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The cross-over fermentation concept and its application in a novel food product: The dairy miso case study

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ABSTRACT

Cross-over fermentations are processes in which a microorganism from one traditional fermentation process is introduced onto a new substrate and/or to a new partner. Here we show that *Aspergillus oryzae* normally used for the production of miso, a fermented soybean paste from Asia, can be applied to a traditional European fermented dairy product quark cheese, produced by fermenting milk with *Lactococcus lactis.* This cross-over fermentation resulted in a product with intense aroma properties, mainly due to high amounts of volatile fatty acids, ethyl esters, higher alcohols and ketones. Active metabolism of *A. oryzae* was required for alcohol production, whereas fat degradation occurred mainly due to enzymatic activities. Traditionally used practices in miso production, like mixing, addition of various amounts of salt and variations in fat content of the substrate altered metabolic and enzymatic properties of *A. oryzae* resulting in differences in final product characteristics. Aroma intensity of the product was shown by comparing volatile organic compounds with blue and white mould cheese. This study showed the potential of cross-over fermentation for novel food products. The enormous diversity of microorganisms used in traditional fermentation processes and the vast number of alternative substrates offer numerous opportunities for further novel fermented product development.

1. Introduction

Fermentation is one of the oldest techniques used by mankind to preserve food products. This ancient food processing technique was already practised around 8000 B.C. in a dairy environment (Fox, 1993), 7000 B.C. for making fermented beverages consisting of rice, honey and fruit (McGovern et al., 2004) and 6000 B.C. for wine production (McGovern, Hartung, Badler, Glusker, & Exner, 1997). Fermentation has evolved all over the world without knowing the role of the microbiological workhorses present in the raw material. It is only after Pasteur discovered the role of microorganisms and their role in fermentation processes that humans could actively start to control the microorganisms used for fermentation (Bordenave, 2003). Modern, large scale fermentations usually rely on single strain or defined starter cultures leading to better control on product quality and consistency (Ross, Morgan, & Hill, 2002). This resulted in a large variety of traditional fermentations being carried out on industrial scale all around the world.

The periodic table of fermented foods by Gänzle (2015) shows this large variety of food fermentations carried out across the globe using diverse microbiological workhorses. This microbiological diversity can be exploited to produce novel fermented food products with improved product characteristics, like an increased aroma content with a decreased ethanol content in beer produced by a co-cultivation of a non-conventional yeast isolated from African fruit with brewer's yeast (van Rijswijck, Wolkers–Rooijackers, Abee, & Smid, 2017) or lupin tempeh with *in situ* vitamin B_{12} fortification by co-fermentation of *Rhizopus oryzae* used for tempeh and *Propionibacterium freudenreichii,* a bacterium usually used in dairy fermentations (Wolkers–Rooijackers, Endika, & Smid, 2018). These examples show that i) new microbial interactions can result in interesting product characteristics and ii) novel substrates can be selected for fermentative processing with use of microorganisms known from traditional fermentation processes. The latter concept is what we call 'cross-over fermentation', in which a microorganism is taken from a traditional fermentation process and is introduced onto a new substrate and/or introduced to a new microbial partner in a mixed culture. In this study, we demonstrate that *Aspergillus*

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oryzae normally used in the production of miso can ferment quark cheese, resulting in a novel fermented food product with intense aroma.

Miso is traditionally produced by fermenting soybeans with *A. oryzae* pre-cultured on rice (called koji) in the presence of salt contents ranging from 55 to 200 g salt/kg product for up to 3 years (Shibasaki & Hessbltine, 1962). In addition to soybeans alone, many variations in grain and bean substitutes can be used for making miso (Shurtleff & Aoyagi, 1976). Koji is made from cooked polished rice grains inoculated with *A. oryzae* incubated at 30–35 °C for 2–3 days whilst regularly mixing. Before the mould starts sporulation, it is inoculated on to the soybeans (Shurtleff & Aoyagi, 1980). *A. oryzae* has been used for solid-state fermentations already since 3000-2000 years ago in China and has a long history of use since 700 B.C. in Japan for production of soy sauce, Japanese spirit (shochu), Japanese rice wine (sake) and miso (Abe & Gomi, 2008). *A. oryzae* is known to secrete many hydrolytic enzymes during solid state fermentations (Machida, Yamada, & Gomi, 2008), like lipases (Ohnishi, Yoshida, & Sekiguchi, 1994), proteases and amylolytic enzymes (Maeda et al., 2004). Furthermore, *A. oryzae* has been shown to produce β-galactosidase (Akasaki, Suzuki, Funakoshi, & Tamashina, 1976). These characteristics make *A. oryzae* a potential candidate for fermentation of dairy substrates.

Quark is best described as a soft unripened fresh cheese. Quark is made from heat-treated milk which is inoculated with starter lactic acid bacteria, usually *Lactococcus lactis* and in some cases rennet, resulting in acidification to a pH of ~4.5 and gelation. Traditionally, the whey is removed from the curd using linen cloth bags, whereas in industrial processes this is usually replaced by mechanical methods. The result of this process is a smooth creamy white product with a fresh and mildly acidic taste (Farkye, 2017, pp. 1103–1110).

In this study we exchanged the traditional salted soybeans for quark as the substrate for fermentation by *A. oryzae*. We show how the fungus behaves in a dairy environment and how its biological activity is affected by important process parameters like salt content, headspace environment and fat content. This re-fermentation of quark cheese results in a product, which we call 'dairy miso', with a particularly strong aroma showing the great potential and the need for other 'cross-over fermentations' to be explored.

2. Materials and methods

2.1. Strain

A commercial koji-kin starter (Brouwland, Belgium) was inoculated onto malt extract agar plates by stabbing the powder into three crosses on the plates. Plates were incubated at 25 °C for three days upon sporulation. *A. oryzae* spores were harvested with L-shaped spreaders by adding 10 mL 4 °C ACES buffer containing 0.2 g/L polysorbate 80 (referred to as buffer from now on) to the plates and scrubbing. Contents were harvested from the plates using a sterile syringe and transferred to 50 mL tubes. The syringe was washed with 20 mL buffer bringing the total volume up to 30 mL. The suspension was centrifuged at 4 °C for 5 min at 1100×*g* and the pellet was re-suspended in 30 mL buffer. Centrifugation was repeated and the pellet was re-suspended in 5 mL buffer. Spores were stored in aliquots of 1 mL consisting of 0.35 mL 875 mL/L glycerol and 0.65 mL spore suspension at −80 °C until further use.

2.2. Preparation of rice koji

Polished white rice grains were washed multiple times in a vessel until the water did not turn opaque. Subsequently, the rice was boiled in an open vessel with a weight ratio of 1:2 rice to water for 15 min and cooled down in a flow cabinet for 15 min. Sterile petri plates were filled with 32 g boiled rice to make koji plates. Plates were inoculated with sterile loops by running a loop full of spore suspension across the koji plate from end to end two times. Inoculated plates were incubated at 30 °C for three days.

2.3. Quark cheese, blue mould cheese and white mould cheese

Commercially available full-fat quark cheese (Jumbo huismerk, The Netherlands) or skimmed quark cheese (Jumbo huismerk, The Netherlands) were bought. Full-fat quark was used in all experiments unless stated otherwise. Blue mould and white mould cheese were bought from an artisanal cheese vendor at a local market (Wageningen, the Netherlands).

2.4. Dairy miso production

Dairy miso was prepared in 200 mL glass jars with screw top lids. Prior to use jars were autoclaved at 121 °C for 15 min. Sixteen g of koji (half a petri plate) was inoculated into 50 g of quark together with 60 g/kg NaCl except for the salt variation experiments in which 0, 20, 40, 60, 80, 100, 150 and 200 g/kg were used. Jars were incubated aerobically unless stated otherwise at 25 °C up to 68 days and 2 g sample was taken at regular intervals and stored at −20 °C until further analysis. For anaerobic incubation, anaerobic jars were made anaerobic using Anaerocult A sachets (Merck millipore).

2.5. Analysis of substrates and extracellular metabolites

Lactose, glucose, ethanol, lactate, citrate, acetate, pyruvate, glycerol, propionate and butyrate were quantified by high performance liquid chromatography (HPLC) as described by van Mastrigt, Abee, Lillevang, and Smid (2018).

2.6. Volatile organic compound analysis by HS SPME GC-MS

During fermentations 2 g of dairy miso was transferred to 5 mL GC-MS vials and frozen at (−20 °C) until analysis by headspace solid phase microextraction gas chromatography mass spectrometry (HS SPME GC-MS) according to the method used by Dank, Smid, and Notebaart (2018).

2.7. Water activity

Water activity of dairy miso was measured using Labmaster a_w (Novasina). The machine was calibrated using SAL-T humidity standards according to the manufacturer's protocol. Samples were measured according to the manufacturer's protocol.

2.8. Inhibition of fungal growth with natamycin

Fungal growth during dairy miso production was inhibited by adding Delvocid containing 500 g/kg natamycin in varying contents to the fermentation jar along with the koji, quark cheese and 60 g/kg NaCl. The chosen natamycin contents were 0, 25, 50, 100, 200, 400 and 800 mg/kg.

2.9. Estimation of aroma production rates

Aroma production rates were estimated for the first 10 days of fermentation for each salt concentration by fitting linear models through the mean aroma content of each time point and taking the slope of the linear model. One-way analysis of variance followed by Tukey honest significant differences was performed using R to determine statistically different ($P < 0.05$) aroma production rates amongst the different fermentations.

3. Results

3.1. Dairy miso benchmark trial

To explore the potential of *A. oryzae* in dairy miso production a benchmark miso was produced containing 60 g/kg NaCl, a salt content

used in production of red sweet miso (Shurtleff & Aoyagi, 1976) and a 1:3 koji:quark ratio. Visual characteristics of the miso developed over time from being thick quark with intact rice grains towards a less viscous solution with less prominent grain particles. The formation of volatile organic compounds (VOCs) for the benchmark product was monitored in time for 68 days (Fig. 1).

Formation of VOCs kept on developing over a course of 68 days, although the total amount of VOCs detected in the headspace stabilised after 40 days. A total of 77 individual components were detected, in which the aroma profile was largely dominated by acids and ethyl esters thereof (supplementary file A.1). These aroma compounds were grouped into acids, alcohols, esters, ketones, aldehydes and other components. Of these groups acids, alcohols, esters and ketones were found to be most dominant. It is important to note that the trend found for each of the groups reflected the pattern found for all the individual compounds (supplementary figure A.1).

The profiles of VOCs detected in the dairy miso were compared with those of commercial blue mould cheese and white mould cheese. The total amount of VOCs found in dairy miso exceed the amount detected in blue and white mould cheeses with up to 3 times more total response (Fig. 2). This mainly is due to the high alcohol and ester content of dairy miso compared to the mould cheeses. Compared to blue mould cheese dairy miso has very strong sweet and floral notes, coming from the higher alcohol and esters formed in the product and less pungent ketone notes.

3.2. Effect of salt on volatile organic compound formation and primary metabolism

3.2.1. Volatile organic compound formation

In traditional miso production various sodium chloride contents are used to produce different kinds of miso flavours (Shurtleff & Aoyagi, 1976). Therefore we studied the effect of different NaCl contents (0–200 g/kg) on flavour formation in dairy miso. The addition of salt resulted in a corresponding decrease in water activity (a_w) of the product (Table 1).

A first visual observation when following the fermentations in time was that samples prepared with a high salt content (>100 g/kg salt) remained more viscous throughout the fermentation, indicating less proteolysis. Formation of VOCs was monitored for a period of 30 days (Fig. 3).

Total production of VOCs followed similar trends up to 80 g/kg NaCl (Fig. 3). At 100 g/kg, the rate of VOCs formation declined, although significant amounts were still being produced after 40 days of incubation. To quantify the differences in VOC production, the production rates in the first 10 days was estimated by fitting linear models for each replicate at each salt content (Fig. 4). Indeed significantly different VOC production rates were found between the <80 g/kg salt miso and ≥100 g/kg salt miso ($P < 0.01$ for ≥100 g/kg salt compared to 0, 20, 40 g/kg salt, $P < 0.05$ for ≥100 g/kg salt compared to 60 g/kg salt, supplementary file A.2).

To further elucidate on the effect of NaCl on VOC formation in dairy miso, the compounds were analysed per chemical category (acids, alcohols, esters and ketones) (Fig. 5). Specific production rates per compound type were calculated for the first 10 days for acids and esters (supplementary file A.2). Ester and acid formation was found to decrease at NaCl contents of 100 g/kg and higher.

3.2.2. Primary metabolism

Substrate consumption and metabolite production of *A. oryzae* was monitored throughout the fermentation of quark with NaCl contents ranging from 0 to 200 g/kg. The main carbon source in quark is lactose. At all NaCl contents lactose was degraded from its initial concentration 102 ± 20 mmol/L to 21 ± 2.5 mmol/L in a course of 30 days (Fig. 6). Notably, galactose was not detected in dairy miso. Initial glucose content was found to be 78 ± 18 mmol/L (Fig. 6). During fermentation glucose was consumed and produced simultaneously. At 0 and 20 g/kg NaCl, glucose consumption seems higher compared to higher salt contents, resulting in lower glucose pools in dairy miso. The main metabolite produced during the fermentations was found to be ethanol (Fig. 6). Lactate did not increase during fermentation, indicating no growth of LAB. Ethanol reached concentrations up to 400 mmol/L at various salt contents and production was not significantly different at increasing salt contents. Next to ethanol also pyruvate, acetate, citrate, butyrate, glycerol, and propionate were detected. Ethanol, pyruvate and citrate production was not notably affected by higher salt contents. Butyrate (Fig. 6), glycerol and propionate (supplementary figure A.2) production decreased at high salt contents.

3.3. Effect of natamycin on volatile organic compound formation

To study whether the fungus needs to be metabolically active in dairy miso for aroma production, dairy miso with a natamycin content

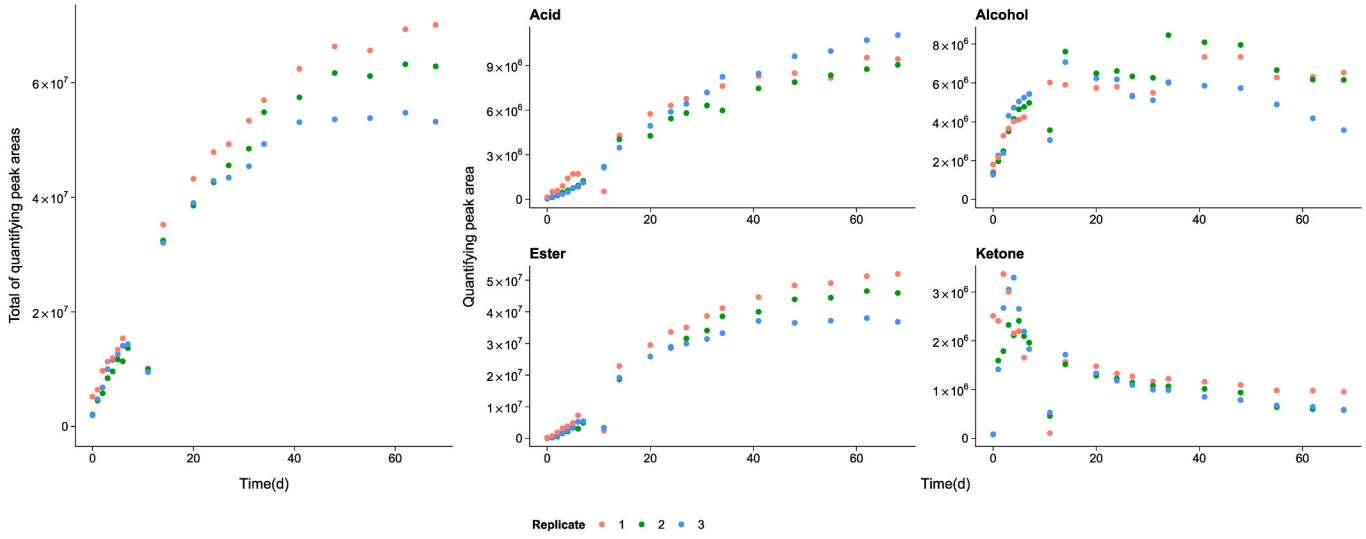

Fig. 1. Volatile organic compound development in dairy-based miso produced with 60 g/kg NaCl and *A. oryzae*. The total volatile organic compound development is shown on the left. Each volatile organic compound was assigned to a compound type (acids, alcohols, esters and ketones) and summed. The total of each group at each time point is shown on the right. Aroma formation was followed over a course of 68 days. Biological replicates (n = 3) are displayed by different colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

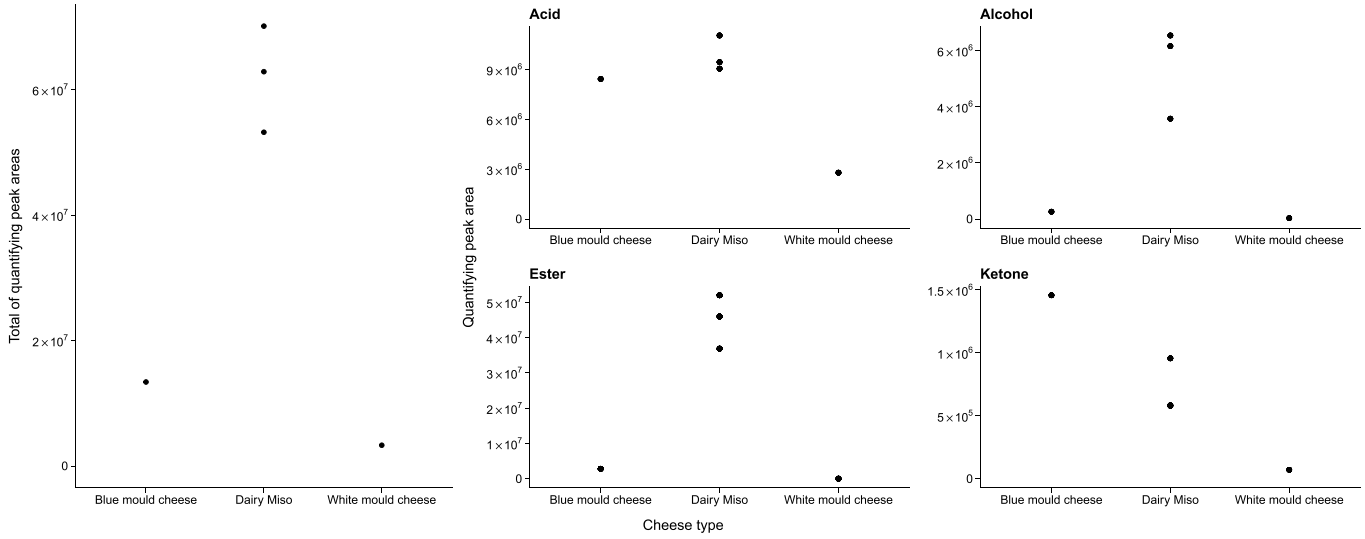

Fig. 2. Comparison of volatile organic compound contents between a commercial blue mould cheese, white mould cheese and dairy miso of 68 days old produced with 60 g/kg NaCl and *A. oryzae*. The total volatile organic compound development is shown on the left. Each volatile organic compound was assigned to a compound type (acids, alcohols, esters and ketones) and summed. The total of each group is shown on the right.

Table 1
Water activity for dairy miso produced with NaCl contents ranging from 0 to 200 g/kg.

NaCl content (g/kg)	0	20	40	60	80	100	150	200
Measured water activity	0.982	0.957	0.939	0.92	0.907	0.88	0.874	0.861

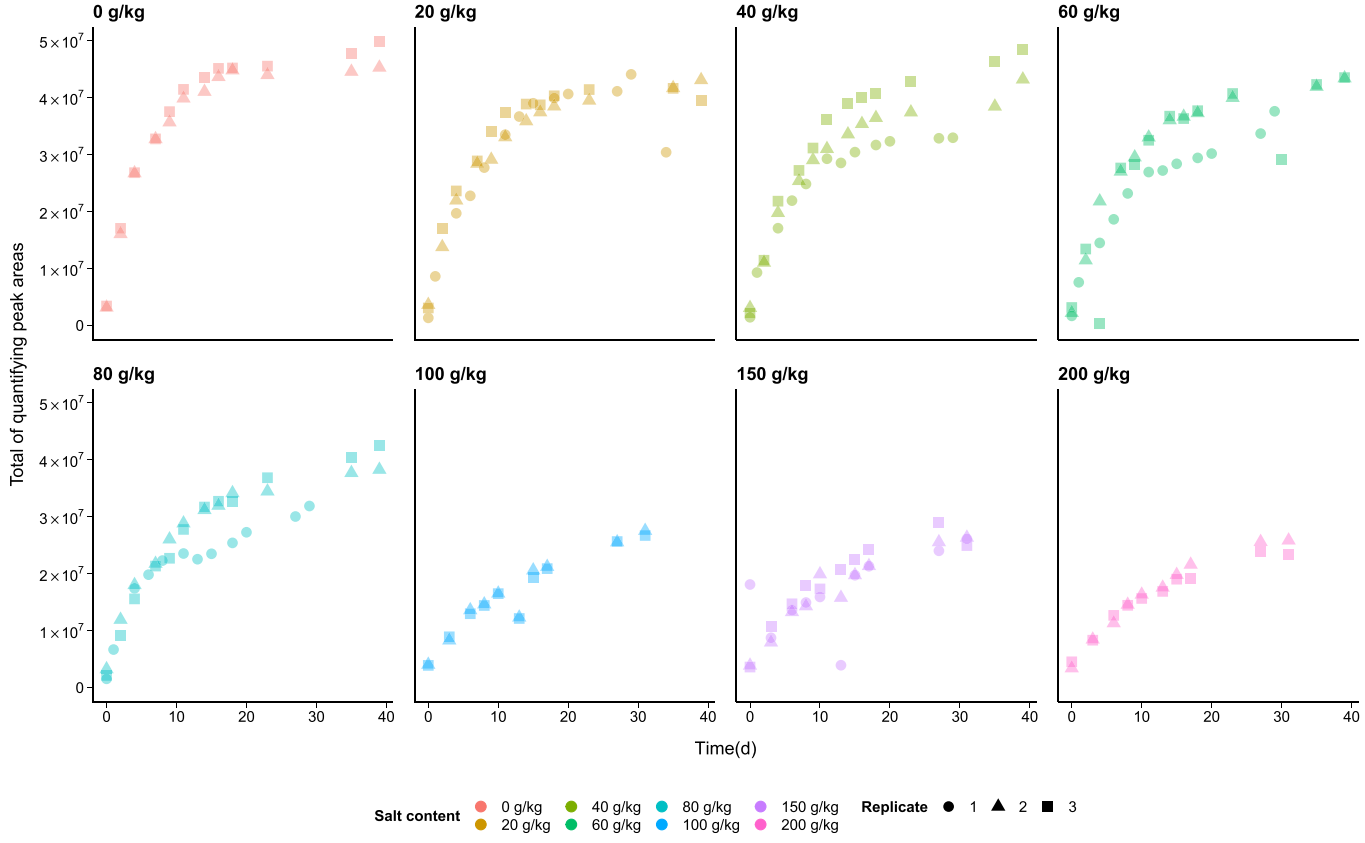

Fig. 3. Total volatile organic compound development for dairy miso produced using NaCl contents ranging from 0 to 200 g/kg over a course of 30 days. Biological replicates (n = 3) are displayed by different shapes.

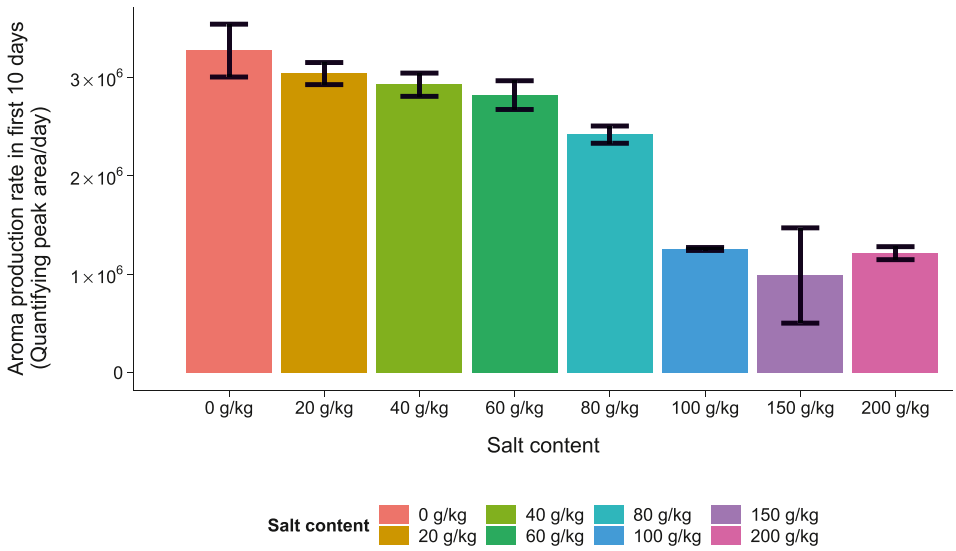

Fig. 4. Volatile organic compound production rates for different NaCl contents estimated by fitting linear models for the first 10 days for biological replicates (0–80 g/kg NaCl n = 3, 100,150,200 g/kg NaCl n = 2). Slopes of the linear models were considered to be the aroma production rate per day.

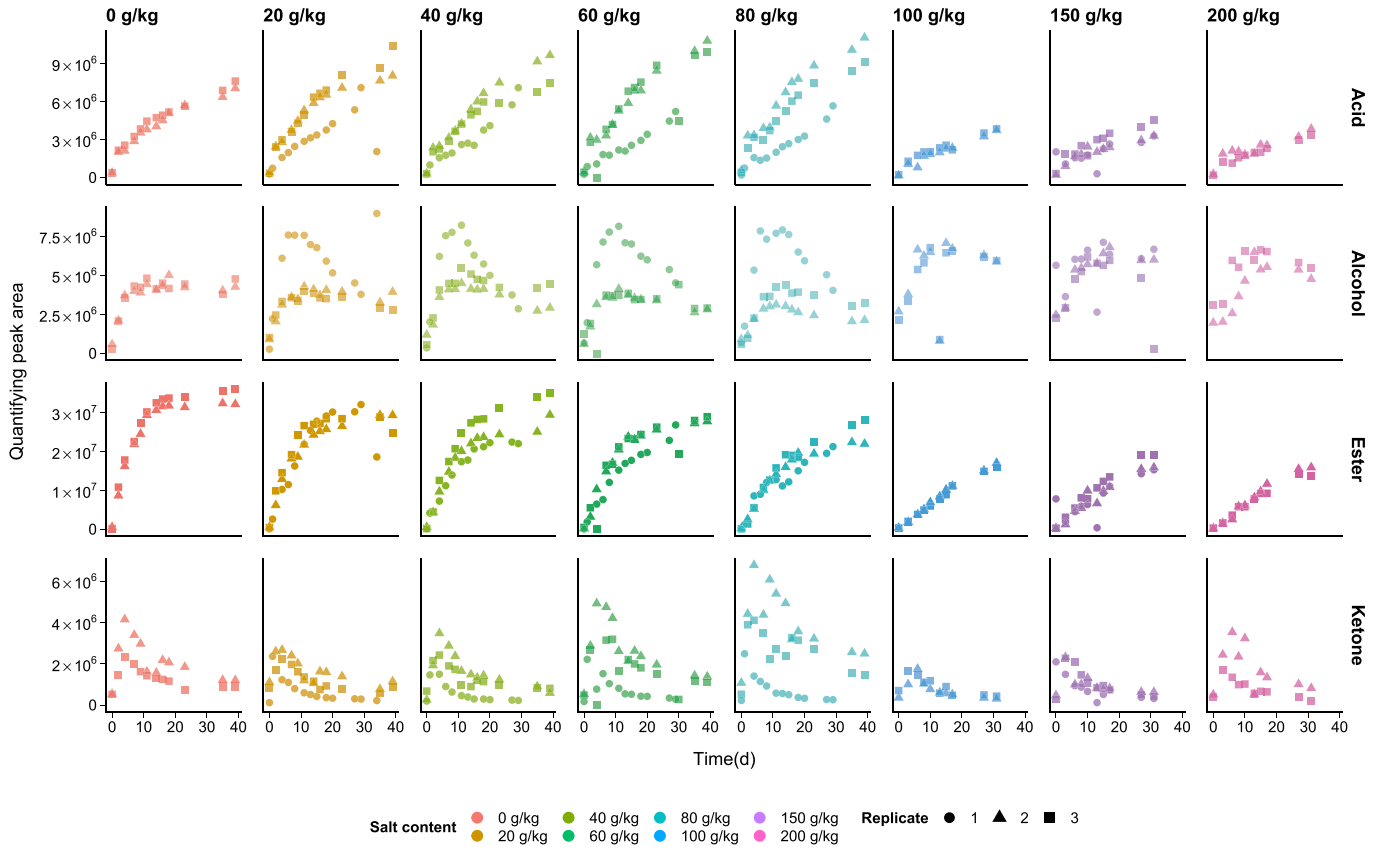

Fig. 5. Volatile organic compound development per compound type for dairy miso produced using NaCl contents ranging from 0 to 200 g/kg over a course of 30 days. Biological replicates are displayed by different shapes (0–80 g/kg NaCl n = 3, 100,150,200 g/kg NaCl n = 2).

ranging from 0 to 800 mg/kg was produced (see Fig. 7). Natamycin is a broad-spectrum antifungal agent for which the mode of action has not been fully elucidated, although it is clear that natamycin can bind to ergosterol and inhibits transport of substrates and ergosterol related protein functions (te Welscher, 2010). Impaired substrate transport leads to reduced metabolic activity of the fungi and therefore aroma formation without active metabolism can be studied.

Compared to the control miso (no natamycin added), aroma production at 25 mg/kg natamycin already decreased, with further decreasing aroma production at increasing natamycin contents. Although natamycin clearly has an effect on the production of aroma compounds, they were still formed. A clear dose-dependent decrease in alcohol and ester production was found after 28 days of incubation (Fig. 7). Moreover, the production of volatile organic acids and ketones

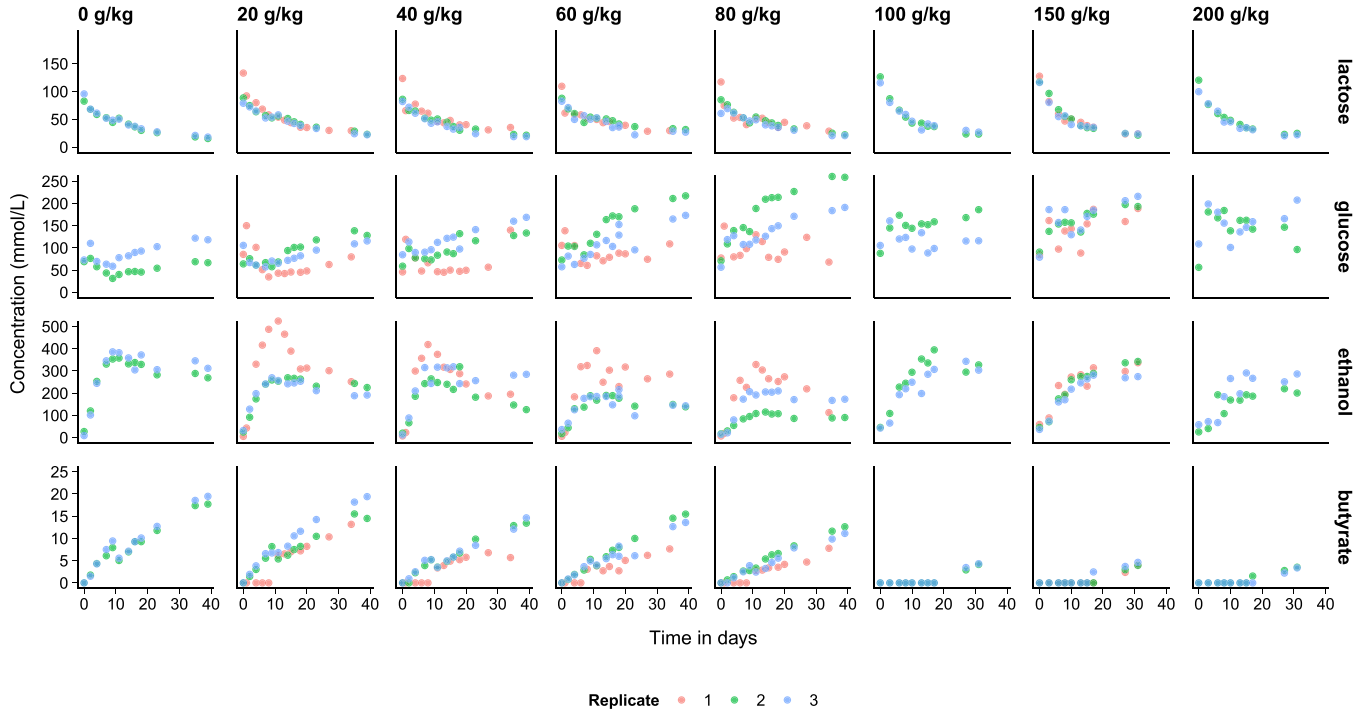

Fig. 6. Metabolite consumption and production during dairy miso production using various NaCl contents and *A. oryzae*. Each miso was followed over a time period of at least 30 days. Biological replicates (n = 3) are displayed by different colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

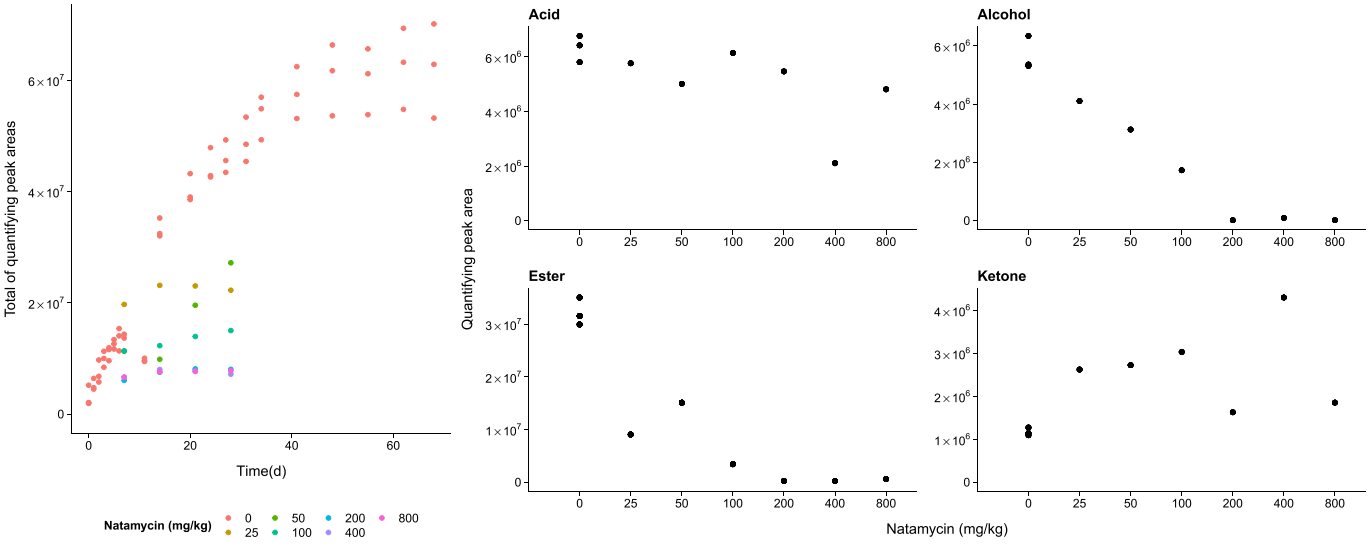

Fig. 7. Volatile organic compound development of dairy miso produced with 60 g/kg NaCl, *A. oryzae* and with natamycin contents varying between 0 and 800 mg/kg. The total volatile organic compound development is shown on the left. Each volatile organic compound was assigned to a compound type (acids, alcohols, esters and ketones) and summed. The total of each group at t = 27 days for 0 mg/kg natamycin and t = 28 days for all other natamycin contents is shown on the right.

seem to be hardly affected by increasing natamycin contents. This suggests that production of alcohols requires an active fungal metabolism. Because of the limited alcohol production also ethyl ester formation is impaired due to reduced fungal metabolism. In contrast, fat degradation, resulting in mainly fatty acids and ketones is independent of fungal metabolism and most likely occurs due to activity of secreted lipolytic enzymes.

A cell-free extract of *A. oryzae* mycelium and intact *A. oryzae* mycelium were applied to quark to confirm this hypothesis (supplementary figure A.3). Similar contents of acids and ketones were found in dairy miso's produced with the cell-free extract and intact mycelium, whereas alcohol and esters were hardly detected in dairy miso made with the cell-free extract. The intact mycelium did show alcohol and ester production, demonstrating active metabolism is required for production of these compounds. Furthermore, microscopic analysis of 2-month-old dairy miso confirmed the presence of intact hyphae, supporting our conclusion that intact metabolising *A. oryzae* is needed for alcohol and ester production. To monitor if fungal growth occurred, fungal DNA was quantified using qPCR. No significant differences in fungal DNA copy numbers were found (data not shown). We therefore

did not find evidence of active growth of the fungus. However, it cannot be excluded that fungal biomass per nucleus increased and thus that fungal growth did occur.

3.4. Headspace environment

During our study it was noticed that dairy miso that was sampled and thus mixed regularly, obtained a stronger aromatic smell. Indeed, GC-MS analysis of dairy miso sampled with a frequency of 3 times per week showed increased aroma formation compared to dairy miso only sampled at the endpoint (data not shown). Therefore we hypothesised that oxygen is required by the fungus to remain metabolically active. To further elucidate on the effect of headspace atmosphere during fermentation, dairy miso was produced with an aerobic and anaerobic headspace.

Higher contents of VOCs were found in samples of dairy miso with an aerobic headspace (Fig. 8). Furthermore volatile organic acids were present at higher levels with an anaerobic headspace while ketones were found at similar contents. Alcohol and ester formation was higher in samples with aerobic headspace compared to anaerobic headspace. These results are in line with results found when studying the impact of natamycin on VOC production and show that headspace environment contribute to the extent of metabolic activity displayed by *A. oryzae* during fermentation.

3.5. Effect of fat content on volatile organic compound formation

The dominant VOCs found in dairy miso produced with full fat quark mainly originate from fat degradation. A low-fat variant was made to study the effect of fat in quark. As expected, flavour formation in low-fat quark was impaired by the lack of fat being present (Fig. 9). Notably, higher alcohol contents were found in low-fat quark compared to full-fat quark, showing alcohol formation during dairy miso production mainly originates from carbohydrate substrates present in the quark and rice, i.e. lactose and glucose.

4. Discussion

Traditionally, miso is made by inoculating boiled soybeans with *A. oryzae* grown on rice (called koji) and NaCl varying between 55 and 200 g/kg, after which it is fermented for periods of time ranging from 10 days up to 3 years (Shibasaki & Hessbltine, 1962; Shurtleff & Aoyagi, 1976) depending on the type of miso. Generally, traditional miso with a low salt content (≤70 g/kg) tends to ferment quickly, resulting in a miso with a sweet taste, whilst miso with a higher salt content (≥100 g/kg) has a more savoury taste (Shurtleff & Aoyagi, 1976). Soybeans are energy-rich substrates consisting of approximately 19 g fat, 41 g protein and 35 g carbohydrates per 100 g on dry weight basis (Medic, Atkinson, & Hurburgh Jr, 2014). Soaked soy beans have a moisture content of approximately 61.5 g per 100 g, and therefore a fat, protein and carbohydrate content of approximately 7.3 g/100 g, 15.8 g/100 g and 13.5 g/100 g, respectively. The quark used in this study contained 8.6 g fat, 7.6 g protein and 4 g carbohydrates per 100 g and also can be considered to be an energy-rich substrate. The combination of the capacity of *A. oryzae* to express lactase (Akasaki, Suzuki, Funakoshi, & Yamashina, 1976) and to have strong lipolytic (Ohnishi et al., 1994) and proteolytic activity (Maeda et al., 2004) showed potential for fermenting dairy substrates, such as quark.

In this study an initial benchmark trial using 60 g/kg salt and a koji to quark ratio of 1:3 resulted in 'dairy miso'. The dairy miso developed

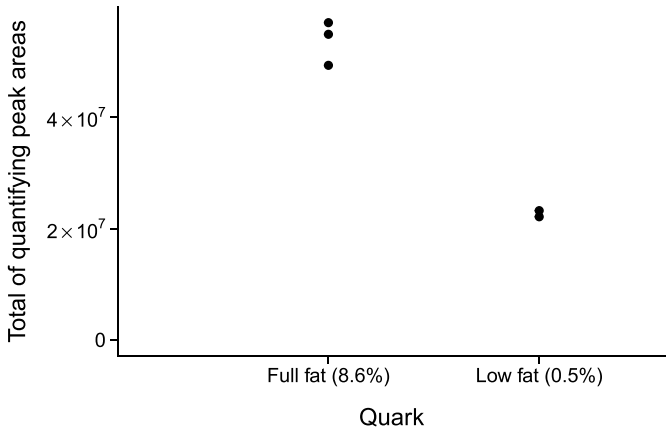

Fig. 9. Effect of initial fat content on volatile organic compound formation of dairy miso after 28 days using 60 g/kg NaCl (n = 3 for full-fat, n = 2 for low-fat quark).

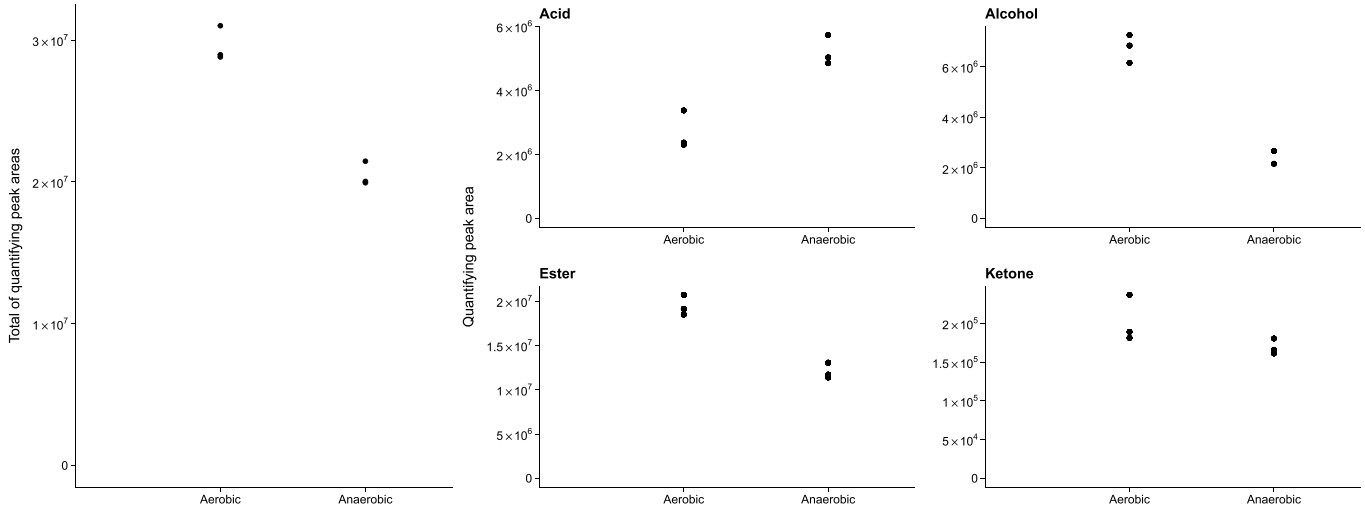

Fig. 8. Volatile organic compound development of dairy miso produced with 60 g/kg NaCl, *A. oryzae* with either an aerobic or anaerobic headspace. Samples were incubated for 30 days without opening the incubation jars. Each dot represents an individual fermentation (n = 3). The total volatile organic compound development is shown on the left. Each volatile organic compound was assigned to a compound type (acids, alcohols, esters and ketones) and summed. The total of each group is shown on the right.

high amounts of VOCs in a course of 68 days. Fat degradation resulted in free fatty acids, ketones, higher alcohols and ethyl ester production. Lactose consumption, amylolytic and proteolytic activity resulted in ethanol, higher alcohols and ester formation. This resulted in a final product with notes of the typical ketone smell of blue mould cheese (Spinnler & Gripon, 2004) in combination with a dominant sweet aroma.

These sweet notes at low salt contents are due to the production of fruity ethyl esters (i.e. ethyl pentanoate(apple), ethyl hexanoate(pineapple), ethyl heptanoate(fruit) and many others, see supplementary figure A.1) derived from fatty acid metabolism and ethanol. In order to form ethyl esters in (dairy) miso, ethanol and acyl-CoA, which derive from free fatty acids, are required together with alcohol O-acetyltransferase activity (Saerens et al., 2006). Thus, both ethanol and free fatty acid concentrations may be the rate determining factors for final ethyl ester content found in dairy miso.

Indeed, dairy miso produced with quark with a low fat content (0.5 g/100 g) had lower ester contents compared to full fat quark (8.6 g/100 g). Products of fat hydrolysis all declined drastically, clearly demonstrating the importance of milk fat hydrolysis in aroma formation of dairy miso and demonstrating the importance of soy bean oil for traditional miso. Accordingly, in blue mould cheeses lipase activity of the moulds is responsible for characteristic aroma due to liberation of free fatty acids and subsequent degradation into ketones (Spinnler & Gripon, 2004).

Many fungi can utilise triacylglycerols as carbon source by expressing extracellular lipases (Kinderlerer, 1993; Ohnishi et al., 1994). Triacylglycerols are degraded into free fatty acids and monoglycerides, after which methyl ketones like 2-nonanone and 2-heptanone are formed by partial β-oxidation of these free fatty acids (Kinderlerer, 1993). Subsequently, methyl ketones can be reduced to higher alcohols, a process which is favoured in oxygen-limited conditions (Kinderlerer & Kellard, 1984). Conversion of medium-chain fatty acids to methyl ketones has been linked to act as a detoxifying mechanism during fat hydrolysis (Kinderlerer, 1993) and reduction of ketones to alcohols is linked to regenerating NAD^+ in oxygen-limited conditions (Kinderlerer & Kellard, 1984). In dairy miso fat degradation by lipolytic activity is clearly demonstrated by the abundance of fat-related volatile organic compounds detected during fermentation. Throughout the fermentation fat is hydrolysed, resulting in a constant release of free fatty acids. Subsequently, in the initial stages of the fermentation methyl ketones are formed to a large extent, after which the ketones are reduced to higher alcohols for regeneration of NAD^+ in oxygen-limited conditions. NAD^+ regeneration from ketone reduction therefore helps restoring redox balance (de Smidt, du Preez, & Albertyn, 2008) from NADH that is generated during β-oxidation in the peroxisomes (Kinderlerer, 1993). During glycolysis, ketone contents therefore may be expected to be lower due to higher reduction to alcohols. This is in line with our findings with natamycin. Fatty acid degradation by lipolytic enzymes remained similar and ketones were found to increase at increasing natamycin contents, due to decreased reduction of ketones to alcohols. Glycolysis, fat hydrolysis by extracellular lipolytic enzymes and β-oxidation in the peroxisome are thus crucial for the strong VOC and thus aroma formation in dairy miso.

In line with traditional miso production (Shurtleff & Aoyagi, 1976), VOC formation was found to differ amongst the fermentations with different NaCl contents. The dairy miso produced with higher salt contents (>100 g/kg) remained more viscous compared to low salt contents, an observation which is also made in blue cheese in which the addition of salt reduces proteolytic activity of *P. roqueforti* (Kinsella, Hwang, & Dwivedi, 1976). Production of ethyl esters and fat degradation products were the most prominently affected by the addition of NaCl. This could be explained by a decreased lipolytic activity, resulting in lower contents of free fatty acids and corresponding degradation products. In blue cheese maximal lipolytic activity was found to occur between 40 and 60 g/kg NaCl (Fox, McSweeney, Cogan, & Guinee, 2004), in line with our results. Furthermore, butyrate, a common milk fat hydrolysis product in blue cheese (Kinsella et al., 1976) and glycerol, a product of fat hydrolysis (Fu, Zhu, Gao, & Duan, 1995), were detected in lower contents at increasing salt contents, clearly showing fat hydrolysis slowed down by the addition of salt. Although the differences in fat hydrolysis rates in the first 10 days were not found to be significant, we clearly demonstrate that fat hydrolysis and corresponding ketone and ethyl ester formation is reduced by the addition of high amounts of salt (>100 g/kg).

Interestingly, at all salt contents lactose was degraded to similar extends. Primary metabolism of sugars was therefore not affected to large extends by increments of salt, resulting in formation of up to 400 mmol/L of ethanol in dairy miso. Indeed, *A. oryzae* has been shown to be able to grow at an a_w of 0.85 (Gibson, Baranyi, Pitt, Eyles, & Roberts, 1994), whereas dairy miso with a salt content of 200 g/kg had an a_w of 0.861. It seems therefore that the main effect of salt is a decreased lipolytic and proteolytic activity, whereas glycolytic activity is not affected at large. Glucose originates from the koji in which it has been liberated from starch by the action of the amylolytic activity of *A. oryzae*. During fermentation glucose was consumed and produced simultaneously.

In general, we showed that by adjusting tradition process parameters like oxygen, salt and fat content it is possible to steer volatile organic compound formation in dairy miso. Targeting both the fungal primary metabolism or extracellular fat hydrolysis by changing these parameters gives an opportunity to steer formation of specific compound groups. Generally, the strong hydrolytic activity and corresponding strong aroma forming capacity of *A. oryzae* makes it a suitable candidate for exploration of novel substrates for interesting new food products.

Our study shows cross-over fermentations can result in novel food products with interesting characteristics, like enhanced aroma profiles. Additionally, cross-over fermentations can enhance nutritional value and impact sustainable food production by replacing imported substrates to locally grown substrates (Wolkers–Rooijackers et al., 2018). Furthermore cross-over fermentations can aid in replacing meat protein by plant protein, thereby potentially aiding the protein transition from meat to plant needed to produce sufficient amounts of food to feed the world in the future (Aiking & de Boer, 2018).

5. Conclusion

In this study we show that a microorganism traditionally used on a different substrate in a different environment can be applied to new environments for cross-over fermentations. We show that *A. oryzae*, which is traditionally used in plant-based fermentations in Asia, can be applied in a traditional European fermented dairy product to produce a novel fermented product with intense aroma properties. Active metabolism of *A. oryzae* was required for alcohol production, whereas fat hydrolysis resulting in acid and ketone formation is mainly due to secreted extracellular enzymes. In order to form high amounts of esters both active metabolism for alcohol production and lipolytic activity for free fatty acids production is required. The predominant aroma active VOCs present in dairy miso were related to fat hydrolysis, ethanol production and corresponding ethyl ester formation.

Traditionally used practices in miso production, like aeration, adding salt and variations in substrate (fat/sugar contents) all affect either fat hydrolysis and/or ethanol formation and therefore can be and are already used traditionally for producing qualitatively different miso. Our study shows that interesting novel food products can be produced by taking microorganisms from their traditional environment into a new environment and that the final product can be altered by using traditional practices. We demonstrated the potential of cross-over fermentation for novel food products. The enormous diversity of microorganisms used in traditional fermentation processes and the vast number of alternative substrates offer numerous opportunities for novel fermented product development.

Declaration of interest

Declarations of interest: none.

CRediT authorship contribution statement

Alexander Dank: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Oscar van Mastrigt:** Formal analysis, Methodology, Data curation. **Zhaoying Yang:** Investigation. **Varun M. Dinesh:** Investigation. **Søren K. Lillevang:** Resources, Conceptualization. **Christian Weij:** Resources, Conceptualization. **Eddy J. Smid:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition, Project administration.

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Appendix A. Supplementary data

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