The effect of cow's milk on biomass growth in *Schizophyllum commune.*

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Abstract

Fungi have long been used in a wide variety of industries. In recent years, there has been renewed interest as the demand for bio-based materials increases. Here, cow's milk was used as a growth medium for model species *Schizophyllum commune* in liquid shaking cultures. The addition of cow's milk showed a 3.0- to 3.4-fold increase compared to conventional growth medium. Harvested cultures were analysed using a combination of FTIR spectrometry, Bradford protein assays, SDS-PAGE and TLC to assess the mechanisms underlying the increased production of biomass. Results show *S. commune* is able to utilise most carbohydrates, proteins and part of the lipids present in milk.

Contents

Layman summary

Fungi have been used in the bioindustry for a long time. They help produce a variety of products such as medication, food-additives, biofuel and packaging materials. Learning how these fungi grow can help with the development and improvement of sustainable products. One of the fungi used in both the bioindustry and research is the wood-rot fungus *Schizophyllum commune*. It is commonly found all over the world, and can be easily grown under laboratory conditions, unlike many other fungi.

Here, two strains of *S. commune* were grown in submerged cultures, either in regular growth medium which contained glucose as the main food source, or in medium supplemented with cow's milk instead of glucose. Cow's milk contains large amounts of nutrients, mainly sugar in the form of lactose, proteins such as caseins, and fats. The addition of milk caused the fungus to grow up to three times more biomass compared to the conventional medium without milk. Samples were collected over 21 days from both the fungus and the medium. The samples were analysed using Fourier Transform Infra-red spectroscopy, a technique which uses infra-red light to identify what compounds are present in the fungus as well as the medium. This showed that the main components remaining in the milk medium were proteins.

To see the concentration of proteins remaining in the milk medium it was measured using a Bradford assay. This is a technique where a chemically active dye is added to samples causing them to turn blue when proteins are present. The amount of blue colouration was measured and compared to the milk medium before growth of the fungus. This showed that 95% of all proteins in the milk medium had been consumed. Since proteins were the main component remaining, this meant most of the lipids and sugars had also disappeared. To see what kind of proteins remained, SDS-PAGE was used. This technique makes use of the electrically charged nature of proteins to separate them depending on their mass. This showed that caseins, which make up the main component of milk proteins, where no longer detectable. In addition, a variety of new proteins were seen in the milk after the fungus had grown.

The spectroscopy measurements also showed that most of the fats disappeared from the milk but showed up in the fungus samples. To take a closer look at this, Thin Layer Chromatography was used, a chemical technique that can separate and visualise different types of fats depending on specific properties. This confirmed that there were no fats remaining in the medium after 7 days of growth. In addition, it visualised more fats in the samples taken from the fungus grown in the milk medium compared to those grown in the standard medium. This indicated that not all the lipids were consumed by the fungus.

Here it is shown that *S. commune* consumed most of the nutrients available in cow's milk, causing a threefold increase in biomass compared to growth in standard medium. Understanding how the fungus used these nutrients helps improve current growth methods, which can be of use to applications in both science and industry.

Introduction

Our current textile and leather industries are considered to be inextricably linked to resource depletion and waste accumulation (Parisi *et al.*, 2015; Joseph & Nithya, 2009)[34,18]. Looking to provide these industries with renewable and sustainable alternatives, interest in fungal production systems has risen markedly in recent years. Aside from their invaluable role as part of circular degradation in nature, fungi hold great value for our economy producing food, pharmaceuticals, chemicals and other compounds (Grimm & Wösten, 2018)[10]. Additionally, fungal mycelium is well suited for the production of sustainable materials such as packaging and insulation, as well as textile and leather replacements (Jones *et al.*, 2021)[16].

Basidiomycete *Schizophyllum commune* is a mushroom-forming white-rot fungus with a brown leathery fruiting body (Alexopoulos *et al.*, 1996)[2]. It is known to grow on the dead and decaying plant matter of over 150 genera of woody plants, but can also utilise substrates such as grass and silage (de Jong, 2006)[17]. One of the most commonly found fungi, it has a broad range of viable habitat and is distributed across every continent with the exception of Antarctica. *S. commune* is widely studied and considered a model organism due to its ease of cultivation in a research environment and there are a variety of molecular tools and databases available, including complete genomic sequences (Ohm *et al.*, 2010)[31]. While *S. commune*'s ability to break down cellulose and lignin based substates is extensively documented (Tovar-Herrera *et al.*, 2018; Zhu *et al.*, 2016; Schmidt & Liese, 1980)[41,45,37], little is known about its ability to produce lipases, with primary documentation being lipase homologs found during genomics research (Singh *et al.*, 2014; Kam *et al.*, 2017; Ohm *et al.*, 2014)[39,19,32]. There has been an increasing interest in lipases in recent years, as demand for sustainable alternatives such as biopolymers and biodiesel rises (Jaeger & Eggert, 2002)[14]. For research purposes, *S. commune* is generally grown in solid or liquid cultures using defined *Schizophyllum commune* minimal medium (SCMM) (Dons *et al.*, 1979)[5]. Stemming from 1979, the use of SCMM is prevalent within the scientific community, with little or no changes made to the original formulation. While suited for small batch experiments, production of large quantities of SCMM can quickly become quite costly. As the fungal bioindustry continues to expand, the demand for scalable fermentation systems increases. This in turn has caused an intensified search for cheap, renewable substrates to serve as energy sources for these systems.

In many parts of the world, milk has long been a staple food of the human diet, containing a wealth of essential nutrients. Cows and other mammals have been domesticated for the main purpose of milk production for millennia. Today, the dairy sector is a massive industry, and long-term global projections expect annual milk production to reach 959 million liters by 2028, growing 1.7% per year, faster than most other agricultural products (OECD/FAO, 2022)[30]. Milk is considered a colloidal suspension

containing emulsified lipid globules. The most abundant compounds of cow's milk at a macronutrient level are water (85-87%), lipids (3.8-5.5%), proteins (2.9-3.5%) and carbohydrates (5%). In addition to this, milk contains a wide variety of micronutrients in the form of oligosaccharides, immunoglobulins, amines, organic acids, vitamins, and minerals (Fox *et al.*, 2015)[7]. The composition of cow's milk is dependent on a variety of factors such as genetics of the breed and the individual, the stage of lactation, the health of the cow and environmental conditions such as the climate, feed and terrain (Huppertz & Kelly, 2009)[13]. Despite high industry standards and government administration guidelines, considerable variation remains depending on factors such as country, brand and post-processing due to its organic origin. Initial findings have shown the addition of cow's milk to SCMM to be highly effective in stimulating mycelial growth for *S. commune*. The addition of cow's milk to liquid cultures was also seen to have an inhibitory effect on the fungus' ability to produce schizophyllan, an extracellular β-(1-6)-branched β-(1-3)-glucan secreted by *S. commune* when grown in submerged cultures. Schizophyllan has long been of interest to both research and industry communities due to its material and immunomodulating properties (Zhang *et al.*, 2013)[44]. The exact function of this polysaccharide to the fungus is not fully known, although there are indications it is connected with formation of the fungal cell wall (van Wetter *et al.*, 2000)[43].

The aim of this research is to gain more understanding of the properties and effects of cow's milk when used as a medium for the growth of the filamentous basidiomycete *S. commune*, in the search for a sustainable low-cost alternative to conventional growth media. Biomass and spent medium of *S. commune* strains H4-8A and H4-39 grown in liquid shaking cultures containing cow's milk was harvested and analysed using Fourier transform infrared (FTIR) spectroscopy, Bradford protein assays, SDS-PAGE and thin-layer chromatography (TLC). *S. commune* was found to utilise carbohydrates, proteins and part of the lipid contents of milk to produce up to 3.4 times higher biomass compared to defined media.

Materials & Method

Strains and culture conditions

Monokaryon *Schizophyllum commune* strains H4-8A (FGSC 9210) and H4-39 (FGSC 9216) were provided by the UU Fungal Microbiology research group. Stocks were cultivated in 55 mm Petri dishes containing 10 mL SCMM and 1.5% (w/v) agar. Cultures were grown for 7 days at 30° C in the dark, cut into 50 mm cubes and stored in cryogenic Eppendorf tubes at -80° C for future use. Three different media were used for experiments, SCMM, whole milk medium (WM) and skimmed milk medium (SM). SCMM contains 2% (w/v) glucose as a carbon source, and 0.15% L-asparagine and 0.012% (w/v) thiamine-HCl as nitrogen sources (Dons *et al.*, 1979)[5]. WM and SM consist of a 1:1 ratio 2x SCMM without glucose, and whole milk or 0% fat milk (Albert Heijn, The Netherlands), respectively. 50 mL

tubes (Greiner AG, Kremsmünster, Austria) containing 20 mL SCMM were inoculated using one cube of -80° C stock and grown for 5 days at 30° C and 50 rpm in the dark. Cultures were moved to blender cups along with 80 mL SCMM and macerated for 30 seconds at 18.000 rpm using a Waring Blender (Waring Laboratory, Torrington, England). Contents of the cups was transferred to 250 mL Erlenmeyers and grown for 24 h at 30° C and 200 rpm in the dark. Next, precultures were once more macerated for 30 seconds at 18.000 rpm, and biomass wet weight per volume was determined before inoculation of experimental units.

Biomass & growth measurements

250 mL Erlenmeyer flasks containing 100 mL SCMM or WM were inoculated with strains H4-8A and H4-39 preculture biomass amounting to 0.1% (w/v) wet weight and grown in duplicate for 4, 7, 10, 14 and 21 days at 30° C and 200 rpm in the dark. Biomass and spent medium were separated by vacuum filtration through Miracloth (Merck Millipore, Burlington, Massachusetts) using a 11 cm Ø Büchner funnel connected to a Divac 1.2 L vacuum pump (Leybold, Cologne, Germany). Filtrate was collected and its pH measured using a C1000 pH meter (Consort, Turnhout, Belgium), then homogenised by sonication for 10 seconds at 50% output using a Sonifier 450 (Branson, Danbury, Connecticut) and stored in aliquots at -80° C. Mycelium was washed by filtering 750 mL tap water through the Büchner funnel containing the residue, then transferred to pre-weighed Petri dishes and dried at 60° C overnight after which measurements were taken using an Entris II precision balance (Sartorius AG, Göttingen, Germany). Mycelium was stored in closed plastic bags at room temperature.

Fourier Transform Infrared spectroscopy

Mycelium samples were taken in triplicate from different parts of stored biomass. For day 0 samples, mycelium of precultures was filtered before the second maceration step and dried at 60° C overnight. Aliquoted frozen medium was first lyophilised using a freeze dryer (Ilshin BioBase, Gyeonggido, South Korea) before sampling. To account for possible influence of spent preculture medium added during inoculation of experiments, day 0 medium samples were obtained by adding filtered spent preculture medium to unspent medium. The volume of spent preculture medium added was determined by taking the average volume used to inoculate experimental units: 5% and 7.5% (v/v) for H4-39 and H4-8A preculture medium, respectively.

To prepare samples for FTIR analysis, 1 mg of mycelium or 2 mg of medium sample was added to 250 mg KBr (FT-IR grade, ≥99% trace metals basis) and dried for 24 h at 60° C. Samples were ground down using mortar and pestle and pressed into pellets (1.5 m, 8 t) using a manual press (Specac, Orpington,

England). Measurements were taken at a wavenumber range of 4000 to 400 cm^{-1} with 1 cm^{-1} wavelength steps using a Frontier spectrometer (PerkinElmer, Waltham, Massachusetts). Light absorption values for each wavelength were graphed and divided into wavenumber (ν) ranges corresponding to different bonds. Signal strength for each wavenumber range was calculated as the total area under the curve within the range, then subtracting the trapezoid area below the graph:

$$Signal\ strength = \left[\sum_{i=a}^{b} \frac{1}{2}(Y_i + Y_{i-1})\,(X_i - X_{i-1})\right] - \frac{1}{2}(Y_a + Y_b)(X_a - X_b)$$

Where *a* and *b* are the first and last wavenumber in each range. Relative signal strength was calculated for each range as a percentage of the total relevant signal range (Sup. Table 3). Due to the sizes of the wavenumber ranges chosen, some signal strengths calculated resulted in values below 0. To avoid underestimation of the other signals, negative signal strengths were treated as 0.

Bradford assay

S. commune strain H4-39 was grown in 250 mL Erlenmeyers containing 100 mL SCMM, WM or SM for 7 days at 30 ° C and 200 rpm in the dark. Spent medium was filter separated using Miracloth (Merck Millipore, Burlington, Massachusetts) using a 11 cm Ø Büchner funnel connected to a Divac 1.2 L vacuum pump (Leybold, Cologne, Germany), then homogenised by sonication for 10 seconds at 50% output using a Sonifier 450 (Branson, Danbury, Connecticut) and snap-frozen to minimise protein degradation using liquid nitrogen. Immediately preceding the assay, samples were thawed at room temperature and a series of dilutions was made using physiological saline solution (PSS). 100 µL of each sample and equal volume of Bradford reagent (Bradford, 1976)[4] was added to 96-well plates and homogenised by up-and-down pipetting. A calibration curve was created using Bovine Serum Albumin (BSA) standard (Thermo Fisher, Waltham, Massachusetts). PSS was used as a blank, and SCMM -Glc as negative control. Mixtures were allowed to rest for 5 minutes before absorbance was measured at 595 nm using a Synergy HT plate-reader (BioTek, Winooski, Vermont).

SDS-PAGE

Samples were obtained as described for the Bradford assay. Samples were thawed at room temperature immediately preceding SDS-PAGE experiment, vortexed for 5 seconds at 1500 rpm using a mini vortex mixer (Yu Shuo Da, Liaoning, China) and added in a 1:1 ratio to 2x Laemmli buffer (Laemmli, 1970)[24]. Samples and buffer were homogenised by vortexing for 5 seconds at 1500rpm, then immersed in boiling water for 2 minutes to completely dissociate proteins before loading onto gel.

Mini-PROTEAN Tetra Cell systems (Bio-Rad, Hercules, California) were filled with premade SDS Buffer (Bio-Rad, Hercules, California) containing 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS at pH 8.3. Gels were hand cast and consisted of a 4.5% stacking gel and 12% running gel and run for 1.5 hours at 100 V. Gels were stained overnight using staining solution containing 1% (w/v) colloidal Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid and destained using a 25% (v/v) methanol, 7.5% (v/v) acetic acid solution. Gels were visualised using a GelDoc imaging system (Bio-Rad, Hercules, California).

Lipid extraction

Lipid extraction protocol was adapted from Li *et al.*, 2021[25]. Cultures were grown as described for the growth and biomass measurements. Immediately after filtration, all samples were lyophilised using a freeze drier (Ilshin BioBase, Gyeonggido, South Korea). 0.25 mg of each sample was added to cryogenic microtubes together with 100 µg 0.5 mm Ø glass beads (QIAGEN, Hilden, Germany) and 500 µL extraction solution consisting of 2:1 (v/v) chloroform-methanol. Mechanical lysis was performed for 60 seconds using a TissueLyser II bead mill (QIAGEN, Hilden, Germany). Samples were centrifuged for 5 minutes at 10,000 x g using a Microcentrifuge (Eppendorf, Hamburg, Germany) and 350 µL of supernatant was transferred to a new microtube. This extraction step was repeated 3 times and aspirated supernatants merged. Next, extraction solution was evaporated over a period of 8 hours using a heatblock at 50° C in a fumehood. Obtained sediment was redissolved in 100 µL chloroform and stored at -20° C.

Thin Layer Chromatography

A generic TLC chamber was saturated using a 70:30:1 (v/v/v) hexane, diethyl-ether and acetic acid solvent mixture (Fuchs *et al.*, 2015)[8] for 30 minutes, aided by a thin strip of filter paper reaching up to the top of the chamber. 5 µL of each sample and 15 µg of Lipid Standard mix (Merck, Burlington, Massachusetts), containing equal ratios of 1,3-diolein, 1,2-dioleoyl-*rac*-glycerol, glyceryl trioleate and monoolein, was spotted on silica gel 60 glass plates (Merck Millipore, Burlington, Massachusetts) and allowed to dry for 5 minutes. TLC was performed for 15-20 minutes depending on the speed of elution, after which the silica plate was taken out of the TLC chamber and allowed to dry for 20 minutes at room temperature. The plate was then stained with a staining solution containing 0.03% Coomassie Brilliant Blue R-250 and 20% (v/v) methanol for 15 minutes, de-stained with a 20% methanol (v/v) solution for 10 minutes and allowed to dry for 30 minutes (Nakamura & Handa, 1984)[27]. Bands were visualised using a GelDoc imaging system (Bio-Rad, Hercules, California).

Results

Biomass, growth & pH

To compare growth between *S. commune* strains H4-8A and H4-39 in different media, biomass experiments were performed. Growth of mycelium in WM resulted in a more than threefold increase of biomass compared to SCMM (Fig. 1). Maximum biomass of H4-39 in WM was 29.25 ±1.48 g L^{-1} at 10 days, compared to 8.50 ±1.13 g L^{-1} in SCMM at 7 days. Maximum biomass of H4-8A in WM was 28.55 ±0.78 g L^{-1} at 7 days, and 9.50 ±2.40 g L^{-1} in SCMM at 7 days (Sup. Table 1). Speed of biomass increase was similar between strains in WM, with H4-39 reaching 77.5% ±2.1% and H4-8A reaching 83.9% ±0.7% of their peak biomass after 4 days. However, in SCMM, H4-39 cultures reached 99.4% ±4.2% of maximum biomass after 4 days, whereas H4-8A cultures contained only 26.8% ±3.7% at that time (Fig. 1, Sup Table 1). Carbon-to-biomass efficiency of H4-8A is 7.7% higher in WM with 0.48 g biomass per g of carbon source, compared to 0.44 g g^{-1} carbon source in SCMM (Sup. Table 2). Similarly, H4-39 efficiency is 23% higher in WM with 0.49 g g^{-1} carbon source versus 0.40 g g^{-1} carbon source in SCMM.

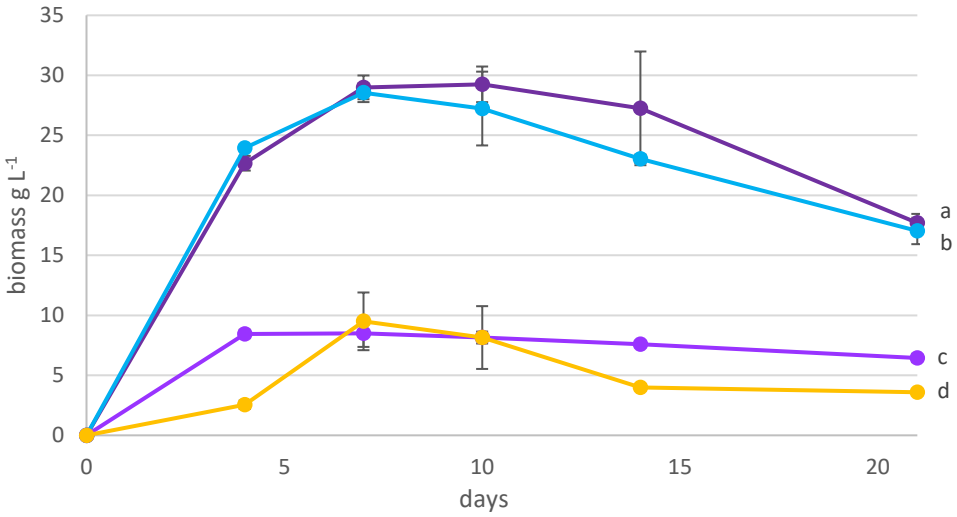

Fig. 1. Growth curves of mycelial biomass harvested from liquid shaking cultures. *Strains H4-39* ***(a,c)*** *and H4-8A* ***(b,d)*** *were grown in WM* ***(a,b)*** *and SCMM* ***(c,d)****. H4-39 peak biomass was measured at day 10 in WM and day 7 in SCMM. For H4-8A, peak biomass was measured at day 7 In both media. Data for H4-8A in SCMM was provided by A. d'Errico. Experiments were performed in duplicate. Error bars denote standard deviation.*

Following filter-separation of cultures, pH of the media was measured immediately (Fig. 2). During growth of both strains in WM, pH of the medium initially acidified until day 4, after which it started basifying until day 21. A similar pattern was seen during growth of H4-39 in SCMM, although the changes were less pronounced. Acidification of the media occurred during initial growth of biomass, while basification coincided with biomass decline (Fig. 1).

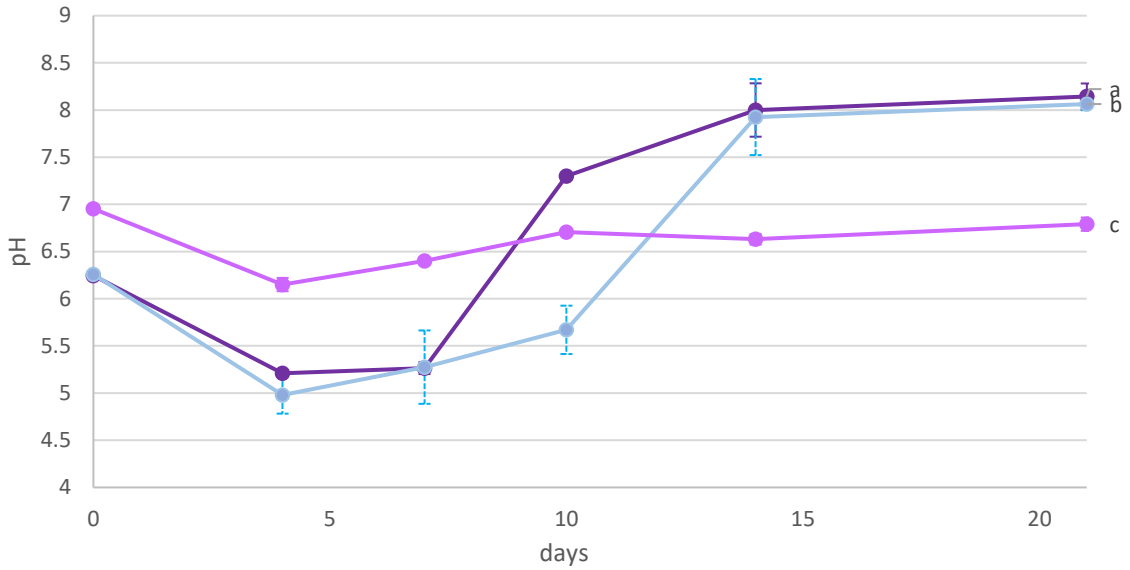

Fig. 2. pH curves of liquid shaking culture media. *pH of H4-39* ***(a)*** *and H4-8A in WM* ***(b)*** *and pH of H4-39 in SCMM* ***(c).*** *No pH measurements were taken during H4-8A growth experiments in SCMM. Experiments were performed in duplicate. Error bars denote standard deviation.*

FTIR spectroscopy

To look at the effects of different media in more detail, FTIR spectrometry was performed on both medium and mycelium. Due to their abundance as both sources of nutrition in the medium and primary components of fungal biomass, the relative signal strength ratio of lipids, proteins and carbohydrates (L:P:C) was used to assess changes in medium and mycelium composition (Fig. 3). In medium the L:P:C ratio was initially roughly equal at day 0 for both strains. After 4 days of H4-39 culture growth, the L:P:C ratio had shifted to 12:23:8, with amide I & II bonds making up 23.4% of the total signal (Fig. 4; I)(Sup. Table 4A). From 7 days onwards the shift in L:P:C ratio had become even more pronounced, reaching 3:25:11 at 21 days (Sup. Table 4A). While the L:P:C ratio did not shift as quickly in growth medium of H4-8A compared to that of H4-39, a similar trend was visible (Fig. 4; II). At 7 days it reached 5:23:12, and amide I & II bonds reached peak signal strength of 29.6% after 10 days, eventually shifting to 3:24:12 at day 21 (Sup. Table 5A).

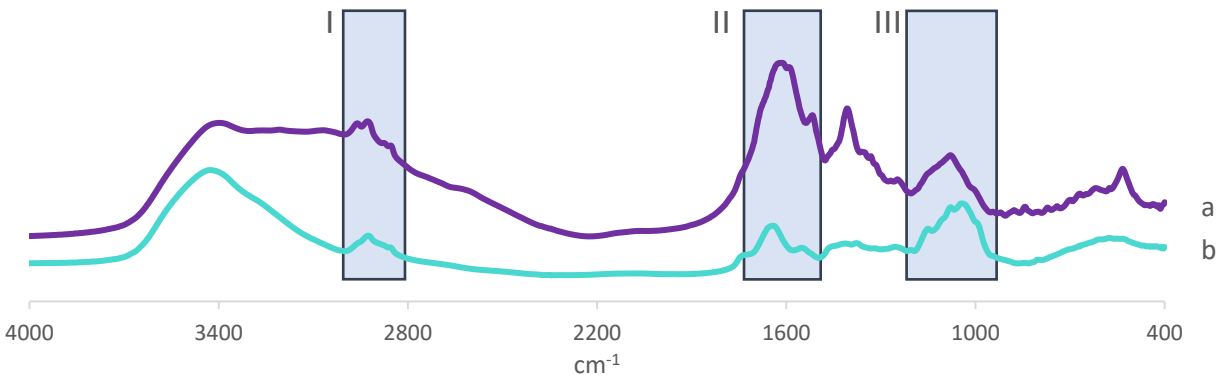

Fig. 3. FTIR spectra of H4-39 in WM (a) and SCMM (b). Marked areas denote *v* ranges for bonds corresponding to specific groups of compounds, lipids **(I)**, proteins **(II)** and carbohydrates **(III)**. See Sup. Table 3 for explanation of *v* ranges.

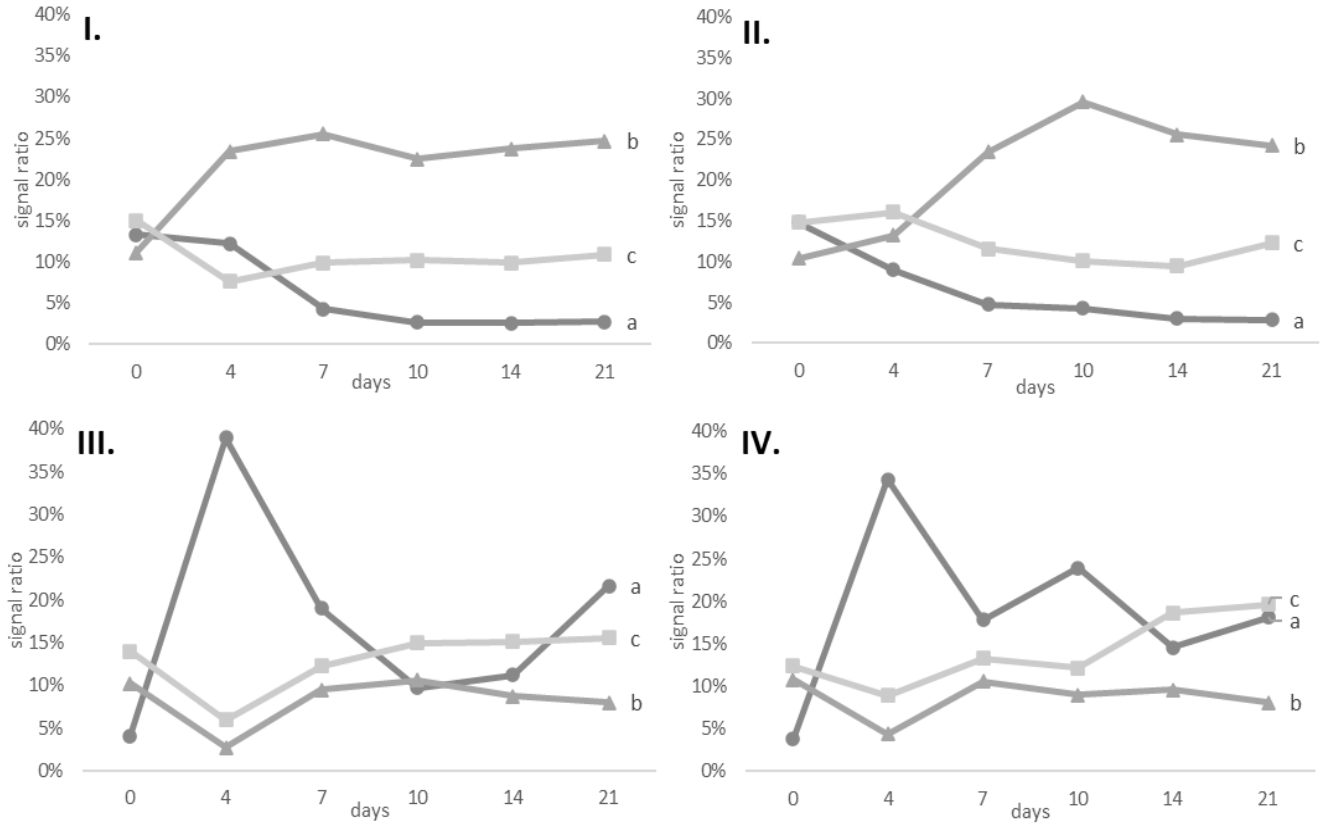

Fig. 4. Change in relative intensity of FTIR signals over time. *Lipids (●), proteins (▲) and carbohydrates (■) in spent medium **(I, II)** and mycelium **(III, IV)** samples of H4-39 **(I, III)** and H4-8 **(II, IV)**. Signal ratio is calculated for each bracket relative to the total signal of each sample (n=2, with 3 technical replicates). In spent medium, a shift in signal ratio favouring proteins was be seen **(I, II)**. In mycelium, an initial large peak in lipids was detected after 4 days, reducing in strength afterwards **(III, IV)**.*

Spectral analysis of both H4-39 and H4-8A mycelium shows a striking shift favouring lipids after the first 4 days of growth (Fig. 4; III & IV). Both strains have similar L:P:C ratios at the time of inoculation, 4:10:14 and 4:11:12 respectively (Sup. Table; 4B, 5B). After 4 days of growth, lipid signal had increased strongly in both strains (Fig. 4; III, IV). In H4-39 mycelium, lipid signal reduced in strength at 7 days, and further at 10 days, after which it increased again to a ratio of 22:8:16 at 21 days (Sup. Table 4B). In H4-8A lipid signal showed a staggered pattern, but declined overall, with L:P:C reaching 18:8:20 at 21 days (Sup. Table 5B).

Bradford assays

To better understand the predominance of protein signals in the FTIR data of WM, proteins were quantified using Bradford assays. Measurements of different media showed almost 10 mg mL^{-1} of protein present before growth (Table 1). In WM 6.1% ±1.8% and in SM 3.6% ±0.6% of the original protein concentration remained after 7 days, indicating consumption by the fungus. No proteins were detected in unspent SCMM. However, after 7 days of growth small amounts of protein were detected in the medium, showing fungal protein excretion. Significantly different protein levels were detected for each biological replicate (BR), and this difference was consistent per replicate for different media

(Fig. 5). Filtered media after growth of replicate B showed higher concentrations of proteins compared to the other replicates, up to 41% higher in WM and up to 28% higher in SM.

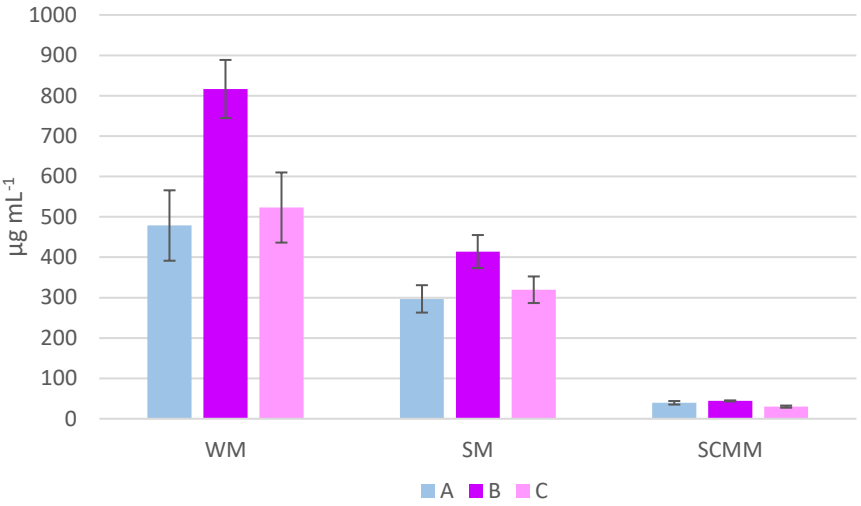

Fig. 5. Protein concentrations (µg mL^{-1}) after 7 days of growth of H4-39 in different media. *A consistent difference in remaining protein concentrations can be seen for each biological replicate* ***(A, B, C)*** *per medium. Error bars denote standard deviation.*

Table 1. Protein concentrations in different media.

BR	day	WM	SM	SCMM
-	0	9994.0	9611.8	-
A	7	478.6	296.8	39.6
B	7	816.6	414.1	44.2
C	7	523.1	319.5	30.0

Bradford assay measurements of protein concentrations (µg mL^{-1}) for multiple dilutions of each biological replicate.

SDS-PAGE

To visualise the spectrum of proteins present in WM and SM, SDS-PAGE was used (Fig. 6). Because of the high concentration of proteins present in both milk media, samples of unspent medium were diluted up to 200 times before being loaded onto the gel. Despite this, some of the sample remained on top of the running gel, indicating incomplete denaturation (Fig. 6). SDS- PAGE showed the presence of at least 3 proteins of different sizes between 26 and 34 kDa in both unspent media, showing the presence of the most prevalent milk proteins, αs1-casein, αs2-casein and β-casein and (Fig. 6a,b,g,h). No bands were visible for β -lactoglobulin or α-lactablumin. After 7 days of growth, these proteins were no longer detected in either medium despite the 100-fold higher sample concentration, suggesting breakdown and possible consumption by the fungus. After 7 days of growth, many different bands ranging from 10 to 170 kDa were seen. Notably, protein profiles of WM and SM are dissimilar (Fig. 6d-

f,i-k). WM showed bands at 170 kDa not detected in SM. Conversely, bands around 130, 26, 20 kDa were visualised in SM but not in WM, suggesting altered protein expression by the fungus. Proteins visualised around 60 kDa are much more abundant in SM than in WM. Spent WM samples showed a large amount of small proteins, partial proteins <10 kDa or peptides and smearing, suggesting reduced consumption of proteins in WM compared to SM. While proteins were being secreted by *S. commune* in SCMM as shown by the Bradford assays, concentrations were so low they were not visualised by SDS-PAGE (Sup. Fig. 1).

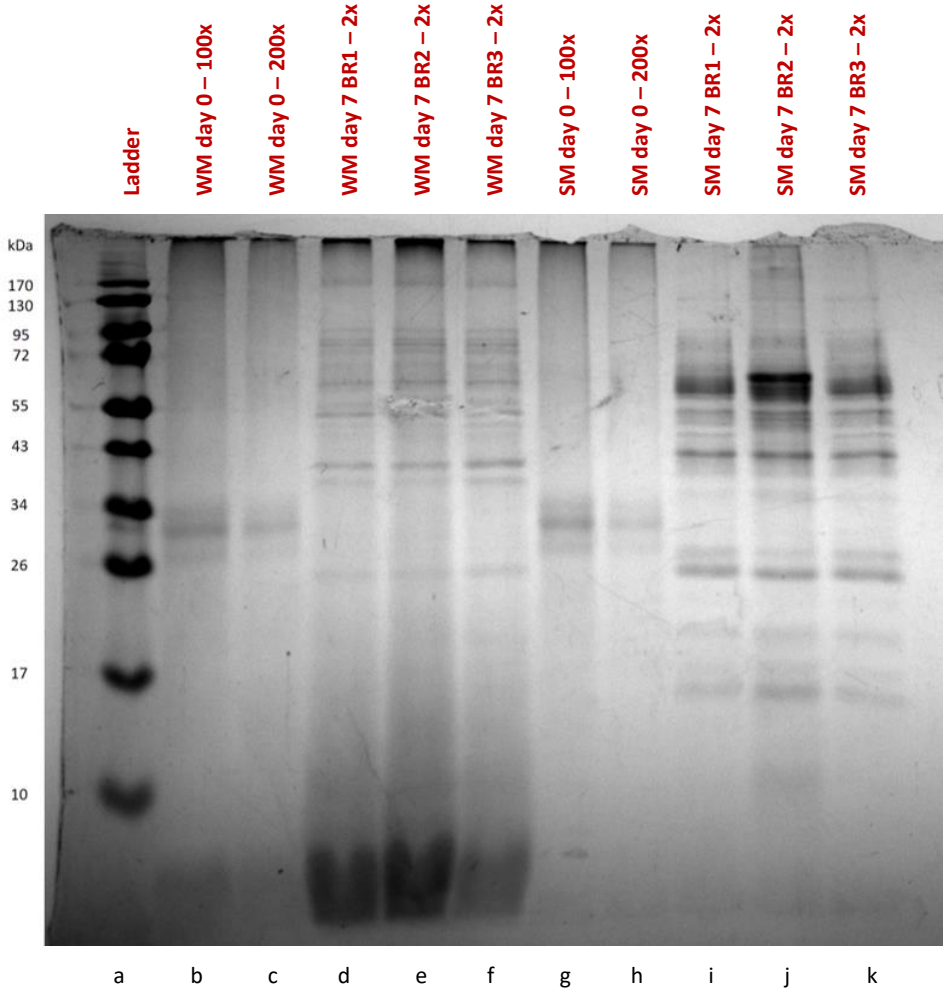

Fig. 6. SDS-PAGE of proteins in WM and SM at day 0 and 7 of H4-39 growth. *Ladder* ***(a)****. WM at day 0 diluted 100x* ***(b)*** *and 200x* ***(c)****. WM filtrate after 7 days of growth, 2x dilution* ***(d, e, f)****. SM at day 0 diluted 100x* ***(g)****, and 200x* ***(h)****. SM filtrate after 7 days of growth, 2x dilution* ***(i, j, k)****.*

TLC

To visualise lipids and their possible consumption in different media, TLC was performed using lipid-extracts of medium at day 0 and 7, as well as of mycelium after 7 days (Fig. 7). Lipids were clearly present in WM at day 0, while none were visible in SM (Fig. 7a,d). After 7 days, no lipids were seen in either medium, but mycelial lipid-extracts showed lipids were present and with a similar spectrum of distribution for both media (Fig. 7c,f). The presence of lipids in mycelium samples grown in SM shows

these are lipids produced by the fungus. Lipids visualised in WM mycelium, located at the height of the di-glyceride standard, are also of fungal origin as shown by their absence in day 0 WM (Fig. 7 arrow). When comparing the lipids present in WM and SM mycelium samples, the WM sample is stained more intensely, which shows more lipids are present in the WM mycelium sample. This could be an indication unconsumed lipids have adsorbed to the mycelium during filtration.

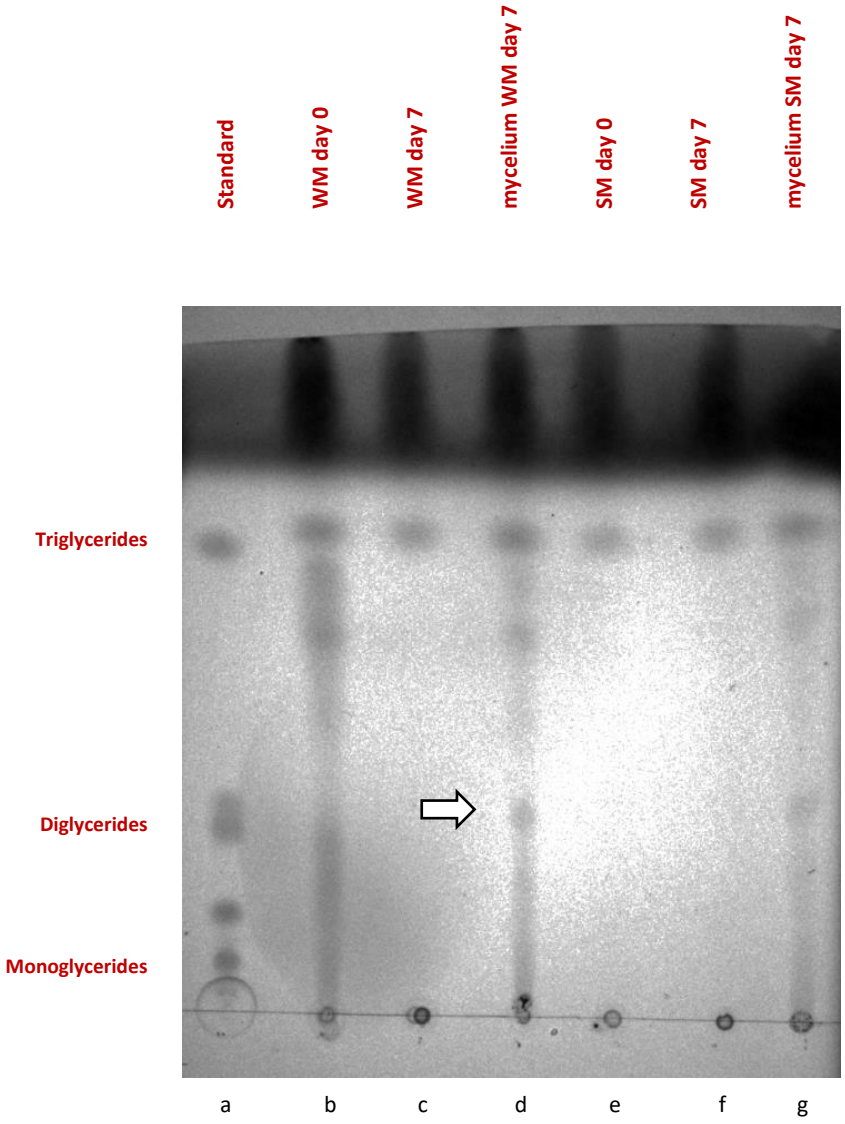

Fig. 7. TLC plate visualising lipid spectra in medium and mycelium. *FLTR: lipid standard containing mono- di- and tri-glyceride mix **(a)**, WM at day 0 **(b)** and 7 **(c)**, H4-39 mycelium grown in WM for 7 days **(d)**, SM at day 0 **(e)** and 7 **(f)**, and H4-39 mycelium grown in SM for 7 days **(g)**. Arrow indicates fungal lipids not found in WM.*

Discussion

A large increase in *S. commune* biomass was seen when cow's milk was utilised as a medium compared to conventional growth medium. Currently, the underlying mechanisms causing this increase in biomass are unclear. Understanding these mechanisms will lead the way towards optimising media for increased biomass yield and mycelium with desirable properties. Additionally, due to the role of *S. commune* as a model organism, gaining further insight into medium consumption and biomass production may benefit a wider range of research and applications. In particular, these findings may aid the optimisation of *S. commune* as a production system for biological compounds such as enzymes, proteins and organic acids. By using FTIR spectrometry in combination with protein spectrum analysis, quantification, and TLC, we attempted to further the understanding of these mechanisms.

Biomass

S. commune strains H4-8A and H4-39 were grown in WM and SCMM for a series of days and biomass was compared showing differences depending on strain and medium used. While both WM and SCMM contain roughly equal amounts of carbohydrates, WM predominantly contains lactose, while SCMM only contains glucose. Interestingly, not all types of carbohydrates may result in an equal increase of biomass, with lactose being one of the least effective at promoting vegetative growth (Alam *et al.*, 2018)[1]. In SCMM, 93.6% of carbon is provided in the form of glucose, with L-asparagine making up the remaining 6.4%. In WM however, carbon is provided by a variety of sources including Lactose, L-asparagine and a wide range of proteins, lipids and other carbohydrates such as glucose and galactose (Foroutan *et al.*, 2019)[6]. Both *S. commune* strains H4-8A and H4-39 are able to capitalise on these carbon sources, respectively showing a 3.0- and 3.4-fold increase in biomass in WM compared to SCMM.

Conventional carbon-to-biomass efficiency calculations divide total biomass by total carbon source mass, resulting in a higher efficiency of carbon-to-biomass in WM compared to SCMM for both H4-8A and H4-39. However, due to the difference in primary carbohydrate source, as well as the wide variety of additional carbon sources present in WM, the conventional calculations are likely insufficient, leading to incorrect conclusions. A more detailed calculation may be warranted, where instead of total carbon source mass, carbon molarity is used to represent the carbon part of the equation. While this is a straightforward calculation for defined media such as SCMM where the exact quantity is known (0.7 M Carbon), WM requires a different approach. Although accuracy of the total carbon approximation improves with increasingly detailed calculations, the simplest approach assumes all carbohydrates to be lactose, all lipids to be triglycerides with an average of 50 C-atoms, and mass of all proteins to consist on average of 53% carbon (Rouwenhorst *et al.*, 1991)[36]. The triglyceride

molecular weight is based on a rough average of the triglycerides present in cow's milk, and a triglyceride chemical formula of $C_nH_{2n}O_6$ (Foroutan *et al.*, 2019; Garton 1963)[6,9]. Using this approach, the estimated total carbon in WM amounts to 2.8 M. This augmented calculation leads to rejection of the hypothesis that carbon-to-biomass efficiency is higher in WM and instead shows it is higher in SCMM (Sup. Table 2).

While early growth in WM was largely similar for both strains, H4-8A appeared to lag behind H4-39 in SCMM during the initial growth phase. This delay was also seen during inoculation of experimental units, where inoculation required consistently higher volumes of H4-8A pre-culture macerate compared to H4-39. There is little documentation to be found about physiological differences between *S. commune* strains in literature, but H4-8A is known to produce more schizophyllan compared to H4-39 (unpublished data). The increased amounts of this extracellular polysaccharide seen in H4-8A cultures indicates the fungus preferentially invests a larger amount of the nutrients available in the production of schizophyllan compared to H4-39. In a resource-limited medium such as SCMM, this may lead to a lag in growth of biomass, as most of the available glucose may first be turned into schizophyllan before it is repurposed or incorporated into biomass. In WM the abundance and diversity of nutrients may be the cause of increased early growth in H4-8A relative to SCMM. While lactose is less effective at promoting vegetative growth (Alam *et al.*, 2018)[1], the amount of schizophyllan produced relative to biomass is also much lower compared to glucose (Kumari *et al.*, 2008)[23]. While the details of this mechanism are not known, it may enable or induce the fungus to produce increased amounts of biomass relative to schizophyllan. Furthermore, shearing stress by shaking is an important factor for the production of schizophyllan, with production of the polysaccharide relative to biomass increasing with higher agitation up to a maximum, after which both are reduced (Rau *et al.*, 1992)[35]. Increased availability of nutrients in WM may further shift production in favour of biomass, as the higher amount of biomass in the shape of fibrous pellets may act as a shock cushion, reducing agitation within the culture. For future research, the incorporation of viscosity measurements would be beneficial in further understanding the role of schizophyllan in the production of *S. commune* biomass.

Consumption of milk nutrients

Produced biomass and medium were separated and analysed using FTIR spectrometry, to gain insight into the composition of mycelium and spent medium. From the data obtained by FTIR analysis a signal ratio was calculated to determine the relative amounts of lipids, proteins and carbohydrates. While L:P:C signal ratios are initially roughly equal in WM, the protein signal becomes dominant after growth of *S. commune*. This data, combined with the results of the Bradford assays which show that protein concentrations have decreased by roughly 95%, infers that the remaining lipid and carbohydrate concentrations must also have decreased by at least the same amount. This confirms our expectations

that most if not all carbohydrates and proteins are utilised by the fungus for the production of biomass. To verify this, future studies may wish to include 3,5-dinitrosalicylic acid assays (DNSA) to assess the depletion of reducing sugars in media. For lipid quantification the selection of an appropriate method is paramount, as the ability to quantify specific lipid types vary strongly between techniques. For media incorporating milk, which contains lipids predominantly in the form of triglycerides, the use of NMR may be most appropriate (Khoury *et al.*, 2018)[22].

Due to spatial limitations, growth experiments were performed with 2 biological replicates for each day, and subsequent FTIR sampling was done in triplicate. While technical replicates were fairly consistent (data not shown), biological replicates showed a high degree of variation (Sup. Table 1; unpublished data). Because of this, the ability to generalise our findings was limited. A reiteration of biomass experiments using a larger sample size (n≥10) would provide a more robust dataset and likely generate more leads for future research.

pH of the medium

pH measurements were taken at each timepoint during biomass experiments. In both WM and SCMM, pH acidified during growth of *S. commune*, coinciding with the initial growth phase until peak biomass was reached. *S. commune* is known to produce large amounts of malic acid, as well as smaller amounts of succinic and fumaric acid (Takao, 1965)[40]. It is also able to produce oxalic, gluconic, ascorbic, maleic, citric, tartaric and epoxysuccinic acid when in contact with black slate, which is rich in organic carbon (Kirtzel *et al.*, 2019)[21]. After 4 days, acidification halted, and both media basified. This may in part be explained by subsequent consumption of the produced acids as part of the citric acid cycle (Wessels, 1959)[42]. However, this does not explain the basification beyond its original values. Since basification of SCMM does not progress beyond original pH values as seen in WM, it may be connected to proteolytic activity by *S. commune*. Basification of the medium is achieved by some fungi through active secretion of ammonia as a result of protease activity and deamination of amino acids (Jennings, 1989)[15], however this has not been described in *S. commune*.

Proteins remaining in the medium

Protein content of spent media was quantified using Bradford assays and analysed using SDS-PAGE. While the Bradford assays indicated that most proteins had disappeared from the medium after 7 days, the SDS-PAGE results showed that the spectrum of remaining proteins was highly complex. The absence of bands between 26-34 kDa shows all caseins have disappeared from both WM and SM. The remaining bands may be attributed to possibly undigested milk proteins, proteinases naturally present in milk (Kelly & McSweeny, 2003)[20], fungal enzymes excreted to facilitate the breakdown of milk

components, partial proteins resulting from enzymatic activity, and autolytic enzymes. Upregulation of proteins excreted by *S. commune* in response to the presence of protein may include GF-aminopeptidase, X-AAPF-endopeptidase and metalloproteases (Hummel *et al.*, 1998)[12].

Several factors are known to influence the outcome of SDS-PAGE. WM was pasteurised before acquisition and autoclaved before the experiment. Heat treatment of cow's milk causes formation of micelle-bound aggregates, which do not migrate properly during PAGE, and stay at the top of the lanes (Sharma *et al.*, 2021; Guyomarc'h *et al.*, 2003)[38,11]. Furthermore, the absence of urea during the SDS-PAGE likely reduced separation of milk proteins dramatically. If neither β-mercaptoethanol nor urea are present, αs2-casein and κ-casein will not show up as individual bands (Andrews, 1983)[3]. It is also indicated κ-casein may not be visualised at all on conventional SDS-PAGE (Pardo & Natalucci, 2002)[33]. With only β-mercaptoethanol present, it may expose hydrophobic regions on the milk proteins, stopping them from properly migrating during PAGE (Sharma *et al.*, 2021)[38]. This issue may be resolved by adding urea, which causes extensive intermolecular hydrophobic bonding in milk caseins, reducing the hydrophobicity and allowing movement (Fox *et al.*, 2015)[7].

At the start of this research the complexity of adequate visualisation of milk proteins was not fully appreciated. Thus, these results may serve as a first step towards a better understanding of the mechanisms of protein degradation by *S. commune*. We suggest future research includes both urea-PAGE and tricine-SDS-PAGE, each giving different insights into the degradation of milk proteins. Urea-PASGE is well suited for larger proteins such as caseins, while tricine-SDS-PAGE is better suited to study proteins of lower molecular mass (Sharma *et al.*, 2021)[38]. As a follow-up, proteins may be isolated and identified using protein mass spectrometry. In addition, complementing SCMM with purified proteins will allow a more focussed analysis of proteolysis by the fungus. Since knowledge on protein degradation by *S. commune*, this approach may lead to interesting results.

Partial consumption of lipids

Mycelium grown in WM and SCMM was analysed using a combination of FTIR spectrometry and TLC to assess the ability of *S. commune* to digest lipids present in cow's milk. Both FTIR and TLC analyses show lipids disappearing from the medium, but if this is due to consumption by the fungus or merely adsorption to biomass during filtration is not immediately clear. FTIR data of mycelium grown in SCMM shows little change in composition over time. This suggests that the strong lipid signal observed in WM mycelium at 4 days is not part of the fungal biomass and is likely due to adsorption of milk lipids to the mycelium during filtration. Due to the relative nature of the FTIR data, the perceived reduction of signal strength between days 4-10 can be partially explained by the increase in fungal biomass during that time. As biomass increases, the relative amount of adsorbed lipids decreases and signal strength diminishes, even if the absolute amount of lipids does not change. However, at 21 days the total

biomass is 22% lower than at day 4, while the lipid signal has diminished by 45% (Fig. 4, III). If no lipids had been consumed, lipid signals should have returned to similar values to those measured at day 4. While TLC results show an increased staining of lipids extracted from WM mycelial samples compared to SCMM samples, staining is less pronounced compared to unspent WM (Fig. 7b,d,g). Since no staining is visible in spent WM after 7 days, this reduction in staining shows the consumption of lipids. Additionally, while staining intensity is quite similar between unspent WM and WM grown mycelium for the mono- and di-glyceride areas, staining around the triglyceride area is notably less pronounced. Thus, regardless of irregularities in total lipid amount applied to the TLC plate, a reduction of lipids can be seen in WM mycelium compared to unspent WM.

Due to the limited time available during this research, it was opted to explore a single TLC approach for the detection, quantification and qualification of lipids. However, the large number of eluent candidates and staining techniques available complicate its application. This is especially critical when attempting to separate and visualise compounds in a complex medium such as WM, and a single eluent mix is unlikely to give a complete view of all lipids present. The interpretation of our findings are further complicated by the likely deterioration of our lipid standard, which contained 4 distinct lipid groups but eluted into 6 separate stains. Future steps should be taken to redefine the TLC protocols in order to gain a deeper understanding of which lipids are being degraded. Instead of attempting to visualise all lipids on one plate, separate experiments using eluent mixtures designed for specific milk lipid groups may yield far clearer results. Additionally, to combat the confounding influence of the milk lipid spectrum, purified lipids, may be added to a defined medium to gain a clearer view of lipid consumption by *S. commune*.

Conclusion

Here it is shown *S. commune* is able to capitalise on most of the complex nutrients available in cow's milk for the production of biomass. The fungus readily consumes proteins and carbohydrates and is likely able to digest part of the lipid contents. These results are an important step towards understanding how to better optimise *S. commune* growth media and the discovery of novel alternatives to currently established nutrient sources.

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Supplementary data

Sup. Table 1. Biomass in g L^{-1} (n=2, ±s.d.).

	WM		SCMM	
day	**H4-39**	**H4-8**	**H4-39**	**H4-8***
0	-	-	-	-
4	22.68 (0.61)	23.95 (0.21)	8.45 (0.35)	2.55 (0.35)
7	29.00 (0.99)	28.55 (0.78)	8.50 (1.13)	9.50 (2.40)
10	29.25 (1.48)	27.23 (3.07)	8.15 (0.49)	8.15 (2.62)
14	27.25 (4.74)	23.05 (0.35)	7.60 (0.00)	4.00 (0.00)
21	17.70 (0.75)	17.03 (1.10)	6.45 (0.35)	3.60 (0.14)

H4-8 SCMM data provided by A. d'Errico.

Sup. Table 2. Carbon-to-biomass efficiency.

	WM	**SCMM**
Carbon source (g L^{-1})	60.00	21.50
Total Carbon (M)	2.75	0.71
H4-8A		
peak biomass (g L^{-1})	28.55	9.50
efficiency (g g^{-1})	0.48	0.44
revised efficiency (g M^{-1})	10.37	13.35
H4-39		
peak biomass (g L^{-1})	29.25	8.50
efficiency (g g^{-1})	0.49	0.40
revised efficiency (g M^{-1})	10.62	11.95

Conventional carbon-to-biomass efficiency is calculated as maximum biomass divided by total carbon source mass. Suggested revised efficiency is calculated by maximum biomass divided by M carbon present in media. Total carbon content of WM was approximated based on most prevalent milk compounds (lactose, triglycerides & caseins).

Sup. Table 3. wavenumber regions and corresponding bonds & macromolecules (adapted from Naumann, 2009)[29]

v range (cm^{-1})	**Bonds**	**Macromolecules**
3700-2996	O-H, N-H	various
2996-2800	C-H	lipids
1800-1720	C=O	esters
1720-1485	Amide I+II	proteins
1485-1185	CH2, CH3, P=O	various
1185-900	C-O-C, C-O-P	carbohydrates
900-400	weak signals	

Sup. Table 4. Relative FTIR signal ratios - H4-39 in WM.

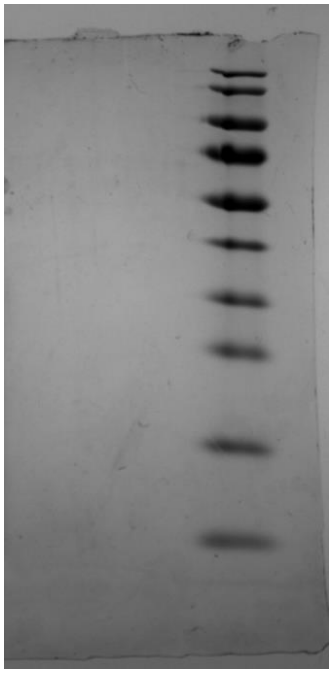
A. spent medium							
v range (cm^{-1})	**Bonds**	**0 days**	**4 days**	**7 days**	**10 days**	**14 days**	**21 days**
3700-2996	O-H, N-H	47.5%	42.2%	44.9%	45.3%	43.2%	45.4%
2996-2800	C-H	13.3%	12.2%	4.3%	2.6%	2.6%	2.7%
1800-1720	C=O	2.7%	0.7%	0.0%	0.0%	0.0%	0.0%
1720-1485	Amide I+II	11.1%	23.4%	25.5%	22.5%	23.8%	24.7%
1485-1185	CH_2, CH_3, P=O	3.1%	5.1%	6.9%	8.4%	9.4%	7.6%
1185-900	C-O-C, C-O-P	15.0%	7.7%	9.9%	10.2%	9.8%	10.9%
900-400	Weak signals	7.2%	8.7%	8.5%	11.0%	11.2%	8.8%
B. mycelium							
v range (cm^{-1})	**Bonds**	**0 days**	**4 days**	**7 days**	**10 days**	**14 days**	**21 days**
3700-2996	O-H, N-H	61.6%	36.7%	52.9%	57.0%	59.1%	48.2%
2996-2800	C-H	4.1%	39.0%	19.0%	9.7%	11.2%	21.7%
1800-1720	C=O	0.0%	5.8%	2.6%	0.6%	0.8%	1.8%
1720-1485	Amide I+II	10.3%	2.7%	9.5%	10.6%	8.7%	8.0%
1485-1185	CH_2, CH_3, P=O	3.8%	2.1%	3.6%	3.7%	2.0%	0.0%
1185-900	C-O-C, C-O-P	14.0%	5.9%	12.2%	15.0%	15.1%	15.6%
900-400	Weak signals	6.3%	7.8%	0.0%	3.5%	3.0%	4.8%

Sup. Table 5. Relative FTIR signal ratios - H4-8A in WM.

A. spent medium							
v range (cm^{-1})	**Bonds**	**0 days**	**4 days**	**7 days**	**10 days**	**14 days**	**21 days**
3700-2996	O-H, N-H	45.6%	49.7%	46.0%	42.1%	43.8%	44.6%
2996-2800	C-H	14.8%	9.0%	4.7%	4.2%	3.0%	2.8%
1800-1720	C=O	3.3%	0.6%	0.0%	0.0%	0.0%	0.0%
1720-1485	Amide I+II	10.4%	13.3%	23.4%	29.6%	25.6%	24.3%
1485-1185	CH_2, CH_3, P=O	2.7%	5.1%	7.2%	7.5%	7.9%	8.8%
1185-900	C-O-C, C-O-P	14.8%	16.0%	11.5%	10.1%	9.4%	12.3%
900-400	Weak signals	8.4%	6.3%	7.1%	6.5%	10.3%	7.1%
B. mycelium							
v range (cm^{-1})	**Bonds**	**0 days**	**4 days**	**7 days**	**10 days**	**14 days**	**21 days**
3700-2996	O-H, N-H	63.2%	41.4%	46.8%	46.4%	49.4%	48.7%
2996-2800	C-H	3.7%	34.4%	17.8%	24.0%	14.6%	18.1%
1800-1720	C=O	0.0%	2.6%	0.9%	1.3%	0.6%	1.2%
1720-1485	Amide I+II	10.8%	4.4%	10.5%	9.0%	9.6%	8.0%
1485-1185	CH_2, CH_3, P=O	3.6%	5.1%	6.1%	5.6%	1.7%	0.3%
1185-900	C-O-C, C-O-P	12.4%	8.9%	13.2%	12.1%	18.6%	19.6%
900-400	Weak signals	6.3%	3.2%	4.6%	1.7%	5.5%	4.1%

Sup. Table 6. Relative FTIR signal ratios - H4-39 in SCMM.

A. spent medium							
v range (cm^{-1})	**Bonds**	**0 days**	**4 days**	**7 days**	**10 days**	**14 days**	**21 days**
3700-2996	O-H, N-H	68.7%	67.9%	63.1%	64.0%	64.4%	64.4%
2996-2800	C-H	3.6%	5.3%	4.2%	4.0%	4.1%	3.6%
1800-1720	C=O	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
1720-1485	Amide I+II	1.9%	1.2%	1.0%	0.6%	0.0%	0.8%
1485-1185	CH_2, CH_3, P=O	4.3%	1.8%	0.9%	0.8%	1.0%	0.6%
1185-900	C-O-C, C-O-P	20.2%	23.9%	30.8%	30.7%	30.6%	30.6%
900-400	Weak signals	1.2%	0.0%	0.0%	0.0%	0.0%	0.0%
B. mycelium							
v range (cm^{-1})	**Bonds**	**0 days**	**4 days**	**7 days**	**10 days**	**14 days**	**21 days**
3700-2996	O-H, N-H	61.6%	67.2%	65.8%	67.4%	64.7%	70.5%
2996-2800	C-H	4.1%	4.0%	3.7%	3.6%	3.6%	3.6%
1800-1720	C=O	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
1720-1485	Amide I+II	10.3%	5.6%	5.6%	5.7%	6.4%	7.0%
1485-1185	CH_2, CH_3, P=O	3.8%	3.4%	3.5%	2.9%	3.4%	2.5%
1185-900	C-O-C, C-O-P	14.0%	16.2%	16.3%	14.6%	15.7%	14.4%
900-400	Weak signals	6.3%	3.7%	5.1%	5.8%	6.1%	1.9%



Sup. Fig. 1. SDS-PAGE of SCMM after 7 days of growth of H4-39.
No bands were visible. Experiment performed in triplicate.