MB1 and MB2) are displayed and the difference in the flavour between biological replicates that is rather close to significance is marked with an asterisk (Wilcoxon test, p value=0.059).



Supplementary Figure S2. Comparison of sensory data of lupine moromi with wheat versus buckwheat. The intensity of aroma and flavour attributes of the replicates of moromi with wheat (MW1 and MW2), and moromi with buckwheat (MB1 and MB2) are displayed and no significant differences were measured.

A.5 Full publication: Genome Sequence of the Diploid Yeast *Debaryomyces hansenii* TMW 3.1188

The following full text of the research article was originally published in the Journal Microbiology Resource Announcements.

Citation:

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GENOME SEQUENCES

Genome Sequence of the Diploid Yeast *Debaryomyces hansenii* TMW 3.1188

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Tobias Link and Rebekka H. Lülf contributed equally to the manuscript.

ABSTRACT *Debaryomyces hansenii* TMW 3.1188 is a halotolerant diploid yeast that was isolated from lupine moromi fermentation. Here, we report on the 24.77-Mbp genome of a diploid strain of the species *D. hansenii*.

Debaryomyces hansenii is a yeast that is commonly found in marine environments or in food fermentations such as cheese or doenjang (1–5). Strain TMW 3.1188 was isolated from a spontaneous lupine moromi fermentation with 10% NaCl (wt/vol) after 12 weeks (6). The identity of the isolate was initially verified via 28S rDNA sequencing using the primers V9G (7) and LR5 (8) (GenBank accession number OP179623). However, because the *ACT1* gene was reported to have greater variability within *Debaryomyces* species, both alleles from TMW 3.1188 were used to verify the affiliation with the *D. hansenii* species (Fig. 1) (9, 10). Isolation of the genomic DNA of TMW 3.1188 was carried out with harvested cells that had been grown for 24 h at 30°C in DSMZ 90 medium with 5% NaCl (wt/vol). DNA was isolated using R-zymolyase (Zymo Research), followed by phenol-chloroform extraction and enzymatic digestion of proteins with proteinase K.

Quantification and fragment distribution analysis of the DNA were performed with a Qubit fluorometer (Life Technologies) and a Femto Pulse electrophoresis system (Agilent). The library was created with the SMRTbell Express template preparation kit v2.0 (Pacific Biosciences [PacBio]). The library was size selected to >17.5 kbp using a BluePippin device (Sage Science) and then sequenced using the Sequel sequencing kit v3.0 (PacBio) according to the manufacturer's instructions. The read mode was set to continuous long reads (CLR) on a PacBio Sequel instrument.

A total of 10,724,419,855 bases were sequenced. The mean subread length was 14,003 bases, with an N_{50} value of 19,076 bp. The mean of the longest subreads was 14,126 bases, with an N_{50} value of 19,076 bp. After sequencing, data quality was checked via the PacBio single-molecule real-time (SMRT) Link software. The genome was assembled with HGAP4 in SMRT Link v10.0.0.108728 with default parameters, except that the estimated genome size was set to 12 Mbp. The assembly generated 26 contigs, with a total sequence length of 24,773,645 bp, an N_{50} value of 1,604,673 bp, and a GC content of 36.23%.

Genomic comparisons using FastANI v1.3 (11) and genomediff in GenomeTools v1.6.2 (12) revealed that TMW 3.1188 has the greatest similarity to strain CBS767^T (GenBank assembly accession number GCF_000006445.2), with an average nucleotide identity (ANI) value of 95.72%, while the K_r distance, which estimates the number of substitutions per site between two unaligned DNA sequences, has a value of 0.055 (13). The ANI value with respect to the type strain of the species *Debaryomyces fabryi*, CBS789^T (GenBank assembly accession number GCF_001447935.2), is 85.33%, and the K_r distance is 0.102. The ANI values with respect to strains J6 (GenBank assembly accession number GCA_001682995.1) and MTCC 234 (GenBank assembly accession number GCA_000239015.2) are only 84.5%. The K_r distance from TMW 3.1188 to J6 is 0.119 and that to MTCC234 is 0.129.

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The authors declare no conflict of interest.

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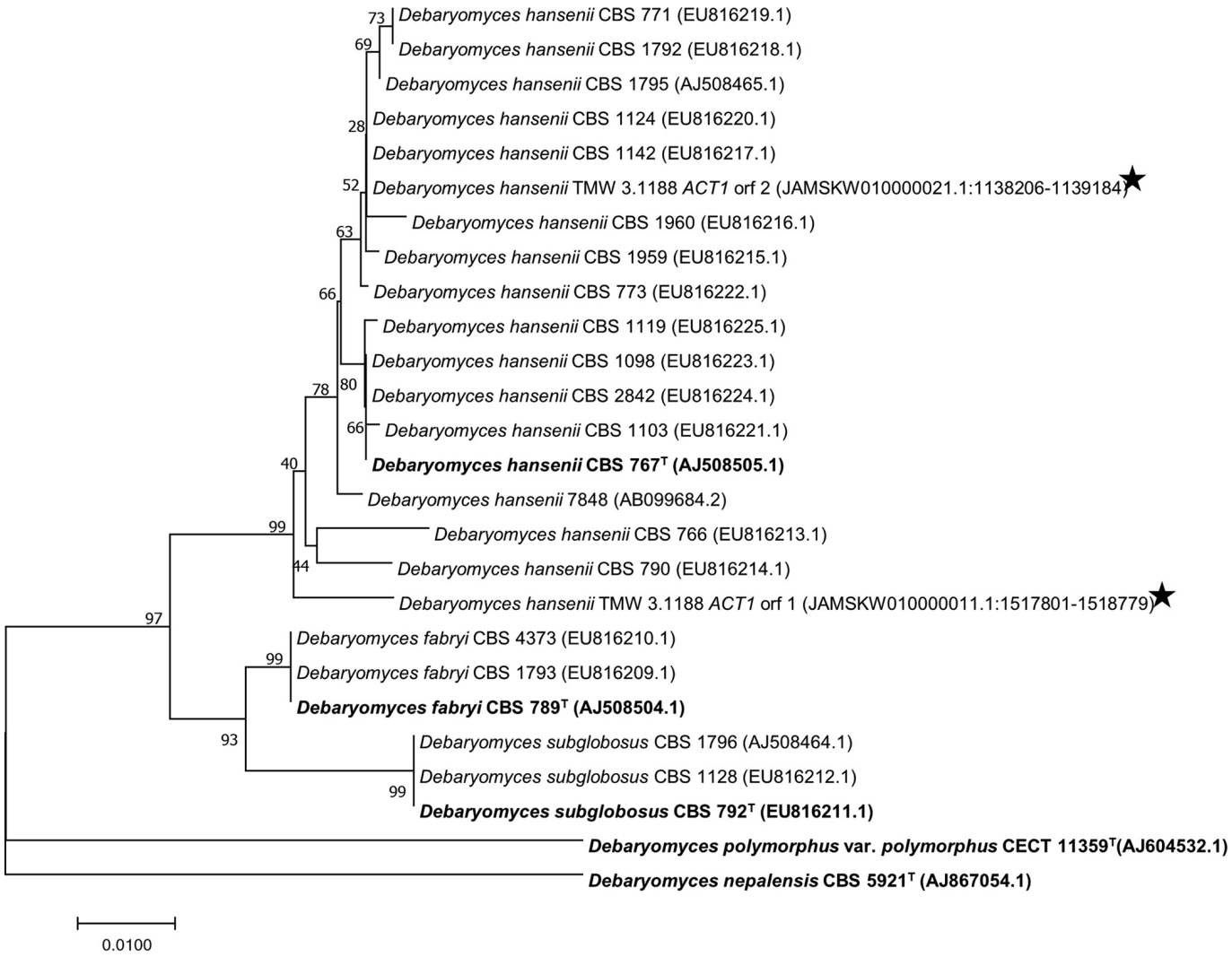

FIG 1 Phylogenetic tree of *Debaryomyces* species. The evolutionary history was inferred using the neighbor-joining method (15). The optimal tree, with the sum of branch lengths of 0.23095609, is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (16). The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (17) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 752 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (18). Type strains were indicated by bold letters with superscript T. Black stars indicate the positions of the sequences from TMW 3.1188. The accession numbers and positions in the chromosome are indicated in parentheses.

Furthermore, genomic comparison using the D-GENIES (14) online tool revealed that each chromosome of CBS767^T had two contigs of TMW 3.1188 aligning to it, one with a higher ANI value (>57%) and one with a lower ANI value (34 to 40%), proving the existence of two chromosomal sets. Contigs 10, 12, 13, and 26 of TMW 3.1188 align to the mitochondrial genome of *Debaryomyces hansenii* (GenBank assembly accession number GCF_000006445.2).

Data availability. The assembled genome is available under assembly accession number GCA_024256405.1, with BioProject accession number PRJNA841823. The raw reads are available under SRA accession number SRR19753073. The 28S rDNA sequence is available under GenBank accession number OP179623.

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A.6 Full publication: *Chromohalobacter moromii* sp. nov., a moderately halophilic bacterium isolated from lupine-based moromi fermentation

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Chromohalobacter moromii sp. nov., a moderately halophilic bacterium isolated from lupine-based moromi fermentation

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ABSTRACT

Three moderately halophilic strains, TMW 2.2308^T, TMW 2.2299 and TMW 2.2304, were isolated from a lupine-based moromi fermentation. Initial identification based on their low molecular sub-proteome using mass spectrometry showed relation to the genus *Halomonas*, however, low score values indicated novelty. The comparison of 16S rRNA gene sequences placed these strains within the genus *Chromohalobacter* with *C. japonicus* CECT 7219^T (99.67% 16S rRNA sequence similarity to strain TMW 2.2308^T), *C. canadensis* DSM 6769^T (99.54%) and *C. beijerinckii* LMG 2148^T (99.32%) being their closest relatives. However, average nucleotide highest identity values of TMW 2.2308^T to *C. beijerinckii* LMG 2148^T of 93.12% and 92.88% to *C. japonicus* CECT 7219^T demonstrate that it represents a novel species within the genus *Chromohalobacter* with additional strains TMW 2.2299 (96.91%) and TMW 2.2304 (96.98%). The isolated strains were non-spore-forming, motile and able to grow at temperatures from 5 to 45 °C with an optimum at 37 °C. Growth of TMW 2.2308^T occurs at 5 to 25% (w/v) NaCl with optimum growth between 10 and 12.5%. The genome of TMW 2.2308^T has a size of 3.47 Mb and a G + C content of 61.0 mol%. The polyphasic evidence lead to the classification of TMW 2.2308^T, TMW 2.2299 and TMW 2.2304 as members of a novel species of the genus *Chromohalobacter*. We propose a novel species as *Chromohalobacter moromii* sp. nov., with TMW 2.2308^T (=DSM 113153^T =CECT 30422^T) as the type strain.

Introduction

Halomonadaceae is a family of 14 genera of (mostly) halophilic *Gammaproteobacteria* including *Chromohalobacter*. The former (not approved) species *Chromobacterium marismortui*, was isolated from the Dead Sea by Elazari-Volcani [1] and reclassified by Ventosa *et al.* [2] as the type species of the new genus *Chromohalobacter*. Currently, the genus includes eight species which are besides *C. marismortui*, *C. beijerinckii* (described by Hof [3] and reclassified by Peçonek *et al.* [4]), *C. canadensis*, and *C. israelensis* (both described by Huval *et al.* [5] and reclassified by Arahal *et al.* [6]), *C. salexigens* [7], *C. sarecensis* [8], *C. nigrandesensis* [9], and the most recent species *C. japonicus* described by Sánchez-Porro *et al.* [10]. Unlike most of the species that were isolated from salterns and seas, *C. beijerinckii* and *C. japonicus* were food associated as they were isolated from salted beans and Japanese salty food, respectively. Therefore, all *Chromohalobacter* species are moderately halophilic, with optimal growth in medium containing between 8 and 10% NaCl. As a major stress response against the osmotic pressure, Vargas *et al.* (2008) found *C. salexigens* to produce compatible solutes as ectoine and hydroxyectoine [11]. As *C. canadensis* shows the ability to form extracellular polymeric substances composed of polyglutamic acid and hetero exopolysaccharide [12], and *C. beijerinckii* can form putrescine, a biogenic amine [13], this genus should be considered in high-salt food fermentations and other biotechnological applications.

In a previous study, we investigated the microbiota of a seasoning sauce fermentation based on two step process with a lupine-based koji and a moromi fermentation [14]. Since identification of some bacteria failed, using subproteome-profiling by matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry, we further investigated these isolates via 16S rDNA and whole genome sequencing. Out of 54 *Chromohalobacter* isolates in total, three strains were differentiated and not related to the described species of this genus. In this study, we propose strain TMW 2.2308^T (=DSM 113153^T =CECT 30422^T), TMW 2.2299, and TMW 2.2304 as the novel species *Chromohalobacter moromii*.

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Table 1
Genome characteristics Strains: 1 TMW 2.2308 (JAHXDI000000000); 2 *C. salexigens* DSM 3043^T (NC_007963.1); 3 *C. israelensis* DSM 6768^T (JQNW01000000.1), 4 *C. canadensis* DSM 6769^T (JAKGAL000000000), 5 *C. marismortui* DSM 6770^T (NZ_SOBR00000000.1), 6 *C. beijerinckii* LMG 2148^T (JAKGAK000000000), 7 *C. nigrandesensis* DSM 14323^T (JAKGAM000000000), 8 *C. sarecensis* DSM 15547^T (JAKGAN000000000), 9 *C. japonicus* CECT 7219^T (JAKGAJ000000000), 10 *C. moromii* TMW 2.2304 (JAHXDD01000000000), 11 *C. moromii* TMW 2.2299 (JAHXCZ01000000000).

Characteristics	1	2	3	4	5	6	7	8	9	10	11
contigs	6	1	28	39	23	60	50	28	73	11	11
length	3,470,002	3,696,649	3,660,991	3,546,649	3,553,220	3,340,801	3,636,986	3,490,490	3,436,593	3,457,785	3,421,152
G+C content	61.0	63.9	63.7	60.9	61.7	61.1	60.9	61.0	61.0	61.0	61.0
Coverage	365	11.5	213	708	214	814	434	913	816	404	377
ANIb [%]	–	83.90	83.77	91.20	86.44	93.12	88.26	88.66	92.88	96.98	96.91
16S rRNA sequence similarity [%]	–	96.89	97.00	99.61	97.89	99.35	98.70	99.09	99.79	100	100
isDDH (formula 2) [%]	–	27.5	27.3	44.1	31.6	52.3	36.0	36.7	51.3	75.1	74.3

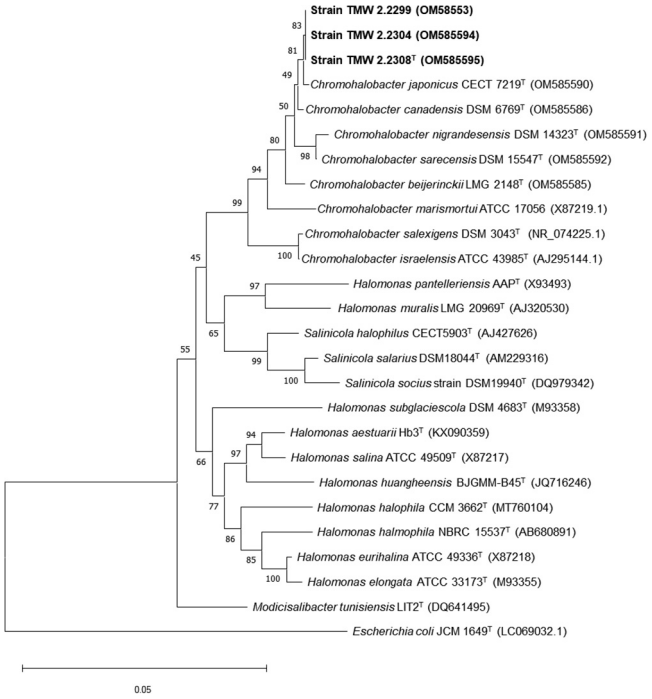

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence. The phylogenetic positioning of *C. moromii* sp. nov. strains was generated using MegaX with the ClustalW algorithm [23] and displayed in a phylogenetic neighbour-joining tree [22]. The numbers at each node represent bootstrap values in percentage based on 1000 replicates [24]. *Escherichia coli* JCM 1649^T was used as an outgroup. Genebank accession numbers are given in parentheses. The scale bare refers to a phylogenetic distance of 0.05 nucleotide substitutions per site.

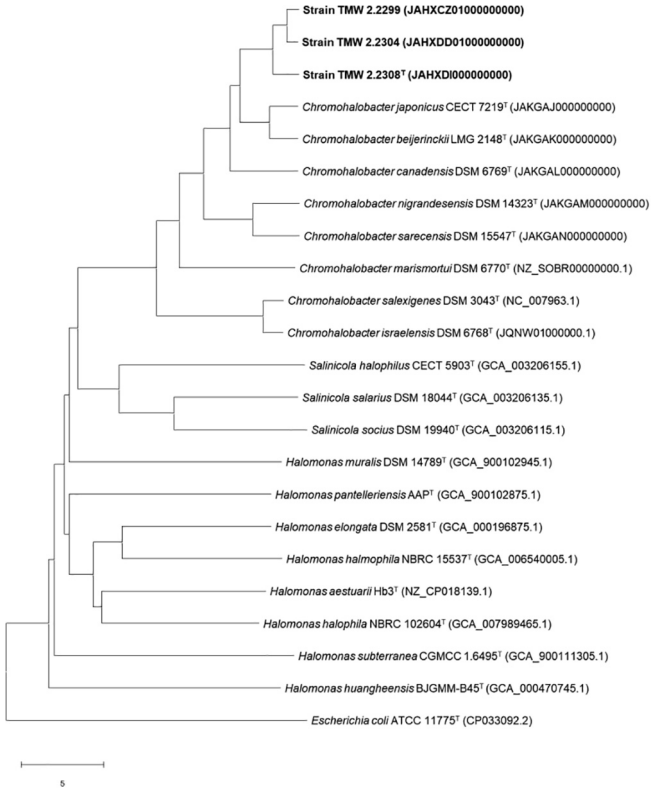

Fig. 2. Phylogenetic tree based on ANIb values. The phylogenetic positioning of *C. moromii* sp. nov. strains was generated using JspeciesWS web version 3.9.0 for ANIb calculation. The tree phylogenetic reconstruction was performed via MegaX implemented neighbour-joining method from the resulting pairwise distance matrix [22]. *Escherichia coli* JCM 1649^T was used as an outgroup. Genome accession numbers are given in parentheses. The scale bare refers to a phylogenetic distance of 5 nucleotide substitutions per site.

Materials and methods

Isolation and cultivation

Three different strains were isolated from a lupine-based moromi and proposed as a novel member of the genus *Chromohalobacter* [14]. They were isolated from a lupine moromi after eight weeks of incubation at 20% NaCl (TMW 2.2308^T, TMW 2.2304) and 15% NaCl (TMW 2.2299). Therefore, samples were diluted in ringer's solution with 5% additional NaCl, plated on malt-extract agar with 5% NaCl (malt extract 20 g/L, soy peptone 2 g/L, NaCl 50 g/L, pH 5.6) and incubated under aerobic conditions at 30 °C. They were cultivatable in SW10 ([10], pH 7.2), MH10 (CECT Medium 218, pH 7.5) and tryptic soy broth with 10% NaCl (TSB10, casein peptone 15 g/L, soy peptone 15 g/L, yeast extract 3 g/L, NaCl 100 g/L, pH 7.3). Experiments were performed under aerobic conditions at 37 °C in the recommended medium supplemented with 10% NaCl or TSB10 medium, or on agar plates with 1.5% agar.

Morphological, biochemical, and physiological tests

The colony morphology was characterised on TSB10 agar plates after 24 h of incubation at 37 °C. For cell size and spore-forming ability, these cultures were observed using a BX61 microscope (Olympus). General phylogenetic traits like Gram-staining behaviour, oxidase and catalase activity were tested according to classic microbiological methods. Growth was examined under anaerobic conditions (AnaeroGen, Thermo Scientific, Netherlands) and microaerobic conditions (CampyGen Compact, Oxoid AGS, United Kingdom). For the determination of the hydrolysis of starch, Tween 80 and casein appropriate agars were used [15]. The usage of citrate was determined by using Simmons' citrate agar (Merck, Germany). Incubation in Sulphide Indole Motility medium (2% casein peptone, 0.6% meat extract, 0.03% sodium thiosulphate,

0.02% ammonium ferric citrate, 10% NaCl und 0.35% agar) showed the motility and ability of H_2S production. The test kits API 20NE and API ID 32E (bioMérieux) were used for multiple tests, whereby all solutions and media were supplemented with 10% (w/v) NaCl.

Optimal temperatures were determined incubating the strains at 4, 15, 20, 25, 30, 37, 40, 42, and 45 °C on agar plates. For the determination of the optimum salt concentration, TSB medium was supplemented with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, and 30% (w/v) NaCl. For the pH range, appropriate buffers (citrate buffer, potassium phosphate buffer, Tris-HCl buffer and carbonate-bicarbonate buffer) were used to adjust TSB10 medium pH to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. The strains were precultivated overnight in TSB10 at 30 °C and tested in 96-well plates using a Spectrostar Nano (BMG Labtech, Germany) to detect growth.

Chemotaxonomic analysis

Analysis of cellular fatty acids were carried out by DSMZ services (Braunschweig, Germany) from active growing cultures via saponification, methylation and extraction according to Kuykendall *et al.* [16] and Miller [17] with minor modifications. Samples were analysed via gas chromatography with a flame ionisation detector and the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.) for identification of the fatty acid methyl esters.

Phylogenetic analysis

Genomic DNA was isolated and purified using gDNA columns (Macherey-Nagel, Düren, Germany) and sequencing was carried out by Eurofins (Constance, Germany) using an Illumina HiSeq 2500 Sequencer. The annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline [18–20]. JspeciesWS web version 3.9.0 was used to calculate average nucleotide identity (ANI) values between all *Chromohalobacter* species via ANI blast (ANIb) [21]. The resulting pairwise matrix was used to perform a phylogenetic reconstruction using neighbour-joining method implemented in MegaX [22]. 16S rRNA gene sequences could be extracted from the genome analysis after annotation. A multilocus sequence analysis (MLSA) was performed with a concatenated sequence of five housekeeping genes: *rpoD* (RNA polymerase sigma factor), *secA* (preprotein translocase subunit A), *gyrB* (DNA topoisomerase (ATP-hydrolyzing) subunit B), *groEL* (chaperonin), and *infB* (translation initiation factor IF-2). These sequences were compared with sequences of closely related type strains provided by the National Center for Biotechnology Information (NCBI) using *Escherichia coli* JCM 1649^T as an outgroup. For the phylogenetic analysis, sequences were aligned using MegaX with the ClustalW algorithm [23] and a phylogenetic tree was reconstructed using neighbour-joining method [22] with 1000 bootstrap replicates [24].

For *in silico* DNA-DNA hybridisation (isDDH) the GGDC 3.0 service by the DSMZ was used to calculate values using formula 2 [25,26].

Distinction of the three strains was enabled via randomly amplified polymorphic DNA (RAPD) PCR (Supplementary Fig. S1). Therefore, primer M13V (5′GTT TTC CCA GTC ACG AC-3′) and method described by Ehrmann *et al.* [27] were used.

Results and discussion

Genome characteristics

The assembled genomes of TMW 2.2308^T, TMW 2.2299 and TMW 2.2304 yielded 3,470,002, 3,421,152, and 3,457,785 nucleotides, respectively. The G + C content was determined to be 61.0 mol% for all of them. The genomes were assembled in 6, 11 and 11 contigs, respectively. General genome characteristics including similarity indices were provided in Table 1.

Phylogenetic positioning and genetic fingerprinting

The phylogenetic analysis of 16S rRNA gene sequences placed the three strains TMW 2.2308^T, TMW 2.2299 and TMW 2.2304 into the genus *Chromohalobacter* (Fig. 1). The highest sequence similarity to TMW 2.2308^T was to *C. japonicus* CECT 7219^T at 99.79%, *C. canadensis* DSM 6769^T at 99.61% and to *C. beijerinckii* LMG 2148^T at 99.35% (Table 1).

The calculated isDDH and ANIb values were below the threshold of species delineation and therefore confirmed that these strains represent a novel species within the genus *Chromohalobacter*. The highest isDDH values for TMW 2.2308^T was 52.3% to *C. beijerinckii* LMG 2148^T and 51.3 % to *C. japonicus* CECT 7219^T, which are below the threshold of 70 % for bacterial species differentiation [28]. The highest ANIb values for TMW 2.2308^T were observed with *C. beijerinckii* LMG 2148^T with 93.12%, *C. japonicus* CECT 7219^T with 92.88%, and *C. canadensis* DSM 6769^T with 91.20% (Fig. 2), which are below the threshold for species differentiation with ANIb values at 94% [29]. The strains TMW 2.2299 and TMW 2.2304 showed isDDH values of 74.3 % and 75.1% and ANIb values of 96.91% and 96.89%, respectively. This indicates that the tree strains belong to the same species *C. moromii* sp. nov. but are not identical clones.

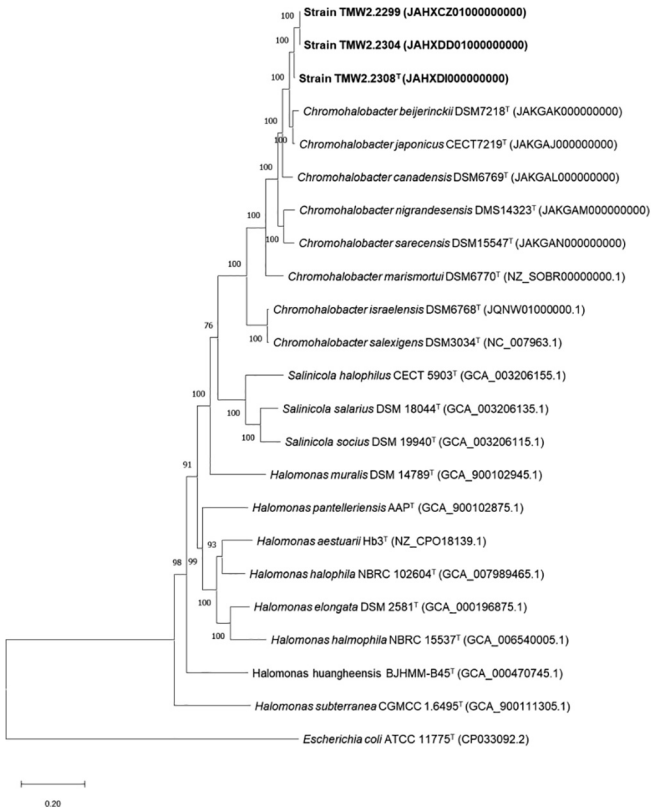

Fig. 3. Phylogenetic tree based on concatenated sequences of rpoD, secA, gyrB, groEL, and infB. The phylogenetic positioning of C. moromii sp. nov. strains was generated using MegaX with the ClustalW algorithm [23] and displayed in a phylogenetic neighbour-joining tree [22]. The numbers at each node represent bootstrap values in percentage based on 1000 replicates [24]. *Escherichia coli* ATCC 11775^T was used as an outgroup. The scale bare refers to a phylogenetic distance of 0.2 nucleotide substitutions per site.

Table 2
Comparison of phenotypical characteristics of strain TMW 2.2308 and other type strains of the genus *Chromohalobacter*. Strains: 1 TMW 2.2308; 2 *C. salexigens* DSM 3043^T; 3 *C. israelensis* DSM 6768^T, 4 *C. canadensis* DSM 6769^T, 5 *C. marismortui* DSM 6770^T, 6 *C. beijerinckii* LMG 2148^T, 7 *C. nigrandesensis* DSM 14323^T, 8 *C. sarecensis* DSM 15547^T, 9 *C. japonicus* CECT 7219^T, 10 *C. moromii* TMW 2.2304, 11 *C. moromii* TMW 2.2299. All strains were tested simultaneously to TMW 2.2308^T and the cell sizes were obtained from literature [4,6–10].

Characteristics	1	2	3	4	5	6	7	8	9	10	11
colony pigmentation	cream	white-cream	cream	white	cream	cream	black	cream-brown	Cream	cream	cream
Cell length [µm]	0.6–2	2–3	1.5–4.2	2–3.8	1.5–4	2.8	2.3–4.2	0.8–6.1	1.2–4.5		
Cell width [µm]	0.4	0.7–1	0.6–0.9	0.6–1.2	0.6–1	0.5	0.35–0.5	0.5–0.8	0.4		
NaCl range (%, w/v)	5–25	5–25	5–27.5	5–22.5	5–22.5	5–22.5	5–30	5–30	5–25	5–22.5	5–25
NaCl optimum	10–12.5	7.5–10	5–10	7.5	7.5–10)	7.5	10	10–12.5	7.5–12.5	7.5	7.5
Temperature range (°C)	4–45	4–45	4–45	4–45	4–45	15–42	4–45	4–42	4–45	4–45	4–45
Temperature optimum	37	37–42	37–40	37	37	37	30	37	37	37–40	37–40
pH range	6–9	6–9	6–9	5–8	7–9	6–9	6–9	6–9	6–9	5–8	5–9
pH optimum	7	7–8	7–8	7–8	7–8	7–8	7	7–8	7–8	7–8	7
Catalase	+	+	+	+	+	-	+	+	+	+	+
Oxidase	-	-	-	+	-	-	-	-	-	-	-
Arabinose	+	-	-	-	-	+	+	-	-	+	+
Maltose	-	+	-	-	-	-	-	-	-	-	-
Sucrose	-	+	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	+	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-
Simmon's Citrate	+	+	+	+	-	+	+	-	+	+	+
Nitrate reduction	+	+	+	+	-	+	-	-	+	+	+
H_2S production	-	+	-	-	-	-	-	-	-	-	-
Indole production	-	+	+	+	-	-	-	-	-	-	-
Casein	-	-	-	-	-	-	-	-	-	-	-
Aesculin	+/-	-	+	+	-	-	-	-	-	-	+/-
Gelatin	-	-	-	-	-	-	-	-	+	-	+/-
Tween 80	-	-	-	-	-	-	-	-	-	-	-
Starch	-	-	+	-	-	-	-	-	-	-	-
Lysin decarboxylase	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-

+ positive reaction
- negative reaction
+/- weak reaction

Table 3
Cellular fatty acid compositions (%).

Fatty acid	Strain TMW 2.2308^T	*C. japonicus* CECT 7219^T	*C. canadensis* DSM 6769^T
$C_{10:0}$	2.9	3.3	1.9
$C_{12:0}$	4.4	5.0	3.5
$C_{12:0}$ 3-OH	7.0	7.5	5.8
Sum in feature 3[a]	10.9	10.1	6.1
$C_{16:0}$	18.3	17.8	25.8
$C_{17:0}$ cyclo	3.4	4.0	10.5
$C_{18:1}$ ω7c	37.7	35.6	11.6
$C_{19:0}$ cyclo ω8c	13.7	14.9	32.5

[a]Summed feature 3 consists of $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c.

The MLSA supported the phylogenetic positioning of *C. moromi* sp. nov. with the closest related species *C. beijerinckii* and *C. japonicus* (Fig. 3).

The RAPD fingerprinting method revealed distinct patterns, enabling the distinction of the three strains (Supplementary Fig. S1).

Phenotypical and cultural characteristics

Colonies of TMW 2.2308^T on TSB10 agar plates were cream-coloured, circular with entire margins and convex. The cells were rod-shaped, straight, or slightly curved, 0.4 × 0.6–2 µm in size and occur singly or in pairs. They are Gram-negative, motile, and non-spore-forming and grow under aerobic and microaerophilic conditions. Growth occurs in TBS media containing 5 to 25% (w/v) NaCl with optimum growth between 10 and 12.5% (w/v) NaCl. They grow at temperatures from 4 to 45 °C, optimally at 37 °C. They grow at pH 6.0 to 9.0, optimally at pH 7.0.

The strains TMW 2.2308^T, TMW 2.2299 and TMW 2.2304 were able to produce catalase but no oxidase. They were tested negative for the production of urease, hydrogen sulfite and indole. They were positive for nitrate reduction and Simmons' citrate test. No reaction could be detected for lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, β-galactosidase and the hydrolysis of casein, aesculin, gelatin, Tween 80 and starch. TMW 2.2308^T could not produce acid from L-rhamnose, D-mannitol, D-sorbitol, sodium glucoronate, sucrose, D-trehalose, 5-ketogluconate, palatinose, and colistin. This strain was able to produce acid from D-glucose, L-arabinose, ribitol, D-cellobiose, D-melibiose, D-mannose, D-maltose, and D-galacturonic acid. TMW 2.2308^T could use D-glucose, L-arabinose, D-mannitol, potassium gluconate, malic acid, and trisodium citrate and could not use following compounds as sole carbon and energy source: D-raffinose, D-mannose, N-acetyl-glucosamine, D-maltose, capric acid, adipic acid, and phenylacetic acid. Phenotypical characteristics of the novel strains and all *Chromohalobacter* type strains are displayed in Table 2.

Chemotaxonomic characteristics

The major fatty acids from TMW 2.2308^T were C18:1 ω7c, C16:0, C19:0 cyclo ω7c and C16:1 ω7c (s. Table 3). For comparison, the fatty acids of *C. canadensis* DSM 6769^T were C19:0 cyclo ω7c, C16:0, C18:1 ω7c and C17:0 cyclo ω7c, and for *C. japonicus* CECT 7219^T C18:1 ω7c, C16:0, C19:0 cyclo ω7c and C16:1 ω7c were revealed as major fatty acids.

Conclusion

The phylogenetic and phenotypic analysis indicated that the three strains TMW 2.2308^T, TMW 2.2299, and TMW 2.2304 isolated from lupine-based moromi with 20% NaCl in Freising, Germany in 2020 represent a novel species placed in the genus

Table 4
Description of *Chromohalobacter moromii* sp. nov.

Genus name	*Chromohalobacter*
Species name	*Chromohalobacter moromii*
Specific epithet	*moromii*
Species status	sp. nov.
Species etymology	(mo.ro.mi.i. N.L. gen. n. moromii, of moromi, of a fermentation mash)
Description of the new taxon and diagnostic traits	Colonies on TSB agar with 10 % NaCl are cream, circular with entire margins and convex. The cells are rod-shaped, straight or slightly curved, 0.4 × 0.6–2 μm in size and occur singly or in pairs. They are Gram-negative, motile and non-spore-forming and grow under aerobic and microaerophilic conditions. Predominant fatty acids are C18:1 ω7c, C16:0, C19:0 cyclo ω7c, and C16:1 ω7c. Catalase is produced, oxidase is not produced. Negative for acid production from L-rhamnose, D-mannitol, D-sorbitol, sodium glucoronate, sucrose, D-trehalose, 5-ketogluconate, palatinose, and colistin. Positive for acid production from D-glucose, L-arabinose, ribitol, D-cellobiose, D-melibiose, D-mannose, D-maltose, and D-galacturonic acid. Negative for the production of urease, hydrogen sulfit and indole. Positive for nitrate reduction and Simmons' citrate test. Negative for lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, β-galactosidase and the hydrolysis of casein, aesculin, gelatin, Tween80 and starch. They use D-glucose, L-arabinose, D-mannitol, potassium gluconate, malic acid, and trisodium citrate and do not use following compounds as sole carbon and energy source: D-raffinose, D-mannose, N-acetyl-glucosamine, D-maltose, capric acid, adipic acid, and phenylacetic acid.
Country of origin	Germany
Region of origin	Freising
Date of isolation (dd/mm/yyyy)	23/04/2020
Source of isolation	Moromi
Sampling date (dd/mm/yyyy)	21/04/2020
16S rRNA gene accession nr.	OM585595
Genome accession number [RefSeq; EMBL; . . .]	GenBank=JAHXDI000000000
Genome status	Complete
Genome size	3.47 Mb
GC mol%	61.0
Number of strains in study	3
Source of isolation of non-type strains	Moromi
Information related to the Nagoya Protocol	Not applicable
Designation of the Type Strain	TMW 2.2308^T
Strain Collection Numbers	DSM 113153^T=CECT 30422^T

Chromohalobacter. Therefore, we propose *C. moromii* sp. nov. with TMW 2.2308^T (=DSM 113153^T and =CECT 30422^T) as the type strain.

The GenBank accession numbers for whole genomes are JAHXDI000000000 (TMW 2.2308^T), JAHXCZ000000000 (TMW 2.2299) and JAHXDE000000000 (TMW 2.2304). The GenBank accession numbers for 16S rRNA gene are OM585595 (TMW 2.2308^T), OM585593 (TMW 2.2299) and OM585594 (TMW 2.2304). A summarised description of the novel species is provided in the protologue Table 4.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2022.126324.

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A.7 Supplementary Data to Section A.6

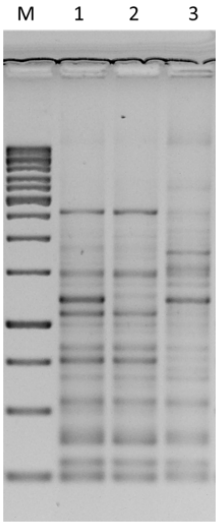

Figure S1 Genetic fingerprints from RAPD PCR. Lane M shows the 1 kb DNA Ladder (Thermo Fisher Scientific). Lane 1 TMW 2.2299 as template. lane 2, TMW 2.2304, lane 3, TMW 2.2308^{T}.

A.8 Additional Data (Thesis publication)

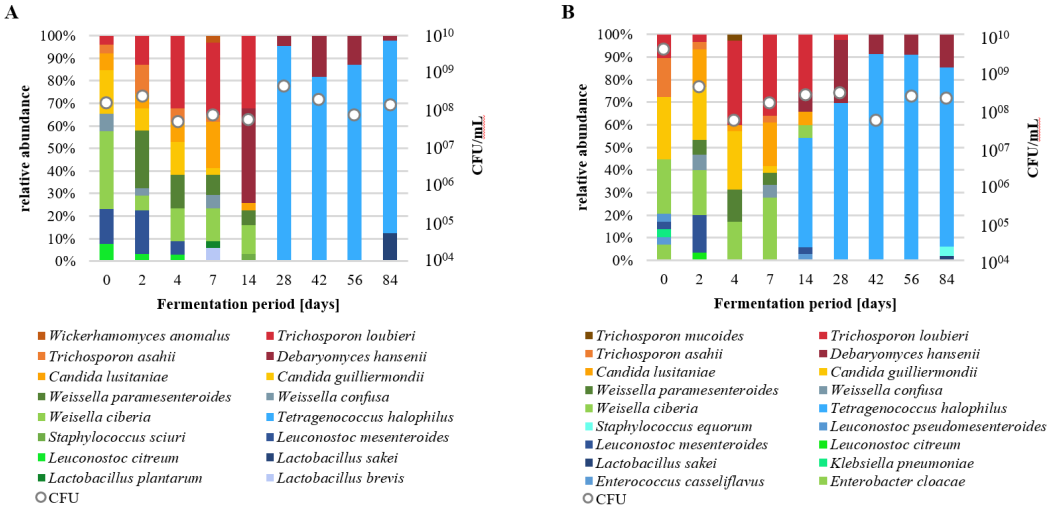

Figure A.1: Microbial diversity of spontaneous and backslopped moromi. The diversity of bacteria and yeast during spontaneous moromi (**A**) and backslopped (**B**) fermentation on MRS agar (without NaCl) via MALDI-TOF-MS. Displayed are relative abundances of species and total number of CFU/mL.

Table A.1: Zones of inhibition due to antibiotic sensitivity of *S. equorum* isolates. Measured diameters are displayed in mm.

Antibiotic	**TMW 2.2497**	**TMW 2.2498**	**TMW 2.2499**
Ampicillin	39	45	35
Penicillin G	30	33	25
Oxacillin	22.5	21	16.5
Tetracycline	39.5	43	45.5
Erythromycin	34.5	39	36.5

B List of Figures

C List of Tables

D List of Publications and Student Theses

Names are underlined to indicate first authorships.

D.1 Peer-reviewed journal articles

Lülf, R. H.*, Vogel, R. F., Ehrmann, M. A.* Microbiota dynamics and volatile compounds in lupine based Moromi fermented at different salt concentrations // International Journal of Food Microbiology. 9 2021, 354. 109316.

Lülf, R. H.*, Hilgarth, M., Ehrmann, M. A. Chromohalobacter moromii* sp. nov., a moderately halophilic bacterium isolated from lupine-based moromi fermentation // Systematic and Applied Microbiology. 2022. 45.

Link, T., ***Lülf, R. H.****, Parr, M., Hilgarth, M., Ehrmann, M. A.* Genome Sequence of the Diploid Yeast *Debaryomyces hansenii* TMW 3.1188 // Microbiology Resource Announcements. 11 2022.

Lülf, R. H.*, Selg-Mann, K., Hoffmann, T., Zheng, T., Schirmer, M., Ehrmann, M. A.* Carbohydrate Sources Influence the Microbiota and Flavour Profile of a Lupine-Based Moromi Fermentation // Foods. 2023. 12.

D.2 Conference contributions

Lülf, R. H.*, Ehrmann, M. A., Link, T., Selg-Mann, K., Vogel, R. F.* Entwicklung regionaler Bio-Würzsaucen auf Lupinenbasis als salzreduzierte, glutenfreie Alternative zu Sojaprodukten // Lupinen Netzwerk-Konferenz (Landesforschungsanstalt für Landwirtschaft und Fischerei). Poster presentation. 2019, Güstrow, Germany

Lülf, R. H.*, Ehrmann, M. A., Vogel, R. F.* Halotolerant Lactic Acid Bacteria in lupine based Seasoning Sauce // 13^{th} International Symposium on Lactic Acid Bacteria. Poster presentation. 2021, online

Lülf, R. H.*, Ehrmann, M. A.* Influence of carbohydrate addition on the microbiome of a lupine-based seasoning sauce // 6^{th} International Conference Microbial Diversity. Poster and Pre-produced Video presentation. 2021, online
This presentation was awarded with the ***SIMTREA award for Food Microbiology*** *by the conference chair.*

Lülf, R. H.*, Ehrmann, M. A.* Analysis of lupine seed-based moromi microbiota reveals a novel salt tolerant *Chromohalobacter* species // Annual Conference of the Association for General and Applied Microbiology. Poster presentation. 2022, online
This presentation was awarded with an ***ePoster Award*** *by the conference chair.*

Lülf, R. H.*, Ehrmann, M. A.* The microbiota of salty fermented lupine seeds // 13th Conference on Halophilic Microorganisms. Poster presentation and Short Talk. 2022, Alicante, Spain

D.3 Student theses

Johanna Fellermeier Einfluss des Proteingehalts auf die Mikrobiota von Buchweizen-Moromi. Internship, 2020

Elena Jauch Charakterisierung der Eigenschaften von *Staphylococcus equorum* zur Eignung als Bestandteil einer Starterkultur für Lupinensoße. Internship, 2022

Franziska Krammer Charakterisierung der Habitat-Anpassung von *Staphylococcus equorum* an Lupinen-Moromi. Bachelor thesis, 2022

Elena Jauch Vorkommen und Diversität von Archaebakterien in Lebensmittelfermentation am Beispiel von Lupinensoße. Bachelor thesis 2022/23