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Interactions between two strains of lactic acid bacteria and *Laetiporus sulphureus* strain FH24 and FH319, and *Wolfiporia cocos* strain FH9 mycelium

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ABSTRACT

This study investigates the interactions between two strains of lactic acid bacteria (LAB; *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides*) and the mycelium of two edible fungal species of the Polyporaceae; *Laetiporus sulphureus* and *Wolfiporia cocos*. Co-inoculation of LAB and mycelia on agar plates increased the growth of both fungal strains. Using different liquid- and solid-state fermentation techniques, combined with HPLC and GC-MS analyses, we discovered that co-fermentation of LAB with the fungi promoted fungal growth and induced morphological changes in the mycelia. Seven volatile compounds were found to differ between samples of monocultures and co-cultures. *Wolfipora cocos* (FH9) seemed to cause metabolic changes in *L. lactis*, in which the production of acetoin and acetate was reduced. Linalool was tested as the inducer compound and high concentrations inhibited bacterial growth. A lower concentration of linalool resulted in no production of acetic acid by *L. lactis* NFICC188, confirming results from GC-MS analysis. The results demonstrated that co-culturing *Wolfipora cocos* (FH9) or *Laetiporus sulphureus* (FH24) with the two LAB strains on agar plates could increase the yield of fungal biomass by 3.3 times on average, 6 days after inoculation. In comparison, agar plates containing commercial acids increased the fungal yield with an average of 3.7 times, 6 days after inoculation. For liquid cultures, the increase in mycelial biomass was generally higher for co-cultures with *Lactococcus lactis* strain NFICC188 than for pre-acidified media. This could be an important future strategy for growing *L. sulphureus* or *W. cocos* mycelia for medicine or human consumption.

1. Introduction

Interspecies communication and interactions have long been known to exist between bacterial and fungal species. Many such interactions have proved essential for certain food products, such as sourdough and cheese (Hansen & Jakobsen, 1997), or for discovering new antibacterial and antifungal compounds (Gong et al., 2019; Morita et al., 2019; Wheatley, 2002; Zhao et al., 2022). Interactions can happen in different ways, for example by secretion of toxins or hormones, by production of otherwise unavailable nutrients, by combating common infectious strains, or by changing the pH or oxygen availability in the surrounding environment (Hansen & Jakobsen, 1997). Interspecies communications can be mutually beneficial for both species, but only a few fungal strains outside of the commercially relevant species have been investigated for bacterial-fungal interactions. Most research on bacterial-fungal interactions has focused on commercial or infectious fungal strains (de Boer, 2017; Deveau et al., 2018; Khalid & Keller, 2021; Lapiere & Richard, 2022).

Laetiporus sulphureus and *Wolfiporia cocos* (also known as *Wolfiporia extensa*) are both fungi belonging to the Polyporales order and are known to cause brown rot in wood (Gründemann et al., 2020; Wang et al., 2013). *L. sulphureus* has been widely consumed and is reported to

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have a taste similar to chicken, lobster, or crab, which is why it has received the nickname "chicken of the woods" (Rapior et al., 2000). The fruiting bodies of *L. sulphureus* have been the subject of many studies, however to the best of our knowledge, cross-kingdom interactions with bacteria have not previously been studied in mycelial cultures. *W. cocos* has been used in traditional Chinese and Japanese medicine for effects claimed to "induce diuresis, excrete dampness, invigorate the spleen and tranquilize the mind" (Kobira et al., 2012; Song et al., 2002). Both fungi are edible, they have been used for medicines, and they degrade cellulosic and hemi-cellulosic material (Gründemann et al., 2020; Wang et al., 2013). This makes them potential candidates for utilizing recalcitrant side-streams from food production to grow mycelium for food or medicinal purposes.

In terms of beneficial bacteria, few orders have been studied as intensely as Lactobacillales. Many cross-kingdom interactions with lactic acid bacteria (LAB) have been investigated, due to their ability to prevent spoilage in fermented foods (Iosca et al., 2023). This is partly due to the fast acidification of substrates during growth, but also due to the production of bacteriocins and antifungal compounds (Abouloifa et al., 2022; Cleveland et al., 2001). Within the LAB domain, there are both homofermentative and heterofermentative species (Doi, 2019). The conversion of hexoses and pentoses for each type has been summarized in Table 1. Homofermentative LAB are strains that can convert one hexose molecule into two molecules of lactate, thereby yielding a net energy production of two ATP molecules (Mora-Villalobos et al., 2020). Furthermore they can convert three pentose molecules into five lactate molecules, yielding seven ATP in the process (Gänzle, 2015). This metabolism means that the majority of the sugars available in a substrate are converted into lactic acid. Formate, ethanol, acetate, and acetoin can also be produced by alternative pathways from pyruvate (Gänzle, 2015). One hexose molecule can be degraded into one molecule of formate, ethanol, and acetate, yielding 3 ATP. When oxygen is present, the glucose can be converted into two molecules of acetate and one molecule of acetoin or butane-2,3-dione (Gänzle, 2015). This metabolism is less acidifying than producing four molecules of lactic acid. It is also energetically favorable, since four ATP and one NADH are generated when the glucose is converted to acetate and acetoin or butane-2,3-dione, compared to two ATP when converted to lactate (Gänzle, 2015).

In contrast, heterofermentative LAB convert one molecule of glucose into one molecule of lactic acid, one molecule of CO_2, and one molecule of ethanol, yielding a net energy production of one ATP molecule (Mora-Villalobos et al., 2020). One molecule of pentose is converted to one molecule of acetate and one molecule of lactate while yielding two ATP molecules (Gänzle, 2015). This means that more acetate, ethanol, and CO_2 are produced by heterofermentative LAB. LAB can be a cheap and effective way to reduce the availability of simple sugars, decrease the pH and introduce new flavors, or beneficial compounds, to a food product.

This paper aims to provide insight into the potential of co-culturing fungi with LAB as a strategy for mycelium production and enhancing fungal properties for sources of novel foods. The effects of co-culturing strains of *L. sulphureus* and *W. cocos* with one homofermentative (*Lactococcus lactis* subsp. *lactis*) and one heterofermentative (*Leuconostoc mesenteroides*) strain of LAB was investigated. This was done by looking into mycelial growth, as well as fungal morphology, on agar plates and in liquid cultures. GC-MS and HPLC analysis were used in order to further assess the mechanisms behind the growth changes observed. To our knowledge, this is the first time that mycelia of *L. sulphureus* and *W. cocos* have been co-cultured with LAB strains.

Table 1
Sugar conversion in homofermentative and heterofermentative LAB. This table was created using information from Gänzle (2015), and Mora-Villalobos et al. (2020).

Strain type	Conversion	Result
Homofermentative	1 hexose → 2 lactate	2 ATP
Homofermentative	3 pentose → 5 lactate	7 ATP
Homofermentative	1 hexose → 1 formate +1 ethanol +1 acetate	3 ATP
Homofermentative	1 hexose + O_2 → 2 acetate +1 acetoin +3 CO_2	4 ATP + NADH
Heterofermentative	1 hexose → 1 lactate +1 ethanol + CO_2	ATP
Heterofermentative	1 pentose → 1 lactate +1 acetate	2 ATP
Heterofermentative	1 hexose +2 NADH → 1 lactate +1 acetate	3 ATP – 2 NADH

2. Materials and methods

2.1. Fungal and bacterial strains

Three strains of *Laetiporus sulphureus* (FH24, FH296, and FH319) and two strains of *Wolfiporia cocos* (FH9 and FH22) were provided by the Institute of Food Chemistry and Food Biotechnology, Justus Liebig University Giessen, Germany. The strains were chosen based on preliminary experiments, in which 31 strains of brown- and white rot fungi were grown in acidified media (Appendix table A1 and appendix figure A1). Of the 31 strains, the two species that grew best on acidified media were chosen. Subsequently, these fungal cultures were grown on malt extract agar plates (containing 20 g/L malt extract (Carl Roth, Germany) and 15 g/L agar (Carl Roth, Germany)) at 27 °C and then stored at 4 °C. They were then used to inoculate second generation plates. Inoculation plugs were picked from actively growing mycelium from second generation plates, which had been incubated at 27 °C for 4–8 days depending on the fungal strain.

Two strains of LAB were provided by the National Food Institute Culture Collection (NFICC), which is located at the Technical University of Denmark, Kgs. Lyngby, Denmark. These were *Lactococcus lactis* subsp. *lactis* (NFICC188) and *Leuconostoc mesenteroides* (NFICC311). For maintenance, glycerol stocks of the bacteria were prepared by growing in liquid potato-dextrose-peptone (PDP) medium at room temperature. The composition of 1 L of PDP media was 4.0 g potato extract (Sigma-Aldrich, Germany), 10 g soy peptone (Carl Roth, Germany), 20 g dextrose (Carl Roth, Germany). The strains were first inoculated and grown overnight at 27 °C. After growth a glycerol stock solution (50% w/w) was added to give a final concentration of 15% w/w glycerol. The glycerol stocks were stored at −20 °C. When used, overnight cultures were prepared in 40 mL PDP medium and incubated at 27 °C overnight under static conditions. For experiments, both bacterial and fungal strains were cultivated on potato-dextrose-peptone agar (PDPA) plates, containing PDP medium and 2% bacteriological agar (Carl Roth, Germany). All experiments in this project were carried out in biological triplicates.

2.2. Co-inoculation with lactic acid bacteria and bacterial analysis

The five fungal isolates were inoculated on to plates containing either lactic acid (laPDPA), or two vertical streaks of lactic acid bacteria (Appendix Figure A2). For the latter, bacterial cultures of NFICC311 (*Lc. mesenteroides*) and NFICC188 (*L. lactis*) were inoculated into liquid PDP and kept at 27 °C overnight. Streaking of them was performed with a sterile inoculation loop (5 μL) from the overnight culture (OD 0.75 of NFICC311 or OD 0.85 of NFICC188). Subsequently, one circular plug (0.6 cm in diameter) of the second-generation growing mycelium was added to each plate. All fungal strains were also grown on non-acidified PDPA media as a control. For plates containing lactic acid, 5 g/L 85% L-(+)-lactic acid (Sigma-Aldrich, Germany) was added to the PDPA. To prevent hydrolysis of the agar during autoclaving, one 1 L bottle containing the agar was filled with demineralized water to 500 mL and one bottle containing the potato-dextrose-peptone with the lactic acid was filled to 470 mL. The bottles were then autoclaved and cooled to 55 °C. Subsequently, 1 M filter-sterilized NaOH was added to the potato-

dextrose-peptone-acid liquid to obtain a final pH of 4.6 and sterilized water was then added to give a total volume of 500 mL. The contents of the two bottles were mixed together and plates were poured immediately. The plates were incubated at 27 °C and mycelial growth was measured every day for eight days after inoculation.

To investigate the difference in acidification of the media by the two bacterial strains, each strain was grown on 12 plates of PDPA. Three plates were harvested on day 2, 4, 6, and 8. Harvesting was done by breaking the agar into small pieces and homogenizing it in 50 mL of demineralized water at 10,000 × RPM with an IKA Ultra Turrex T25 homogenizer (IKA-Werke, Staufen, Germany) for 1 min. Non-inoculated plates were used for measurements of pH for the blank.

For investigation of acid and ethanol production and sugar consumption of the bacterial strains, high-performance liquid chromatography (HPLC) was carried out on liquid PDP with and without LAB inoculation. Overnight cultures of NFICC311 and NFICC188 were centrifuged at 2450×*g* at 10 °C and then they were washed twice with 0.9% NaCl. This was then used to inoculate 10 mL of liquid PDP to give an initial optical density (OD_{600nm}) of 0.01. The cultures were incubated at 30 °C for 18 h. The HPLC method applied by Madsen et al. (2021) was used.

2.3. Impact of pH and acid type

PDPA plates containing acetic acid (pH 4.3), lactic acid (4.3 pH), or hydrochloric acid (pH 4.3) were used to test the impact of pH and acid type on three fungal strains. Preparation of acid-containing PDPA was done as explained for lactic acid PDPA (laPDPA) in section 2.2. For the different types of acids, 5 g/L 85% L-(+)-lactic acid (Sigma-Aldrich, Germany), 5 g/L 99.5% acetic acid (Chemsolute, Renningen, Germany), or 5 g/L HCl 37% (Sigma-Aldrich, Germany) was added to the PDPA. PDPA plates without acid were used as controls. Mycelium growth was measured on day 2, 6, and 8 by using a ruler to measure the diameter at three different points and then the average diameter was calculated.

2.4. Co-cultivation of LAB and fungi in liquid media

The impact of co-culturing the fungal strains with LAB was investigated. Conical flasks of 100 mL volume were filled with 20 mL of medium. Bacterial cultures were inoculated into liquid PDP, and they were kept at room temperature overnight. One circular plug (0.6 cm) of growing mycelium was added to each of the flasks and the media were homogenized at 10,000 RPM with an IKA Ultra Turrex T25 homogenizer for 1 min. The fungal strains were grown as a monoculture in PDP medium (pH 5.9) as a control, and in PDP + HCl (pH 4.2), PDP + NFICC311 (pH 5.9), and PDP + NFICC188 (pH 5.9). For the flasks used for co-cultures, 50 μL of overnight grown LAB (OD_{600nm} of 0.75 for NFICC311 or OD_{600nm} 0.85 for NFICC188) was added after homogenization of the fungal plugs. The flasks were incubated at 24 °C and 150 RPM in the dark, in a shaking incubator. Morphological changes were observed during the experiment. The mycelium was harvested after eight days of incubation, the pH was measured, and the mycelium was pelleted by centrifugation at 6125×*g* for 10 min. The supernatant was discarded, and the wet weight was measured.

2.5. Co-cultivation of LAB and fungi on bi-plates

The influence of volatiles was investigated by growing the fungal strains on agar, which was separated in the middle, but which had a shared headspace (Appendix Figure A3). Bacterial streaks were inoculated onto the agar from overnight cultures grown at 27 °C with a sterile inoculation loop (5 μL). One circular plug (0.6 cm) of growing mycelium was added to each of the bi-plates. The bi-plates were sealed with Parafilm to prevent moisture loss and contamination. They were then incubated at 27 °C and mycelium growth was measured on day 2, 4, and 8.

2.6. GC-MS analysis of volatile compounds

Bacteria and two of the fungal strains were co-cultivated on bi-plates containing PDPA on both sides of the barrier. Bi-plates were inoculated with the methods explained in section 2.5. Each bi-plate was covered with a modified lid (95 mm diameter) attached to a glass pipe (length 4 cm, outer diameter 16 mm, inner diameter 14 mm). The glass pipe was closed off using a screw threaded cap with a PTFE/silicone septum (Agilent Technologies, USA), which enabled non-invasive extraction of volatile organic compounds (VOCs) by SPME (Orban et al., 2019). The space between the lid and the plate was sealed with Parafilm to prevent moisture loss and contamination. VOCs were extracted with a CAR/PDMS/DVB fiber (50 μm/30 μm/30 μm, Sigma-Aldrich, USA), which was exposed to the headspace of culture plates for 30 min at room temperature. Volatiles were measured with gas chromatography-mass spectrometry using an Agilent 7890 B gas-chromatography system coupled to an Agilent 7977 B mass-spectrophotometry detector (Agilent Technologies, USA). The GC system was fitted with a split/splitless inlet and operated in splitless mode at 250 °C and a VF-WAXms column was used (30 m, i. d. 250 μm, film thickness 0.25 μm; Agilent Technologies, Santa Clara, USA). Helium 5.0 (Nippon Gases GmbH, Germany) was used as the carrier gas at a constant flow rate of 1.56 mL/min and the oven temperature was set to 40 °C (3 min) and increased at a rate of 5 °C per min to a maximum of 240 °C where it was held for 7 min. The MS source temperature was 230 °C in scan mode (m/z 33–300, EI 70 eV). The chromatograms were analyzed using Agilent MassHunter Qualitative Analysis version 10.0 and the peaks were identified with the NIST MS Search 2.2 database. A threshold was set to only include peaks with areas over 100,000. Non-inoculated PDPA plates were used as blanks and measured on day 0. All samples including blanks were measured in triplicates. Inoculated samples were measured on 0, 2, 4, 6, and 8 days after inoculation. A standard mixture of alkanes (C_7–C_{30}) and authentic reference compounds were used for compound identification and retention index (RI) calculations, according to van den Dool and Kratz (Arov & Dym, 1963). Purities of references were 90.0% for β-myrcene (Thermo Fisher, US), 92.0% for acetoin (Sigma-Aldrich, US), 99.5% for acetic acid (Chemsolute, Germany), 97.0% for linalool (Acros Organics, Belgium), 99.0% for methyl benzoate (Alfa aesar, Massachusetts, US), 98.0% for methyl 3-methylbutanoate (Alfa aesar, US) and 99.0% for 2-heptanone (Sigma-Aldrich, US).

2.7. Linalool effects on NFICC188 and NFICC311

The minimal inhibitory concentration (MIC) was determined for linalool. PDPA plates containing various concentrations of linalool were inoculated with overnight cultures of NFICC188 (OD_{600nm} of 0.3) and NFICC311 (OD_{600nm} of 0.2). The overnight cultures were first washed twice with 0.9% sterile NaCl. 10 μL of the overnight culture was mixed with 990 μL sterile 0.9% NaCl and 100 μL was plated. Stock solutions of linalool were prepared by mixing 97% linalool with 96 % ethanol at appropriate concentrations, as previously reported (Guo et al., 2021). 30 mL of the mixtures were added to 120 mL of warm PDPA media before pouring, such that the concentration of ethanol was 10% and for linalool they were 5, 2.5, 1.0, 0.5, 0.25 and 0.125 μL/mL on the plates.

Control plates were made containing 30 mL of sterile water or 30 mL of 10% ethanol. The plates were done in triplicate and incubated at 28 °C for 24 h. The lowest concentration of linalool to completely inhibit growth (the MIC) and a quarter of that concentration (¼ of MIC) was used in liquid PDP for testing of metabolic changes in NFICC188. In those experiments, NFICC311 was used as a negative control strain. Overnight cultures of the bacterial strains were washed twice with 0.9% NaCl and resuspended in 10 mL 0.9% NaCl. The cultures were inoculated in 10 mL PDP medium containing either 1 mL sterile water, 1 mL 10% ethanol, 1 mL 10% ethanol and 5% linalool, or 1 mL 10% ethanol and 1.25% linalool. An initial OD_{600nm} of 0.01 for each strain was used. The liquid cultures were incubated at 28 °C for 48 h without shaking.

Subsequently, the cultures were centrifuged at 2450×*g* for 5 min and at 10 °C, the supernatant was then filtered with 0.22 μm PES filters and the filtrate was analyzed for metabolic activity using HPLC with the same parameters as previously mentioned.

2.8. Data analysis of results

Data are shown as mean values with error bars representing the standard deviation. The data were analyzed by ANOVA, carried out using GraphPad Prism 9.5.0. The 2-way ANOVA comparison was carried out on simple effects within rows, comparing the different growth conditions for 1 day at a time. Tukey's post-hoc analysis was used. Only non-significant comparisons ($p > 0.05$) were annotated on graphs to prevent crowding of the results. All figures that are not photographs were prepared in GraphPad Prism 9.5.0. For tables, the significance was tested within the rows or the columns with the same parameters as for figures. Significance was visualized using the same letters ($p > 0.05$) or different letters ($p < 0.05$).

3. Results and discussion

3.1. Co-inoculation with lactic acid bacteria and bacterial analysis

The five fungal strains were inoculated on PDPA plates with and without one of two LAB strains (NFICC311 and NFICC188). On day 6, poor mycelial growth was observed on pure PDPA (pH 6.3), while growth was largest on laPDPA (pH 4.6) for all fungal strains (Fig. 1). *L. sulphureus* mycelium has previously been reported to grow well on plates at pH 6–8 (Luangharn et al., 2014), while others found more acidic conditions to be favorable for liquid cultures (Bergmann et al., 2022). Our results indicate that acidic conditions favor growth on agar plates. For *W. cocos*, a previous study found the optimal starting pH to be between 4 and 6 (Jo et al., 2016). The same study found that the mycelium of *W. cocos* generally grew well on various organic acids, including lactic acid. In the current work, mycelial growth was higher for most fungal strains when grown with NFICC311 compared to NFICC188, except for FH22 where no significant difference in growth was seen in the presence of either of the two LAB strains (Fig. 1A). These trends were also observed on day 8 after inoculation, where strains grown on PDPA plates performed poorly compared to the other media (Fig. 1B). *L. lactis* (NFICC188) is a non-obligate homofermentative organism with lactic acid as the primary metabolite (Cocaign-Bousquet et al., 1996). It has previously been shown to switch between homofermentative metabolism and mixed acid metabolism depending on carbon availability, oxygen level, and substrate composition (Cocaign-Bousquet et al., 1996). During mixed acid metabolism, less pyruvate is converted to lactic acid and more is converted to formic acid, acetic acid and ethanol (Garrigues et al., 1997). *L. mesenteroides* (NFICC311) is an obligate heterofermentative organism, known to produce ethanol, acetic acid and mannitol along with lactic acid (Koduru et al., 2017). Enhanced mycelial growth in the presence of *L. mesenteroides* could be due to faster acidification compared to *L. lactis*. To test this, the pH of PDPA plates was measured during 8 days of growth with the two bacterial strains (Fig. 2).

NFICC311 caused a much faster rate of acidification than NFICC188 and resulted in a much lower final pH at day 8 (4.2 compared to 5.0). Hence, the effects on mycelial growth in Fig. 1 could be due to pH changes, rather than production of metabolites. To gain more insight, liquid PDP was inoculated with the LAB and the concentrations of sugars and metabolites were determined by HPLC (Table 2).

NFICC311 consumed the most glucose and produced the highest total amount of acids. This was expected as NFICC311 had the fastest acidification (Fig. 2). No ethanol production was seen for NFICC188 and only a limited concentration of acetic acid was produced. The above results make it difficult to determine whether the effects of the LAB cultures on mycelial growth are due to simple pH effects or are due to the type of acid that is produced by the LAB strains. To examine this further, one strain of *W. cocos* (FH9) and two of *L. sulphureus* (FH24 and FH319) were cultivated with different acids.

3.2. Impact of pH and acid type

Mycelial growth of the three fungal strains, on different acid-containing agar plate media, was measured on day 6 and 8. All plates containing acid were adjusted to pH 4.3 before inoculation; PDPA plates had a pH of 5.9 (Fig. 3).

A strong correlation between an acidic start condition and growth for all strains was observed, except for plates containing acetic acid. Plates containing the non-organic acid HCl had the fastest mycelial growth, with completely overgrown plates after 6 days. Mycelia grown on plates containing lactic acid grew faster than on PDPA plates and the growth

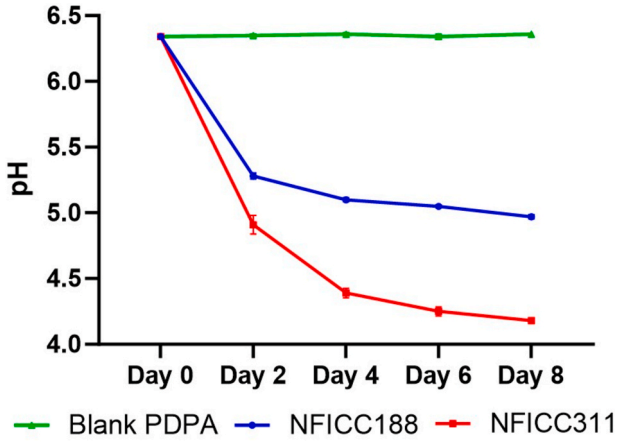

Fig. 2. Difference in acidification of PDPA plates by the two bacterial strains.

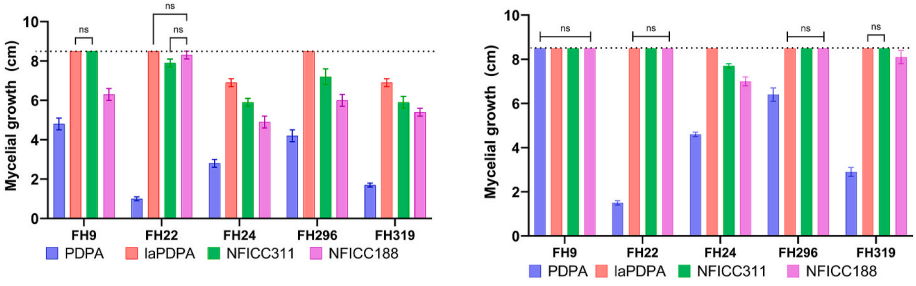

Fig. 1. Difference in mycelial growth of *W. cocos* (FH9 and FH22) and *L. sulphureus* (FH24, FH296 and FH319) in non-acidified media (PDPA), media acidified with lactic acid (laPDPA), and with LAB co-cultures NFICC311 (*L. mesenteroides*) and NFICC188 (*L. lactis*). A) Diameter of mycelium after 6 days growth. B) Diameter of mycelium after 8 days growth. The dotted line at diameter 8.5 cm, corresponds to a full agar plate. ANOVA results of non-significant ($p > 0.05$) comparisons within each strain are annotated with ns.

Table 2
Amounts of glucose and metabolites in PDP after growth of two LAB strains for 18 h. Letters a-c: A difference in letters indicates a significant difference between the two numbers in the same row. n.d. = not detected.

Metabolite	NFICC311 (*L. mesenteroides*)	NFICC188 (*L. lactis*)	Blank (PDP)
Glucose (g/L)	15.86 ± 0.22[a]	19.86 ± 0.08[b]	23.55 ± 0.12[c]
Lactic acid (g/L)	5.67 ± 0.13[a]	6.08 ± 0.06[b]	n.d.[c]
Acetic acid (g/L)	0.58 ± 0.16[a]	0.06 ± 0.01[b]	n.d.[b]
Ethanol (g/L)	2.39 ± 0.08[a]	n.d.[b]	n.d.[b]

rate was faster than for plates co-inoculated with LAB as observed before (Fig. 1). It is therefore likely that the fungal strain benefits from LAB acidifying the media, rather than a specific growth-promoting compound. The lowest growth was seen for plates containing acetic acid, which has previously been shown to be an inhibitor for fungal growth (Kang et al., 2003; Nair et al., 2018; Zinn & Bockmühl, 2020). Fungal strain FH24 showed some tolerance to the acetic acid plates compared to FH9 and FH319, which had no visible growth (0.6 cm was for the inoculation plug) on acetic acid after 8 days. This lack of growth of FH9 (*W. cocos*) is in contrast to what has previously been seen for other *W. cocos* strains, where acetic acid and lactic acid both promoted growth (Jo et al., 2016). However, they used a concentration of 0.1% acetic acid, where we have used a concentration of 0.5%. It is possible that *W. cocos* benefits from low amounts of acetic acid, but is inhibited at higher concentrations. NFICC311 (*L. mesenteroides*) is a known producer of acetic acid and it also produced acetic acid during the liquid fermentation. It is possible that the positive effect of acidification was greater than the inhibition from acetic acid. Interestingly, FH24 (*L. sulphureus*), which showed some tolerance to acetic acid, did not have better growth with FNICC311 (*L. mesenteroides*) than FH319 (*L. sulphureus*), which didn't exhibit acetic acid tolerance.

3.3. Effects on fungal growth in liquid media when co-cultured with LAB

The previous results indicated that co-cultivation of fungi with LAB could lead to increased mycelial growth, although the production of acetic acid from NFICC311 (*L. mesenteroides*) could be a limiting factor. In order to determine if this also applies in liquid media, co-cultivation was carried out in shake flasks (Fig. 4).

On day 8, the mycelia of FH9, FH24 and FH319 changed color depending on the growth conditions. When grown in PDP (pH 5.9) the mycelia for all three strains had a light brown/beige color. When grown with HCl (pH 4.2) the mycelia had a dark-brown color. This could correlate with the start pH where a lower pH could result in darker mycelium. FH9 showed a tendency to merge into one lump when grown with NFICC188 and NFICC311 (Fig. 4). To our knowledge, this has not yet been reported in other studies. This morphological change is interesting, as the merging could be a defensive mechanism for the mycelium, or to retain a specific environment at the core. For FH24, the mycelium was lightest with NFICC311 and darker with NFICC188. Barely any mycelium was collected when FH24 was grown in PDP only. FH319 had an orange color when grown with NFICC188 and a dark-beige color when grown with NFICC311. Other fungal species have previously shown changes in colorant production during growth at different pH (Blechert et al., 2019; Lagashetti et al., 2019). However, the orange color change for FH319 grown with NFICC188 did not seem

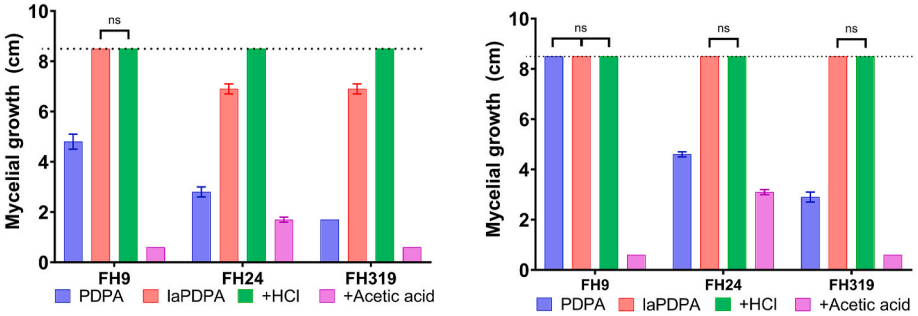

Fig. 3. Mycelial growth of *W. cocos* (FH9) and *L. sulphureus* (FH24 and FH319) on agar plates with different acids. The dotted line is at a diameter of 8.5 cm, corresponding to a full agar plate. ANOVA results of non-significant comparisons ($p > 0.05$) within each strain are annotated with ns. A) Average mycelium diameter after 6 days of inoculation. B) Average mycelium diameter after 8 days of inoculation.

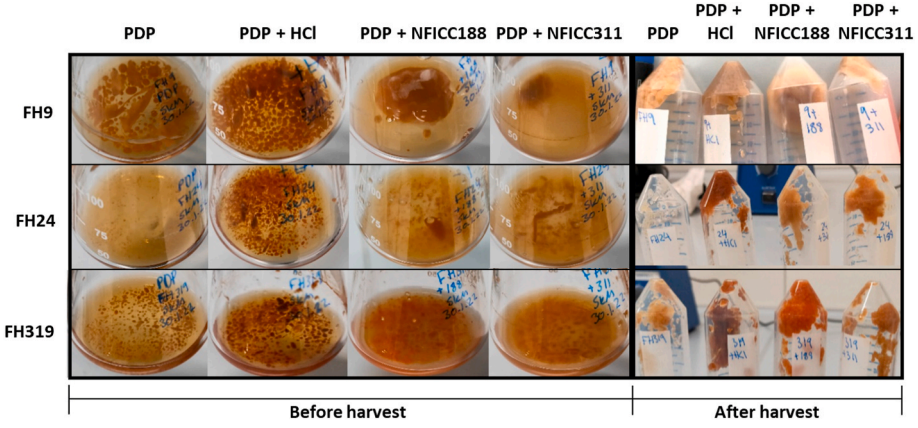

Fig. 4. Morphological differences of *W. cocos* (FH9) and *L. sulphureus* (FH24 and FH319) cultivated in shake flask for 8 days (left hand panel) and after harvesting and centrifugation (right hand panel). Inoculation medium used in both panels was, from left to right: PDP, PDP + HCl, PDP + NFICC188, PDP + NFICC311.

directly related to pH, as the final pH of FH319 with HCl was lower (Fig. 5B). The orange color could be a result of FH319 utilizing, or responding, to a compound produced by NFICC188. Multiple conjugated carboxylic acids of different chain lengths, produced under acidic conditions (pH 3), have previously been reported to be responsible for the orange color of *L. sulphureus* (Bergmann et al., 2022). It is possible that FH319 is using the lactic acid as a carbon source for producing this colorant, as no orange color was observed for HCl acidified cultures.

The pH of the liquid cultures and the wet weight of the mycelia were measured on day 8 (Fig. 5).

An increase in mycelial biomass and a decrease in final pH was seen when FH9 was grown with HCl or NFICC188. When FH9 was grown with NFICC311, the mycelial weight decreased as compared to growth on PDP (Fig. 5A). This could be due to the higher concentration of acetic acid produced by NFICC311 which could inhibit growth, as was seen for plates containing acetic acid (Fig. 3). While NFICC311 increased growth on plates, the inhibitory effect in liquid cultures could be due to an increase in acetic acid produced by the bacteria, which have access to more carbohydrates. On agar plates, the bacteria are restricted to growing at their inoculation spot causing a local depletion of carbohydrates. Growth inhibition was also indicated by the observation that pH only decreased to 3.7, rather than close to 2, as was seen for other cultivations (Fig. 5B). The highest mycelial growth was found when FH9 was grown with NFICC188. This combination also resulted in the lowest pH, which was slightly below 2. This again indicates that the inhibited growth, seen with NFICC311, could be due to acetic acid, as less of this acid was produced with NFICC188 in liquid cultures (Table 2). These results indicate that NFICC311 could potentially be useful for solid-state fermentation or production of fruiting bodies of *W. cocos* as it promotes mycelial growth on plates, but would not be beneficial for growth in liquid fermentations. Mycelia in liquid fermentation could benefit from being cultivated with NFICC188, which promoted growth even when compared with pre-acidified media.

The amount of biomass seemed to correlate with the final pH; the more biomass produced, the lower pH was measured. This was also seen for FH24 and FH319 (Fig. 5). For FH24 the highest mycelial weight was found when grown with HCl and a non-significant ($p > 0.05$) lower growth was seen when grown with NFICC188 (Fig. 5). Increased growth was also seen when co-cultured with NFICC311, which could be due to the acetic acid tolerance seen in the acidic plates (Fig. 3). However, the growth was almost identical to that of FH319 with NFICC311, which didn't exhibit acetic acid tolerance. The least biomass was observed for FH24 alone. For FH319 the highest growth was found when grown with NFICC188 and HCl, while growth with NFICC311 was similar to that in non-acidified media. All three fungal strains showed the best growth when the medium was acidified, although none preferred co-culture with NFICC311. Both FH9 and FH319 showed enhanced growth when co-cultured with NFICC188, while FH24 had a small preference for HCl acidification over co-culturing. The results show a promising possibility for growing *W. cocos* and *L. sulphureus* with strains of *L. lactis* for increasing fungal biomass production without adding additional chemicals to the fermentation.

3.4. Co-cultivation of LAB and fungi on bi-plates

Plates with separated agar were used to screen for interactions, which could be related to volatile compounds. The growth of fungal mycelium was measured on day 2, 4, and 8 (Fig. 6).

After two days, the mycelial growth increased for FH9 when grown in the presence of NFICC311, and decreased when grown near NFICC188 (Fig. 6A). After four days the NFICC311 containing bi-plates had grown to the maximum diameter possible (8.0 cm), while bi-plates with NFICC188 had similar growth to bi-plates without LAB. Interestingly, the mycelium was found to trespass the physical middle barrier, although no growth media was present on the barrier. The same trend was observed for FH24, although the mycelium grew slower for this strain (Fig. 6B). For FH24, the mycelial growth continued to be highest on bi-plates containing NFICC311. For FH319, a significant increase in growth was seen on day 4 in the presence of both LAB strains (Fig. 6C). This was further increased on day 8.

These results indicate possible production of a growth stimulating volatile compound by both LAB strains. FH9 and FH24 was further used for detecting volatiles when grown in the presence of the two bacterial strains. They were chosen since both had a significant increase in growth with NFICC311 compared to NFICC188. Microscope pictures were taken to assess changes of the mycelial structure when the strains were grown alone and with bacterial strains (Appendix figures A4 and A5).

3.5. Measurement of volatile compounds

Volatile compounds were measured with HS-GC-MS for bi-plates containing FH9 or FH24 and LAB. The background volatiles were measured with non-inoculated PDPA plates and controls of pure cultures were measured with each fungal and bacterial strain. Seven VOCs were found to vary between samples and they were identified with authentic reference standards. Heat maps were generated for the peak area of each compound (Fig. 7).

Methyl 3-methylbutanoate (Fig. 7A) was detected almost exclusively in samples containing FH9. The level detected increased when grown with NFICC311 and it decreased when grown with NFICC188. The amount increased to a maximum on day 4 with NFICC311, while increased mycelial growth was measured already on day 2 (Fig. 6). On day 4 the fungal mycelium had started to cross the barrier to the bacterial side (Appendix figure A6). Methyl-3-methylbutanoate is known to have antifungal properties (Gong et al., 2019; Hummadi et al., 2022). It is possible that FH9 expressed this compound as a defensive reaction to

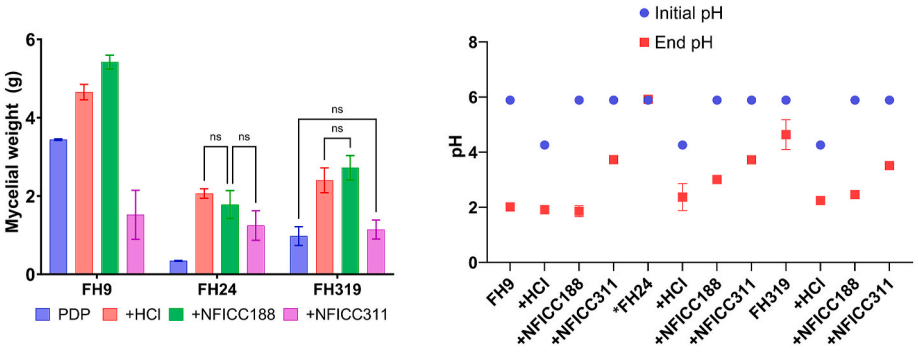

Fig. 5. Mycelial wet weight (A) and pH change (B) of *W. cocos* (FH9) and *L. sulphureus* (FH24 and FH319) after 8 days of fermentation in liquid cultures. The dark blue dots are initial pH after inoculation, and the red squares are pH after 8 days of fermentation. Red error bars indicate SD for end pH. *For FH24 no growth was observed, leaving the end pH data point in the same place as the start pH data point. ANOVA results of non-significant comparisons ($p > 0.05$) are annotated with ns. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

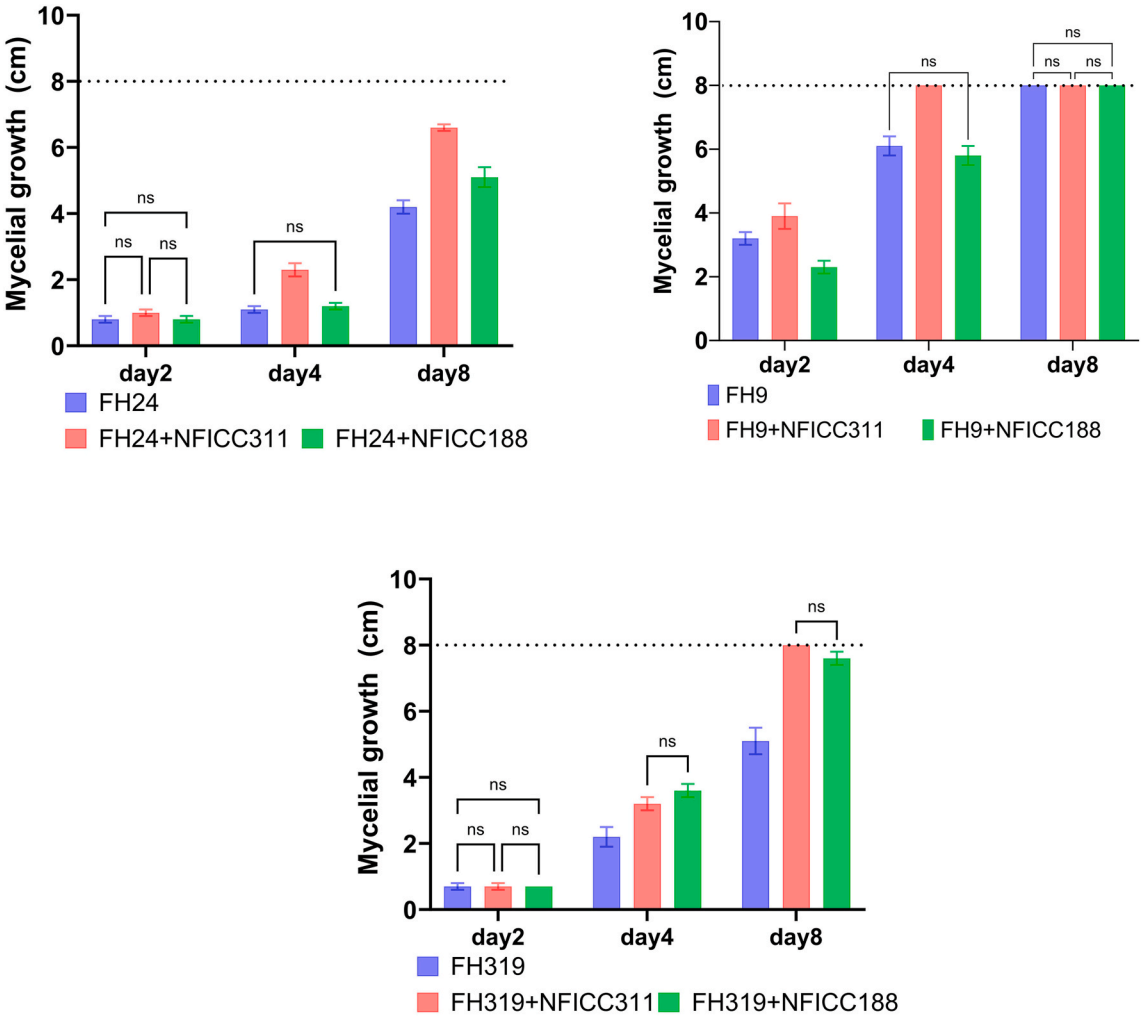

Fig. 6. Mycelial growth of *W. cocos* (FH9) and *L. sulphureus* (FH24 and FH319) in the presence of bacterial strains. The dotted line is at diameter 8.0 cm, corresponding to a full bi-plate. ANOVA results of non-significant ($p > 0.05$) comparisons are annotated with ns. A) Mycelial growth for FH9. B) Mycelial growth for FH24. C) Mycelial growth for FH319.

the production of acetic acid.

2-Heptanone was detected in bacterial samples from day 0 to day 4 in very low amounts and for FH9 only on day 0 in a low amount (Fig. 7B). For FH24 a low amount was observed on day 8, but higher levels were measured when FH24 was grown with NFICC311 and NFICC188. 2-Heptanone is known to be produced in LAB (Gallegos et al., 2017), but to our knowledge it has not been found in *L. sulphureus*. 2-Heptanone has been shown to have an inhibitory effect against different molds when present in the headspace (Calvo et al., 2020). FH24 grew faster with LAB already from day 4, while 2-heptanone was only detected from day 6. On day 6 the fungal mycelium had crossed the middle barrier to the bacterial side (Appendix figure A7).

β-Myrcene was only observed in FH9+NFICC188, where the amount increased from day 4 to day 8 (Fig. 7C). This monoterpene has been found in Basidiomycota including *Schizophyllum commune* (Orban et al., 2023), *Fomes fomentarius* (Fäldt et al., 1999) and *Amanita ovoidea* (Breheret et al., 1997) but not in bacteria. This makes it likely that *W. cocos* produce β-myrcene as a protective compound against NFICC188, since β-myrcene can inhibit bacteria and fungi (Połeć et al., 2020). A previous study found no inhibitory effects of β-Myrcene on brown rot fungi, which could indicate an adaption to this compound's presence (Väisälä, 1974). β-Myrcene can be derived from linalool (Burdock, 2010; Mirata et al., 2008), which was also detected in the highest concentrations in cultures of FH9+NFICC188.

Benzoic acid was only found in samples containing FH24 (Fig. 7D). Its concentration increased in samples with bacterial strains. *L. sulphureus* is known to produce benzoic acid (Nowacka et al., 2014). Benzoic acid is commonly used in foods as a preservative as it harbors antibacterial and antifungal properties (Ogbadu, 2014). Since the compound was detected on day 4 and increased subsequently when grown near bacterial strains, it is likely to be a reaction from FH24 mycelium coming into contact with the LAB. As benzoic acid is most active at low pH (Ogbadu, 2014), it could also be a sign that the pH of the media had reached a level where the compound would be active.

Acetoin was found in samples containing NFICC188 and it was highest when NFICC188 grew alone (Fig. 7E). *L. lactis* has previously been found to be able to produce acetoin (García-Quintáns et al., 2008), being a common metabolite of homofermentative LAB (Gänzle, 2015). The detection of acetoin was highest on day 2 and slowed down afterwards. This indicates again that NFICC188 was slowing down growth activity after day 2 and became less productive. While acetoin production was affected by both fungal strains, a stronger reduction was observed when grown with FH9. As linalool and β-Myrcene were detected on the same culture day, it is possible that one of these induced the change.

Acetic acid was found in large concentrations in all co-fermentations containing NFICC311, with the highest amounts in the sample containing NFICC311 only (Fig. 7F). NFICC188 also showed production of

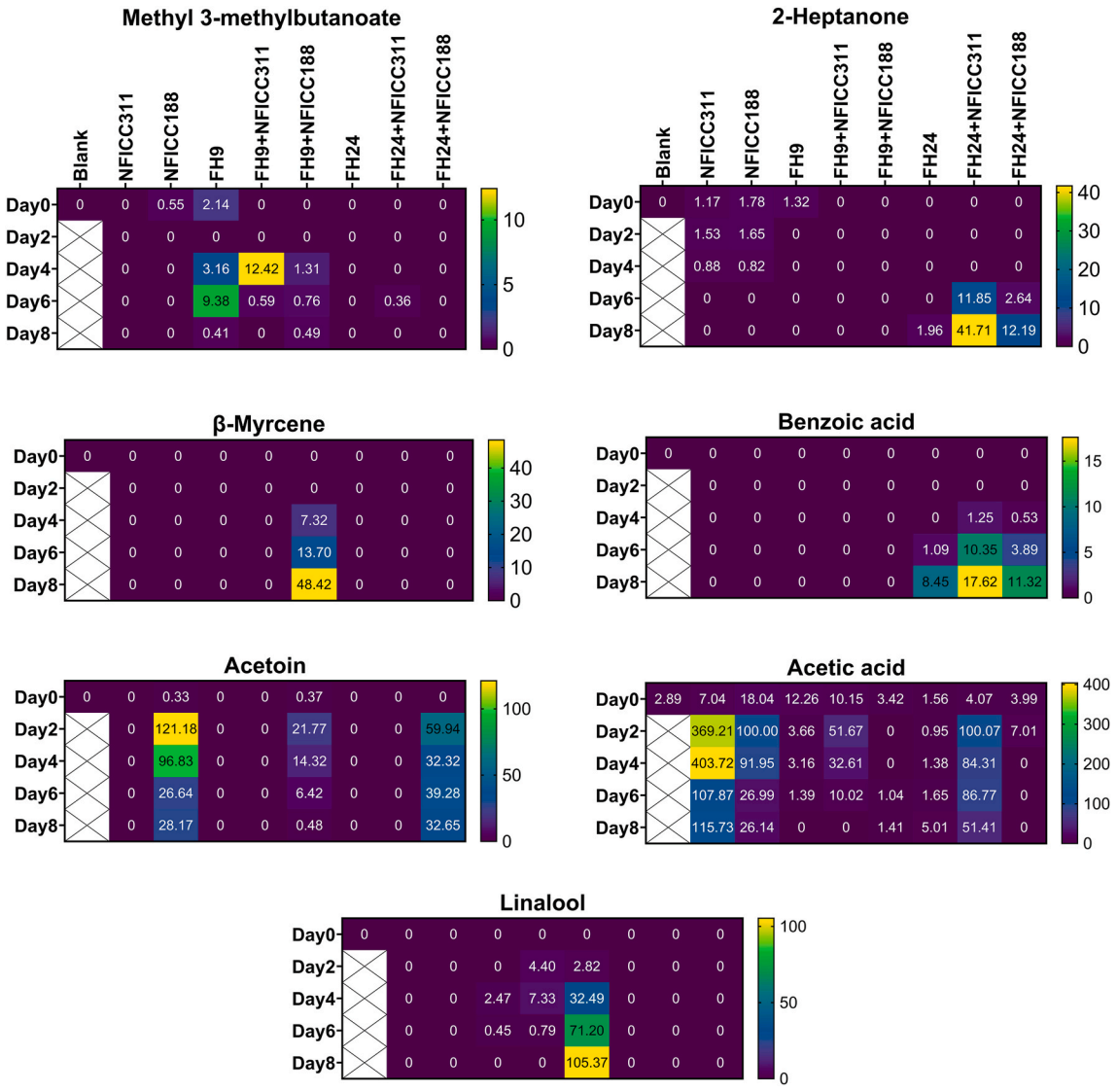

Fig. 7. Heat maps of the seven interesting volatile compounds found in headspace of bi-plates. Numbers represent 10^5-fold peak area averages of triplicates. Detections of A) Methyl 3-methylbutanoate, B) 2-Heptanone, C) β-Myrcene, D) Benzoic acid, E) Acetoin, F) Acetic acid, G) Linalool.

acetic acid when grown alone. Most other samples had amounts comparable to the blank. Homofermentative bacteria such as NFICC188 are known to be able to produce two molecules of acetate and one molecule of acetoin or di-acetyl per molecule of glucose (Gänzle, 2015). Together with the results found for acetoin, it is likely that NFICC188 changed its metabolism as a response to the fungus growing. It is possible that the strain started to produce more lactic acid as a protective measure to lower the pH, as acetate and acetoin formation has previously been shown to be a way for *L. lactis* to prevent acidification (Gaudu et al., 2003). This could also explain the low acidification when grown alone (Fig. 2), as NFICC188 would produce more acetoin and acetate. *Lc. mesenteroides* is usually producing more acetic acid than *L. lactis* (Table 2). It is however normal that *L. lactis* produces more acetic acid under aerobic conditions than during more limited access to air in liquid cultures (Gänzle, 2015).

Linalool was only found in samples with FH9 and in the highest concentration in co-cultures of FH9+NFICC188 (Fig. 7G). Linalool detection has previously been reported in *W. cocos* (Sommer et al., 2021; Wang et al., 2020) and could be produced as a defensive compound by the fungus as its antibacterial properties have previously been demonstrated (Gao et al., 2019). Notably, linalool production was only increased to high concentrations, when the strain was grown with NFICC188 and not with NFICC311. The compound was detected in low amounts on day 2 and increased subsequently. At day 4 the fungal strain had just entered the bacterial side of the barrier, which could explain the increase in linalool as an attack on the bacterial strain (Appendix figure A6). As discussed earlier, the presence of linalool seemed to coincide with the disappearance of acetoin and acetic acid for NFICC188. It was decided to test whether linalool could induce this change in NFICC188 metabolites.

3.6. *Effects of linalool on NFICC311 and NFICC188*

The MIC and ¼ MIC were found to be 5 μg/mL and 1.25 μg/mL on PDPA plates, respectively. To test whether linalool changed the production of metabolites in NFICC188, both bacterial strains were grown in liquid PDP containing MIC and ¼ MIC of linalool (Table 3).

NFICC311 and NFICC188 were both consuming similar amounts of sugars and producing high amounts of lactic acid and some acetic acid in PDP + water and in PDP +1% EtOH media (Table 3). When grown in media containing the MIC linalool concentration, the strains did not produce any lactic or acetic acid and sugar concentrations were similar to the blank. At a ¼ MIC linalool concentration, NFICC311 did not produce lactic acid and had similar sugar concentrations to the blank.

Table 3
Sugar and metabolite concentrations of NFICC311 and NFICC188, when grown in liquid PDP media for 48 h, containing MIC or ¼ MIC of linalool. Letters a-g: A difference in letters indicates a significant difference between the two numbers in the same row. n.d. = not detected.

Sample	Glucose (g/L)	Fructose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)
PDP + water					
Blank (media)	19.46 ± 0.04[a]	1.44 ± 0.02[a]	0.233 ± 0.01[a]	0.168 ± 0.00[a]	n.d.[a]
NFICC311	16.92 ± 0.13[b]	0.74 ± 0.07[b]	3.297 ± 0.20[b]	0.219 ± 0.00[a]	0.10 ± 0.00[a]
NFICC188	16.36 ± 0.09[c]	1.18 ± 0.02[c]	3.829 ± 0.15[c]	0.179 ± 0.00[a]	n.d.[a]
PDP + 1% EtOH					
Blank (media)	19.36 ± 0.02 [ad]	1.44 ± 0.01[a]	0.228 ± 0.01[a]	0.169 ± 0.00[a]	10.80 ± 0.13[bd]
NFICC311	16.96 ± 0.06[b]	1.11 ± 0.00[c]	3.166 ± 0.10[b]	0.201 ± 0.00[a]	10.76 ± 0.01[bd]
NFICC188	16.29 ± 0.07[c]	1.09 ± 0.01[c]	3.886 ± 0.05[c]	0.174 ± 0.00[a]	10.73 ± 0.01[bd]
PDP + 1% EtOH + MIC Linalool					
Blank (media)	19.42 ± 0.01[a]	1.45 ± 0.01[a]	0.230 ± 0.01[a]	0.171 ± 0.00[a]	10.34 ± 0.01[c]
NFICC311	19.26 ± 0.02[d]	1.42 ± 0.02[a]	0.223 ± 0.01[a]	0.172 ± 0.00[a]	10.24 ± 0.02[c]
NFICC188	19.27 ± 0.09[d]	1.42 ± 0.01[a]	0.224 ± 0.01[a]	0.174 ± 0.00[a]	10.24 ± 0.06[c]
PDP + 1% EtOH + ¼ MIC Linalool					
Blank (media)	18.81 ± 0.04[e]	1.40 ± 0.01[a]	0.231 ± 0.01[a]	0.168 ± 0.00[a]	10.81 ± 0.02[b]
NFICC311	18.61 ± 0.03[f]	1.38 ± 0.02[a]	0.234 ± 0.01[a]	0.169 ± 0.00[a]	10.67 ± 0.02[d]
NFICC188	16.01 ± 0.22[g]	1.21 ± 0.01[c]	3.392 ± 0.03[d]	0.169 ± 0.00[a]	10.69 ± 0.02[bd]

NFICC188 had similar consumption of sugars and production of lactic acid as for the PDP + water and PDP +1% EtOH media. However, no acetic acid was produced, when grown with ¼ MIC linalool. This is in accordance with the GC-MS results seen for NFICC188. Linalool was detected on day 2 for FH9, growing with NFICC311 and NFICC188. As NFICC311 did not grow at a ¼ MIC of linalool, it is possible that this strain is more sensitive to linalool than NFICC188. This could be the reason why linalool was detected in higher concentrations for NFICC188 than for NFICC311.

4. Conclusion

In this study, we investigated the effect of two LAB strains on the mycelial growth of *L. sulphureus* strain FH24 and *W. cocos* strain FH9. The two fungal species are known for having edible fruiting bodies and their mycelia are a promising candidate for novel food production. The co-inoculation of the two LAB promoted growth of the fungal strains, which could be partly explained by the acidification of the media. Acidified plates all promoted growth, except for plates containing acetic acid. Fungal strains grown on acetic acid were completely inhibited, except for FH24, which was more resistant. Our findings suggest that *W. cocos* strain FH9 and FH22 and *L. sulphureus* strain FH24, FH296 and FH319 benefit from growing in acidified environments on plates and in liquid cultures. For liquid cultures, the fungal strains benefitted most from growing with the homofermentative LAB, which could be due to the ethanol and acetic acid produced by the heterofermentative LAB. FH319 exhibited a change in color when grown with NFICC188, which wasn't observed on HCl-acidified media. Both LAB strains also induced a morphological change in FH9 when grown in liquid cultures. The mycelium merged into one lump, which wasn't seen when grown on HCl. Future studies should look into the metabolites responsible for these morphological changes and whether other LAB strains could induce them as well. No unknown volatiles were found which had growth-promoting effects on the fungal strains. NFICC311 had the strongest growth-inducing effect on bi-plates, but the only volatile compound found in abundance when co-cultured was acetic acid. The fungal volatile compound linalool was found to inhibit both bacterial strains when added in high concentrations and to change the metabolomics of NFICC188, when added in lower concentrations. Our results show that both strains of *Lc. mesenteroides* and *L. lactis* are promising organisms for co-fermentation with *L. sulphureus* strain FH24 and *W. cocos* strain FH9 on solid media and that *L. lactis* strain NFICC188 is promising for co-culture on liquid media. The results are promising for future production of mycelium for food and medicine purposes.

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CRediT authorship contribution statement

Sanne Kjærulf Todorov: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Axel Orban:** Formal analysis, Investigation, Writing – review & editing. **Andreas Hammer:** Formal analysis, Investigation, Writing – review & editing. **Markus Oberpaul:** Formal analysis, Investigation, Writing – review & editing. **Christopher Back:** Formal analysis, Investigation, Writing – review & editing. **Celia L. Jansen:** Formal analysis, Investigation, Writing – review & editing. **Timothy John Hobley:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Martin Rühl:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Claus Heiner Bang-Berthelsen:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.115891.

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