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Growth and Influence of White-Rot Fungi on the Chemical Composition of Wheat Straw Inoculated under Varying Pre-Conditions

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Abstract: Solid-state fermentation with white-rot fungi is an interesting alternative to chemical straw treatment for ruminant nutrition. However, for practical implementation on farms, feasible handling and its effect on nutritional characteristics have to be tested beforehand. Chopped wheat straw was either soaked and drained or just remoistened to about 24% dry matter without subsequent sterilization. Moist straw was inoculated with *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, or *Volvariella volvacea*. Fermentation lasted up to 42 d with weekly or shorter sampling intervals. Fiber fractions, minerals and elements, and non-starch polysaccharides were analyzed, and microflora was plate counted. Lactic acid bacteria of selected samples were identified by MALDI-TOF. All inoculated fungi grew well under the selected conditions expressed by the visible mycelium and specific smell. *P. ostreatus* developed fruiting bodies in the given time. Initial numbers of lactic acid bacteria were >8.0 log cfu/g. In the beginning, *Weissella confusa/cibaria* dominated. However, neither decrease in lignin nor cellulose concentration was observed during the period in either of the treatments, thus seeming to be inappropriate for ruminant nutrition purposes. Some elements and minerals peaked, especially towards the second and third week (Na, Cl, Mg, Fe, and Mn). Growth conditions for mushrooms were optimized by remoistening the straw with a defined amount of water.

Keywords: solid state fermentation; fungal growth; ergosterol; pH; natural microflora; cell wall polysaccharides; minerals; lactic acid bacteria; pre-treatment

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1. Introduction

In general, straw is a by-product that is abundantly available worldwide. For 2020 the global production of cereal straw was estimated at around 760 Mio t for rice and 610 Mio t for wheat [1]. Straw serves different purposes. Some examples are working straw into the soil for rotting and enhancing organic soil matter [2] or power generation [3]. It is suitable to cultivate certain edible mushrooms [4]. In animal farming, it often serves as bedding [5] and in the swine sector as manipulable material [6]. In ruminant nutrition, straw is mostly used as a fiber component for dietetic reasons and to dilute the energy concentration of the ration [7].

Its low content of metabolic energy, even for ruminants (around 3.4 MJ ME/kg dry matter (DM)), is mainly due to its lignocellulosic complex [8]. However, especially in times of roughage scarcity, solutions have been investigated to improve straw digestibility by detaching lignocellulosic structures.

In this context, biological straw treatment is one approach to improve ruminal digestibility by fungal lignin degradation. Cereal straw is a suitable growth substrate for white-rot fungi, and its chemical and possibly structural composition will be changed by their colonization.

Since the 1990s, various studies have proved that some fungi species and strains are able to degrade lignin under certain conditions [9–12]. White-rot fungi are one type of wood decay fungi [13] and degrade lignin more efficiently than other microorganisms [14]. This is due to their enzymatic activity, namely xylanase, laccase, and manganese peroxidase [15–17].

In this study, three white-rot fungi species and a total of four strains, which were publicly available with the principal ability to degrade lignin, were used. Two defined strains have been described in other investigations before. *Pleurotus ostreatus* (*P.o.*), order of Agaricales, is an edible mushroom. It belongs to the oyster mushrooms, which are the second most cultivated mushrooms worldwide [18]. The species has been included in several trials studying its impact on the straw composition [9,19,20]. The strain CBS 411.71 has been used by, e.g., Salvachua et al. [21], as pretreatment for ethanol generation from straw, and another strain from a commercial provider for home mushroom growing was chosen as an additional representative of the species. *Ceriporiopsis subvermispora* (*C.s.*) CBS 347.63, an order of Polyporales, a non-edible crust fungus, has been proven effective in depolymerizing lignin in several studies [12,22]. *Volvariella volvacea* (*V.v.*), order of Agaricales, also known as paddy straw mushroom, is a typical representative of edible white-rot fungi, especially in tropical regions. It was studied, e.g., on banana leaves for fiber decomposition [23]. Although *V.v.* provided genetic evidence for laccase, it seems to lack an efficient ligninolytic enzyme system [24].

Despite several promising studies on a laboratory scale, to date, to our knowledge, no or at least few practical implementations of SSF targeting animal nutrition have taken place on a larger scale on farms. One of the reasons is the pretreatment, which in most of the scientific studies consist of autoclaving (e.g., [12,21,25]). According to a review [11], timing is a very important factor in fungal treatment to optimize the nutritional value, which can also be an obstacle to practical implementation.

The aim of our study was to evaluate (a) the pretreatment of wheat straw on the development of fungal mycelium of selected strains of *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, and *Volvariella volvacea*; and (b) its effect on the degree of lignification, mineral status, cell wall polysaccharides of the straw and the accompanying microflora at different points in time during solid-state fermentation.

2. Materials and Methods

Winter wheat straw was harvested in July 2018 in Köllitsch, Northern Saxony, Germany, in excellent sensory quality because of preceding dry weather conditions and stored as square bales in a dry ambient. It was chopped to 7–10 cm before the treatments.

An overview of the biological treatments is given in Table 1 and visualized in a flowchart (Figure S1).

Table 1. Biological treatments in the different trials.

		Trial 1—Soak and Drain	**Trial 2—Remoisten and *V.v.***	**Trial 3—Remoisten and *C.s./P.o.***
Inoculant	Strains	*C. subvermispora CBS 347.63*, *P. ostreatus CBS 411.71* and *PO93*	*V. volvacea DSM 6190*, uninoculated control	*C. subvermispora CBS 347.63*, *P. ostreatus CBS 411.71*
	Growth medium	Wheat grains	Wheat grains	Wheat grains
Straw	Soaking	Abundant H_2O	2.7 L H_2O/kg straw (25% target DM)	2.7 L H_2O/kg straw (25% target DM)
	Draining	Yes	No	No
Storage	Ambient °C	21 °C	24 °C	23 °C
Sampling	After d	0, 7, 14, 21, 28, 35, 42	0, 7, 14, 21, 28	0, 5, 7, 10, 14

C.s.—Ceriporiopsis subvermispora; *P.o.—Pleurotus ostreatus*; *V.v.—Volvariella volvacea*.

2.1. Preparation of the Inoculum

Grain Spawn

For Trial 1 (soaked and drained straw), pure cultures of *Ceriporiopsis subvermispora* (CBS 347.63) [12], *Pleurotus ostreatus*, strain CBS 411.71 (Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands) [11,21] and strain PO93 (Pilzhof & Edelpilzzucht Breck GbR, Malschwitz, Germany) were grown on malt extract agar (MEA) (malt extract 20 g/L, K_2HPO_4 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $Ca(NO_3)2 \cdot 4H_2O$ 0.5 g/L; [12]) for 7 d at 24 °C. For the grain substrate, an adaptation to Breck [26] followed: 195 g wheat grains, 5 g chopped wheat straw, 10 g lime (for litter), and 300 mL tap water were autoclaved in a Weck® jar each (850 mL; J. Weck GmbH u. Co. KG, Wehr-Öflingen, Germany) at 121 °C for 20 min. After cooling down, the content of a jar was mixed and divided into two jars each and autoclaved again. The jars were opened on a clean bench and evaporated for 1 h while cooling down.

A piece of malt extract agar (0.5 × 0.5 cm) with mycelium was placed in the middle of the grain surface. The loosely closed jar was incubated at 24 °C. After 8 d, the grains with the grown mycelium were shaken well in the jar and a second time after 11 d. After 14 d, grains were overgrown with mycelium and used for straw inoculation.

In the second and third trial, the inoculum was prepared accordingly. Depending on the temperature requirements of the strains and the velocity of growth, the conditions were slightly varied.

Trial 2 (remoistened straw and *V.v.*): *Volvariella volvacea* (DSM 6190) on MEA for 10 d at 24 °C, incubation on wheat grain at 30 °C for 5 d and "control" with only autoclaved wheat grains as described above (grain substrate).

Trial 3 (remoistened straw and *C.s./P.o.*): *Ceriporiopsis subvermispora* (CBS 347.63) and *Pleurotus ostreatus* (PO93) on MEA for 7 d at 24 °C, incubation on wheat grain at 24 °C for 8–9 d.

2.2. Solid State Fermentation of Wheat Straw

2.2.1. Trial 1—Soaked and Drained Straw

Straw was filled in nylon bags (24 cm × 33 cm, ~400 g dry straw/bag) and soaked in abundant tap water for 20–24 h; afterward, it was drained for 2–3 h.

Twenty g of the spawn was mixed in 1230 g of moist straw each. This relation was adapted from Breck and Breck [27]. The straw was then packed relatively densely in a perforated plastic bag (cast polypropylene, flame perforated) (30 cm × 40 cm) together with a data logger for temperature (Tinytag Transit 2, TG-4080; Gemini Data Loggers Ltd., Chichester, UK) to give around 1200 g moist straw/bag. This bag was placed in another plastic bag (37 cm × 58 cm) on a disc of polystyrene (Ø 15.5 cm; 3 cm thick) to prevent possible standing in water. A triangle (~10.8 cm^2) was cut both in the front and the back of the outer plastic bag to allow for air circulation. After one day, the upper opening of the outer bag was closed with a rubber. The storage room was dark and had a constant temperature, and relative humidity was recorded. The pH value was determined at day 0 and when removed for analysis. The straw was visually examined. Samples were frozen at −20 °C for further analysis.

Trial 1: Ambient temperature was around 21 °C.

There were three passes in total, with one fungus each, delayed by one week. Eighteen bags per fungus were prepared to allow for a weekly sampling of three replicates for a total of six weeks.

2.2.2. Trial 2 and 3—Remoistened Straw

Straw was filled in plastic tubs. Tap water (2.68 L/kg air dry straw) was added to the straw to give around 250 g DM/kg. It was mixed after 6 and 22 h to allow for even soaking. Before inoculation, it was homogenized in a concrete mixer (120 L, 550 W, 230 V; CM120L, HORNBACH Baumarkt AG, Bornheim, Germany).

Straw inoculation and packing were then performed as described for Trial 1 (soaked and drained straw). The storage room was dark and had a constant temperature of around 23–24 °C, and relative humidity was recorded.

In Trial 2 (with *V.v.*), twenty bags per fungus were prepared to allow for a weekly sampling of four replicates for a total of 4 weeks, including day 0. In trial 3 (with *C.s./P.o.*), the quadruplicates were sampled after 0, 5, 7, 10, and 14 d. The pH value was determined at day 0 and when removed for analysis. Selected samples underwent microbial colony counting. The straw was visually examined. Samples were frozen at −20 °C for further analysis.

2.3. Microbial Analysis

From selected samples from the biological treatments, colonies of microbial groups were counted [28]. Aerobic mesophilic bacteria comprising yellow-pigmented bacteria and the genera of *Pseudomonas*, *Enterobacteriaceae*, *Bacillus*, *Staphylococcus*, *Micrococcus*, *Streptomyces*, and others were plate counted on tryptose medium (with triphenyl tetrazolium chloride (TTC)). Yeasts and molds comprising all types of yeasts, the genera of *Dematеaceae*, *Verticillium*, *Acremonium*, *Fusarium*, *Aureobasidium*, *Aspergillus*, *Penicillium*, *Scopulariopsis*, *Wallemia*, and others were counted on Rose Bengal Chloramphenicol Agar (with Tergitol NP 10) and Dichloran Glycerol (DG 18) Agar. Lactic acid bacteria were counted on MRS agar (pH 5.7) (de Man, Rogosa, Sharpe). Likewise, single lactic acid bacteria colonies (103) from Trial 2 and 3 (with remoistened straw) were isolated from 28 straw samples stored for 0, 7, and 14 d, grown on MRS medium at 30 °C for 72 h (MRS agar, pH 5.7, Art. HP65.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at the BfUL (State-owned company for environment and agriculture, Nossen, Germany). They were identified by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) at the RIPAC-LABOR GmbH in Potsdam.

2.4. Chemical Analysis

Samples of untreated and treated straw were analyzed for DM, crude ash, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom), acid detergent fiber expressed exclusive of residual ash (ADFom), acid detergent lignin (ADL), crude protein (CP) (all parameters according to VDLUFA [29]), non-fiber carbohydrates (NFC) = (1000 − (aNDFom + CP + EE + ash)), cellulose = (ADFom-ADL), and hemicellulose = (aNDFom-ADFom) (aNDFom and ADFom values see Tables S1 and S2).

Monomers of non-starch polysaccharides (NSP) were analyzed by the Julius Kühn-Institut in Braunschweig [30]. After starch degradation, acid hydrolysis, and derivatization, the samples were analyzed by gas chromatography (SP-2380 capillary column, 30 m × 0.53 mm × 0.2 µm film thickness, Cat. No. 25319 Supelco, Merck KGaA, Darmstadt, Germany; HP Agilent 6890 GC, Santa Clara, CA, USA).

The BfUL determined minerals and trace elements using X-ray fluorescence (S8 Tiger apparatus, Bruker, Karlsruhe, Germany). Ergosterol from the biological treatments was analyzed according to the following procedure: the air-dry sample (2 mm particle size) was saponified in ethanolic potassium hydroxide solution overnight at ambient temperature. An aliquot of the solution was diluted with a solution of sodium ascorbate. From this solution, ergosterol was extracted and purified by solid phase extraction using a CHROMABONDTM XTR column eluting with cyclohexane (Macherey-Nagel GmbH & Co. KG, Düren, Germany). An aliquot of the eluate was evaporated, dissolved in methanol, and separated on a HyPURITYTM C18 column (Thermo Fisher Scientific, Waltham, MA, USA) via isocratic HPLC. The extinction was measured at 282 nm and quantified by an external calibration using an ergosterol standard (CAS 57-87-4, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

2.5. Statistical Analysis

For the biological treatments, the following effects on chemical composition were tested:

First trial (soaked and drained straw):

$$Y_{ijk} = \mu + Fungus_i + Time_j + Fungus \times Time_{ij} + \varepsilon_{ij} \quad (1)$$

where μ = general mean, i = 1, 2, 3 (*C. subvermispora*, *P. ostreatus* (2 strains)), j = 1, 2, 3, . . . , 7 (0 d, 7 d, 14 d, 21 d, 28 d, 35 d, 42 d fermentation time), and ε_{ijk} = residual error.

Second and third trial (remoistened straw):

$$Y_{ijk} = \mu + Fungus_i + Time_j + Fungus \times Time_{ij} + \varepsilon_{ij} \quad (2)$$

where μ = general mean, i = 1, 2, 3, 4 (control, *V. volvacea*, *C. subvermispora*, *P. ostreatus* PO93), j = 1, 2, 3, . . . , 5 (0 d, (5 d,) 7 d, (10 d), 14 d, (21 d, 28 d) fermentation time), and ε_{ijk} = residual error.

Variance analysis using the procedures univariate and multivariate was performed for the treatments after the respective storage time, and the Tukey test was applied post hoc where indicated. The software IBM® SPSS® Statistics (Version 19, SPSS, Inc., IBM Company©, Chicago, IL, USA) was used.

3. Results

3.1. Temperature Development

No temperature increase $\geq$ 2.0 K above ambient was observed with drained samples. However, with remoistened straw and *V. volvacea* as an inoculant, there was a clear temperature development (Figure 1). The maximum of +11 K above ambient was achieved within the first 7 h and declined thereafter, staying 2 K above ambient even after 4 weeks of storage.

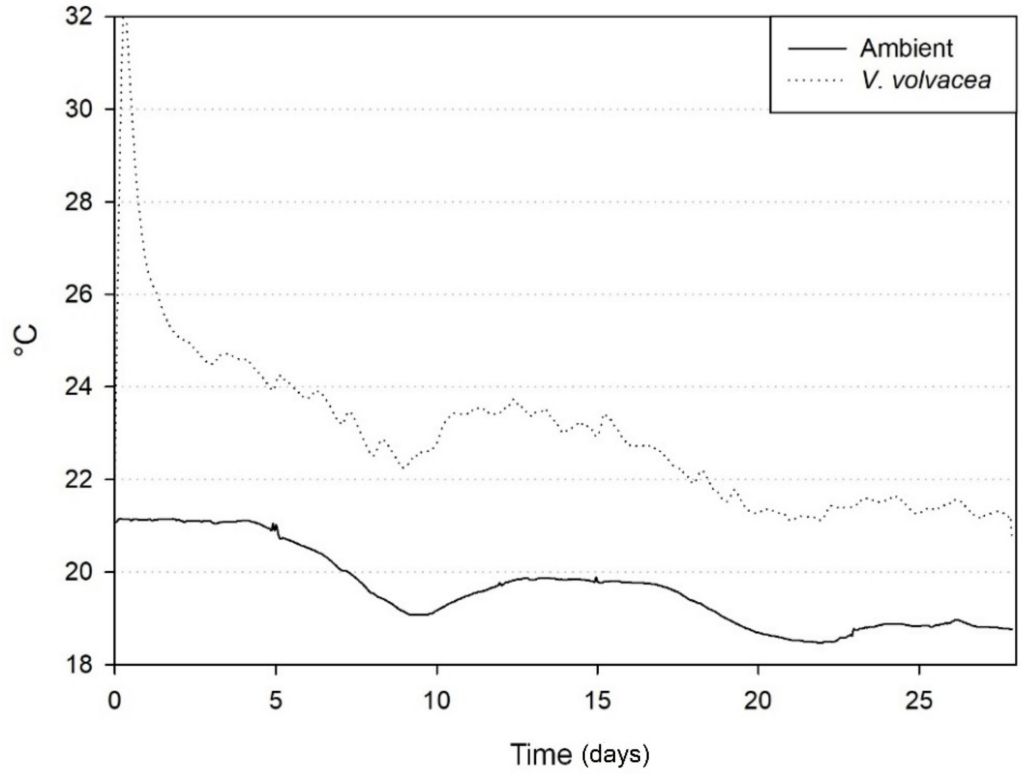

Figure 1. Temperature development in straw inoculated with *V. volvacea* for 28 d.

3.2. Fungal Growth and pH Development

The ergosterol content as an indicator of fungal growth is presented in Figure 2a,b. Fungal strain, as well as storage duration and their interaction, had a significant effect on the ergosterol content (Tables S3 and S4). *C. subvermispora* showed the highest ergosterol

production in the treatments, both drained and remoistened (Figure 2a,b). Only the uninoculated straw with mold growth seemed to achieve similar values (Figure 2b). Overall, the content increased more rapidly in the remoistened samples compared to the drained ones (gradient m = 32.2 or 88.0 for PO93 in the drained or remoistened treatment resp.). After 14 d *C. subvermispora* had produced 291 mg/kg DM more ergosterol in the remoistened straw, and *P. ostreatus* PO 93 266 mg/kg DM more than during the same time in the drained straw, i.e., 2.7 or 3.9 times more, respectively.

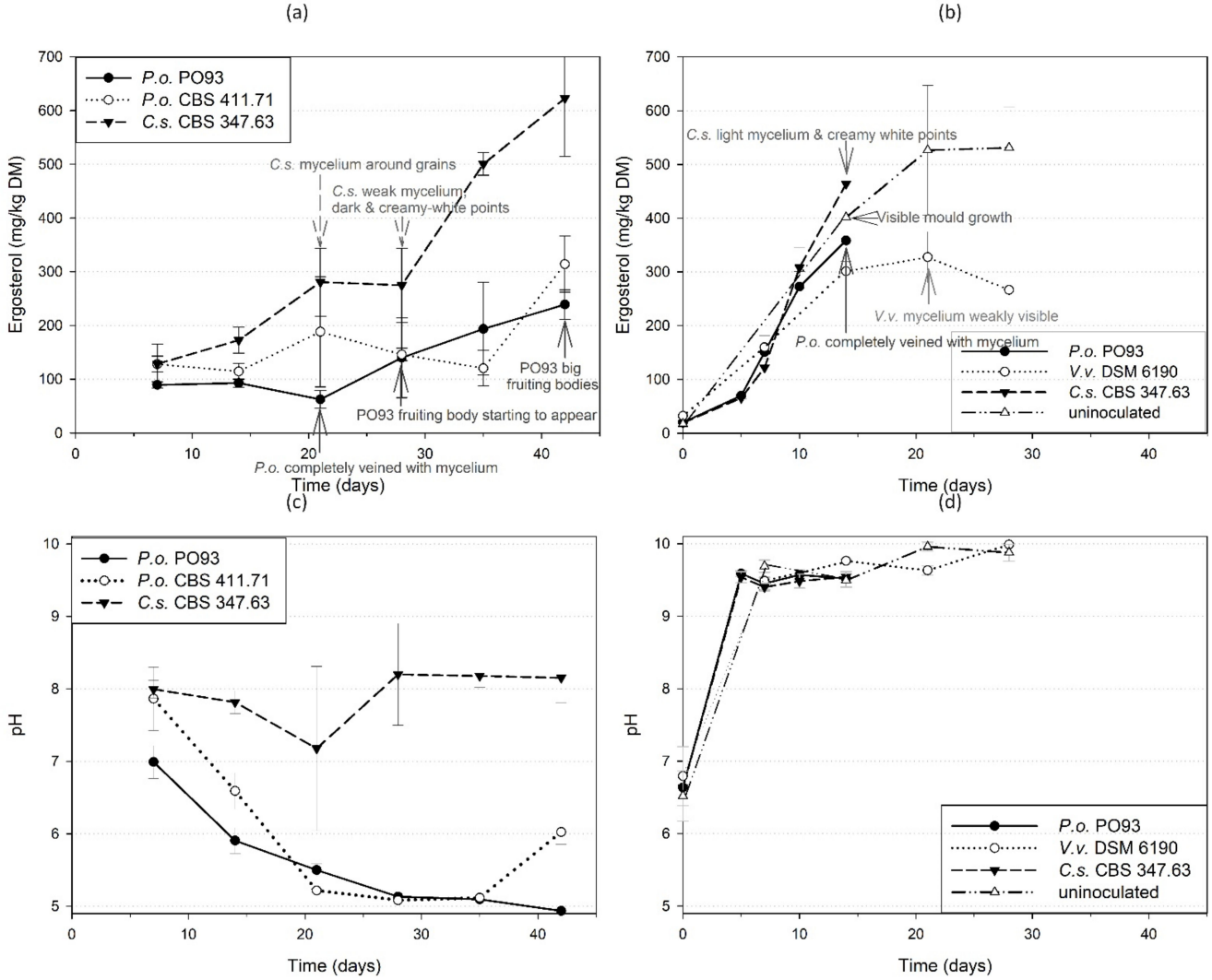

Figure 2. Ergosterol content and pH development in the straw treated with different fungi up to 42 d: ergosterol in (**a**) drained, (**b**) remoistened straw; pH in (**c**) drained, (**d**) remoistened straw. The text attached to the arrows describes the visual appearance and development of the fungi in the straw at the respective time. *P.o.—Pleurotus ostreatus*; *C.s.—Ceriporiopsis subvermispora*; *V.v.—Volvariella volvacea*. Error bars indicate the standard deviation (SD).

This was also reflected in the growth of the mycelium in the straw. A particular observation in *C. subvermispora*-biodegraded straw was the appearance of yellowish-white droplets after 14 d (also in the remoistened straw) to 28 d, which later on turned to dark, hard granules.

Another difference between the drained and the remoistened straw was the pH development, which was also significantly affected by fungal strain and storage duration (Tables S3 and S4). While *P. ostreatus* developed towards an acid pH in the drained straw and *C. subvermispora* was only slightly alkaline, all strains increased rapidly towards an alkaline pH in the remoistened straw (Trials 2 and 3) (Figure 2c,d).

3.3. Accompanying Microflora

In the drained straw, the yeast population presented around 10^5 cfu/g FM on day 7 (Figure 3a). In *C. subvermispora*-biodegraded straw, it declined towards day 14 and increased steeply to $>10^6$ cfu/g FM yeasts at day 21 (Figure 3a). Yeast numbers declined only after 42 d to $<10^4$ cfu/g FM. The yeast population of *P. ostreatus*-biodegraded straw was $>10^4$ log cfu/g FM from day 21 onwards (Figure 3a). Thus, there was a statistical trend of an effect of interaction between fungal strain and storage duration (Table S3). Treatments in remoistened straw presented higher yeast numbers of $>10^6$ cfu/g FM at day 7 and declined thereafter (Figure 3b). However, there was no statistical effect of either fungal 241 strain or storage duration on yeast numbers (Table S2). Yeast numbers of the 242 uninoculated straw remained at a lower level. The mold population in the drained 243 straw was at $>10^5$ cfu/g FM at day 7 with *P. ostreatus* (Figure 3c), while it was at almost 10^7 cfu/g FM in the remoistened treatment (Figure 3d). Total molds in the *P. ostreatus* treatments declined to $<10^5$ cfu/g FM in the drained straw (Figure 3c), while it was $>10^7$ cfu/g FM in the remoistened treatment (Figure 3d). *C. subvermispora* straw remained at a high level of $>10^6$ cfu/g molds from day 21 on in the drained straw (Figure 3c). Thus, only in the remoistened treatments was there a significant effect of storage duration on mold numbers (Table S2).

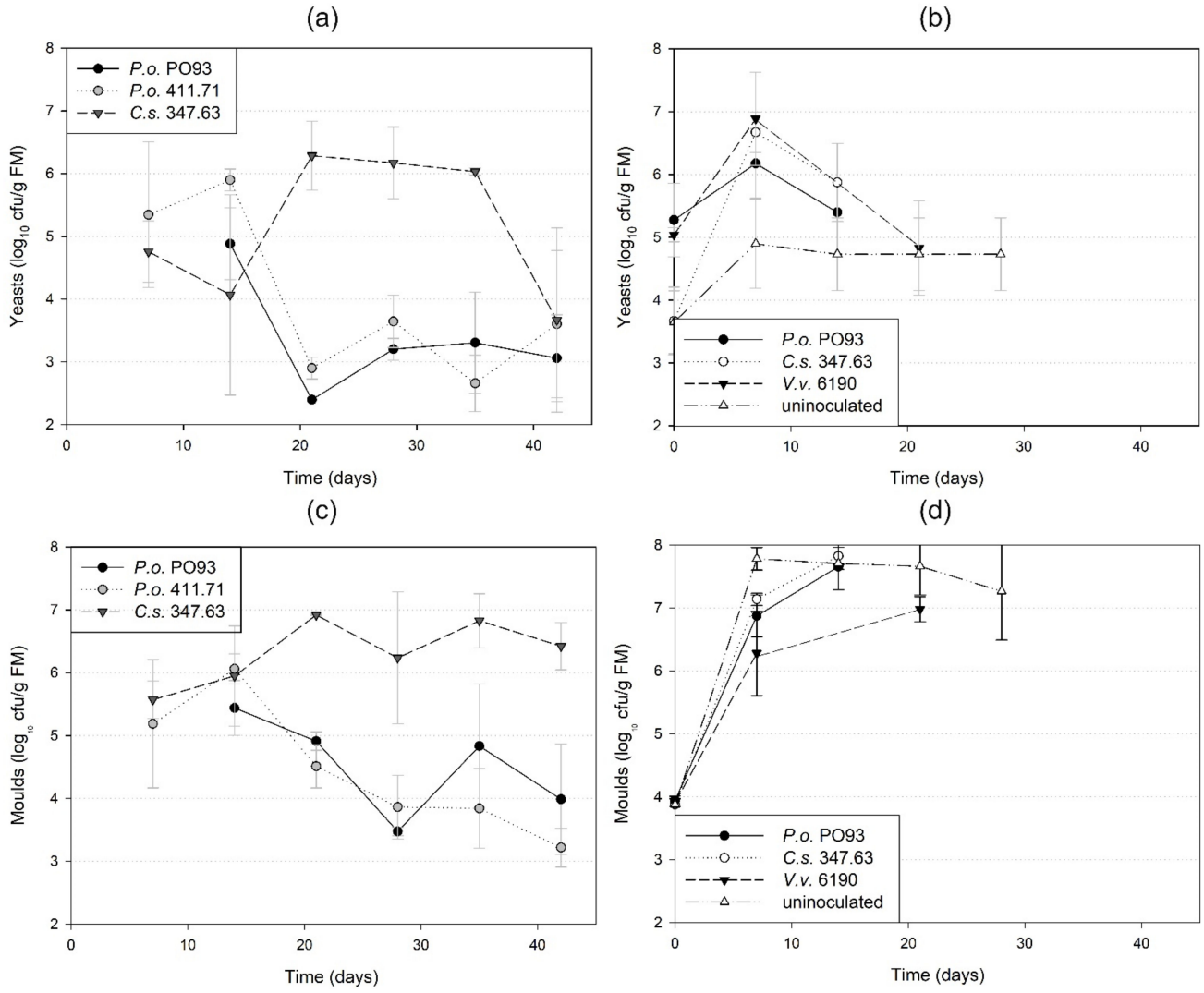

Figure 3. Number of yeasts and molds found on the straw treatments during incubation. Yeasts in (**a**) drained, (**b**) remoistened straw. Molds in (**c**) drained, (**d**) remoistened straw. *P.o.—Pleurotus ostreatus*; *C.s.—Ceriporiopsis subvermispora*; *V.v.—Volvariella volvacea*. Error bars indicate the standard deviation (SD).

In the drained straw, the amount of aerobic mesophilic bacteria remained stable at a high level of >10^8 cfu/g FM when *C. subvermispora* was the inoculant (Figure 4a). The aerobic bacteria leveled at >10^6 cfu/g FM with both strains of *P. ostreatus* (Figure 4a). Still, the slight decline resulted in a significant effect of time (Table S3). In these two latter treatments, the number of lactic acid bacteria rapidly declined after day 14 from initially 10^8 cfu/g FM to finally <10^4 cfu/g FM, while *C. subvermispora*-biodegraded straw remained more or less around 10^7 log cfu/g FM throughout the 6 weeks (Figure 4c). The interaction of time x strain was also highly significant (Table S3). In the remoistened straw, the number of aerobic mesophilic bacteria peaked on day 7 with all three fungi (Figure 4b) (*P. ostreatus*, *C. subvermispora*, *V. volvacea*) at >10^9 cfu/g FM and seemed to decline slightly afterward whereby Trials 2 and 3 stopped after 28 and 14 d (Figure 4b). It was similar in the uninoculated treatment, thus showing only an effect of time (Table S4). The number of lactic acid bacteria was similar for the fungal treatments in the remoistened straw, with slightly higher values for *V. volvacea*, while the uninoculated treatment showed a sharp increase within the first week (Figure 4d). It was only influenced by the inoculant (Table S4).

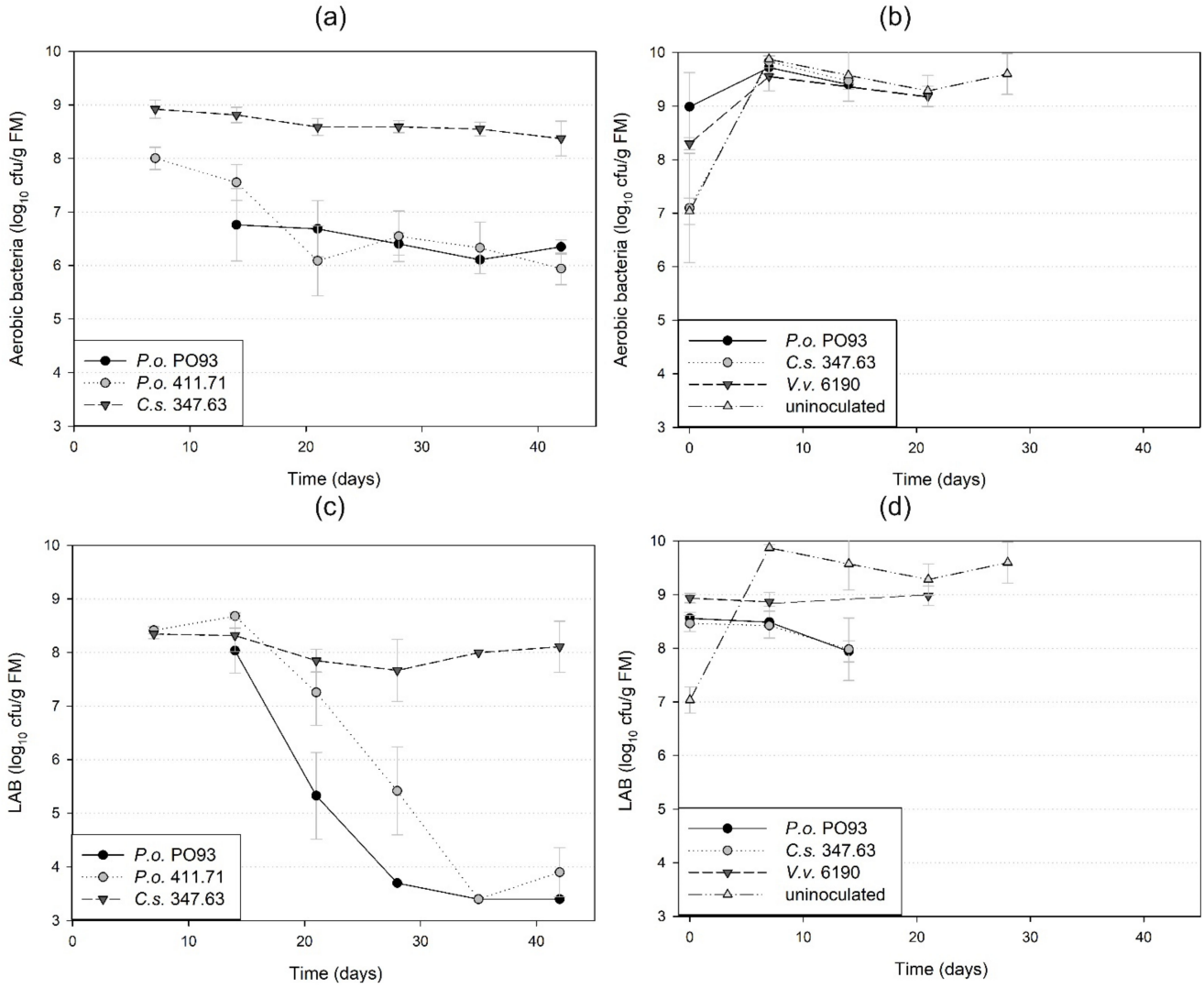

Figure 4. Numbers of aerobic mesophilic bacteria and lactic acid bacteria (LAB) found on straw treatments. Aerobic mesophilic bacteria on (**a**) drained, (**b**) remoistened straw. LAB on (**c**) drained, (**d**) remoistened straw. *P.o.*—*Pleurotus ostreatus*; *C.s.*—*Ceriporiopsis subvermispora*; *V.v.*—*Volvariella volvacea*. Error bars indicate the standard deviation (SD).

Single LAB colonies were picked randomly from MRS plates of Trials 2 and 3 (remoistened straw) and identified by MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry). The numbers of identified genera and species are listed in Table 2, and the relative abundance of genera is shown in the appendix (Figure S2). In the remoistened straw, almost half of the identified colonies belonged to the genus *Weissella* and species *cibaria/confusa*, respectively. This proportion even increased to around 60 % in the *C. subvermispora*-biodegraded straw after 7 and 14 d. The uninoculated treatment with the epiphytic microflora showed a proportion of 43 % *Weissella*. On the contrary, *P. ostreatus* inoculation decreased the *Weissella* population to <10% of the identified genera after 7 and 14 d. Instead, a dominance of *Pedioccus* followed by Lactococcus was seen after 7 d, whereas *Enterococcus* was most abundant after 14 d. In *C. subvermispora*-biodegraded straw, *Pediococcus* was the only other genus that was identified besides *Weissella* on day 14.

Table 2. Lactic acid bacteria species identified by MALDI-TOF and number of their isolates from remoistened straw at different incubation times.

Storage Duration (d)	Treatment	Isolates (n)	Genus (n)	Species (n)
0	Remoistened for 24 h	49	*Enterococcus (2)*	*durans*
				mundtii
			Lactobacillus (12)	*curvatus (6)*
				pentosus/paraplantarum/plantarum (6)
			Pediococcus (8)	*pentosaceus*
			Weissella (24)	*cibaria/confusa*
			unidentified (3)	(3)
7	*C. subvermispora*	8	*Enterococcus (1)*	*casseliflavus*
			Pediococcus (1)	*pentosaceus*
			Weissella (5)	*cibaria/confusa*
			unidentified (1)	
	P. ostreatus PO93	11	*Lactobacillus (1)*	*pentosus/paraplantarum/plantarum*
			Lactococcus (3)	*lactis*
			Leuconostoc (1)	*sp.*
			Pediococcus (4)	*pentosaceus*
			Weissella (1)	*cibaria/confusa*
			unidentified (1)	
	epiphytic microflora (uninoculated)	14	*Enterococcus (5)*	*casseliflavus (1)*
				faecium (1)
				mundtii (3)
			Pediococcus (1)	*pentosaceus*
			Weissella (6)	*cibaria/confusa*
			unidentified (2)	(2)
14	*C. subvermispora*	5	*Pediococcus (2)*	*pentosaceus*
			Weissella (3)	*cibaria/confusa*
	P. ostreatus PO93	16	*Enterococcus (4)*	*casseliflavus(2)*
				gallinarum (2)
			Lactobacillus (1)	*pentosus/paraplantarum/plantarum*
			Lactococcus (1)	*lactis*
			Leuconostoc (2)	*citreum*
			Pediococcus (1)	*pentosaceus*
			Weissella (1)	*cibaria/confusa*
			unidentified (6)	(4), (1), (1)

3.4. Changes in the Chemical Composition

3.4.1. Fiber Fractions

During the first week, in the drained treatments, there was a general trend of increasing cellulose and decreasing hemicellulose contents with all three strains (Figure 5a,b).This

effect of time, also in interaction with fungal strain, was significant (Table S3). Hemicellulose content was significantly lower after 42 d (mean of 291 g/kg DM) compared to the beginning (364 g/kg DM) (Figure 5b). However, ADL decreased numerically only with *C. subvermispora* after 7d (56.0 g/kg DM), which increased from then on almost linearly to 89.5 g/kg DM after 42 d (Figure 5c), which was significantly higher than at day 0. With the two *P. ostreatus* strains, ADL content was more oscillating (Figure 5c). There was a significant effect of inoculant, time, and their interaction on the ADL content (Table S3).

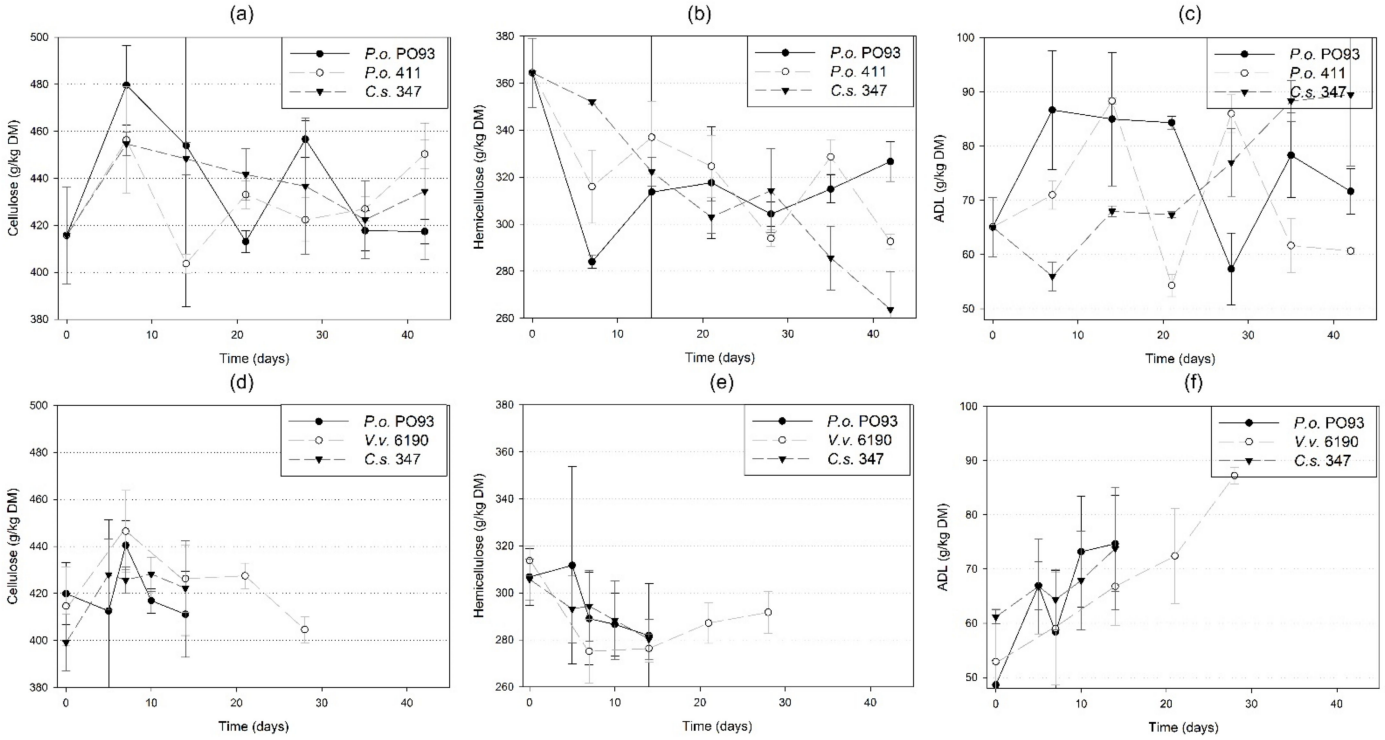

Figure 5. Cellulose, hemicellulose, and ADL concentrations in drained (**a**–**c**) and remoistened (**d**–**f**) straw treatments. *P.o.*—*Pleurotus ostreatus*; *C.s.*—*Ceriporiopsis subvermispora*; *V.v.*—*Volvariella volvacea*. Error bars indicate the standard deviation (SD).

In the remoistened straw, there were no significant differences between the strains concerning cellulose, hemicellulose, and ADL contents (Figure 5d–f). Only in the uninoculated treatment, the hemicellulose concentration decreased from 311 g/kg DM to <240 g/kg DM after 14 d (not shown). Generally, hemicellulose concentration was initially lower than in the drained treatments (Figure 5e). There was a linear increase in ADL with *V. volvacea* from initially 52.9 to 87.2 g/kg DM after 28 d (Figure 5f). Thus, there was only an effect of time on the fiber components here (Table S4).

3.4.2. Non-Fiber Carbohydrates

Soaking and draining alone led to a halving of the NFC content compared to remoistening only (0 d) (Table 3). In the drained straw treatments, it increased again towards day 21 as hemicellulose decreased. In both the drained and remoistened straw fungi, time and their interaction had an effect on NFC content (Tables S3 and S4).

Table 3. Non-fiber carbohydrates (g/kg DM) in the different fungal treatments after varying storage time (d).

Strain	***P.o.* PO 93**	***P.o.* PO 93**	***P.o.* 411**	***C.s.* 347**	***C.s.* 347**	***V.v.***
	drained	remoistened	drained	drained	remoistened	remoistened
Trial	1	3	1	1	3	2
0 d	54.9	127	54.9	54.9	106	106
5 d		109			82.2	
7 d	53.3	113	52.7	41.3	78.3	123
10 d		112			79.6	
14 d	43.7	128	65.3	65.7	81.8	127
21 d	89.3		92.0	77.7		81.4
28 d	95.3		101	51.3		79.0
35 d	99.0		80.0	71.3		
42 d	78.7		98.0	75.0		
mean	76.6	118	81.6	64.3	85.6	103
SEM	1.90	3.09	1.90	1.90	3.09	3.09

P.o.—Pleurotus ostreatus; *C.s.—Ceriporiopsis subvermispora*; *V.v.—Volvariella volvacea*. SEM standard error of the means.

3.4.3. Non-Starch Polysaccharides

In the original straw, 545 g/kg DM NSP was found. More than half of the monomers were cell wall glucose, followed by xylose, and far behind arabinose, galactose, and, last, mannose (Table 4). After 28–42 d, total NSP had reduced by about 8.8 % in DM with all mushrooms in the drained straw (figures not shown). *C. subvermispora*-biodegraded straw presented the highest relative increase in mannose by more than doubling, while the absolutely highest loss was in glucose with 47 g/kg DM. With both *Pleurotus* strains, mannose also increased, however, to a lesser extent (~40% after 42 d), and glucose reduced by 33 g/kg DM. A significant effect of time and its interaction with the inoculant on glucose and mannose content was found (Table S1). The ratio of xylose:arabinose increased significantly by time with *C. subvermispora* towards 11.4 (i.e., a factor of 1.6 compared to the original straw, Table 4) during the weeks 3–6 of fermentation, while with both *P. ostreatus* strains there was only a trend towards a higher ratio with increasing fermentation duration ($p < 0.1$).

Table 4. Content and proportion of polysaccharide monomers in untreated wheat straw.

Monomers	**g/kg DM**	**% of NSP**
Arabinose	28.22	5.17
Xylose	220.35	40.4
Mannose	1.98	0.36
Galactose	6.90	1.27
Glucose	287.96	52.8
Xylose/Arabinose	Ratio 7.01	

NSP Non-starch polysaccharides.

3.4.4. Minerals and Trace Elements

Generally, the crude ash content increased over time: from 56.7 g/kg DM in the drained control to 70.0 g/kg DM in straw with *C. subvermispora* (almost linearly) and 66.0 g/kg DM with *P. ostreatus* PO 93 (oscillating) and 59.0 g/kg DM in *P. ostreatus* CBS 411.71 after 42 d (oscillating) (Trial 1). During the shorter storage period of Trial 3 (and Trial 2), the increase from initially 85.1 g/kg DM in the remoistened straw to a mean of 92.1 g/kg DM after 14 d was not significant (Table S4).

Drained straw with both *P. ostreatus* strains increased significantly in Ca and Si while decreasing in K (Trial 1, Figure 6a,b). Thereby Si rose most with PO 93 from 15.3 to 21.5 g/kg DM by more than one-third within 42 d (Figure 6a). *C. subvermispora* showed

the same general behavior; only the K content remained similar to the control over time (Figure 7c).

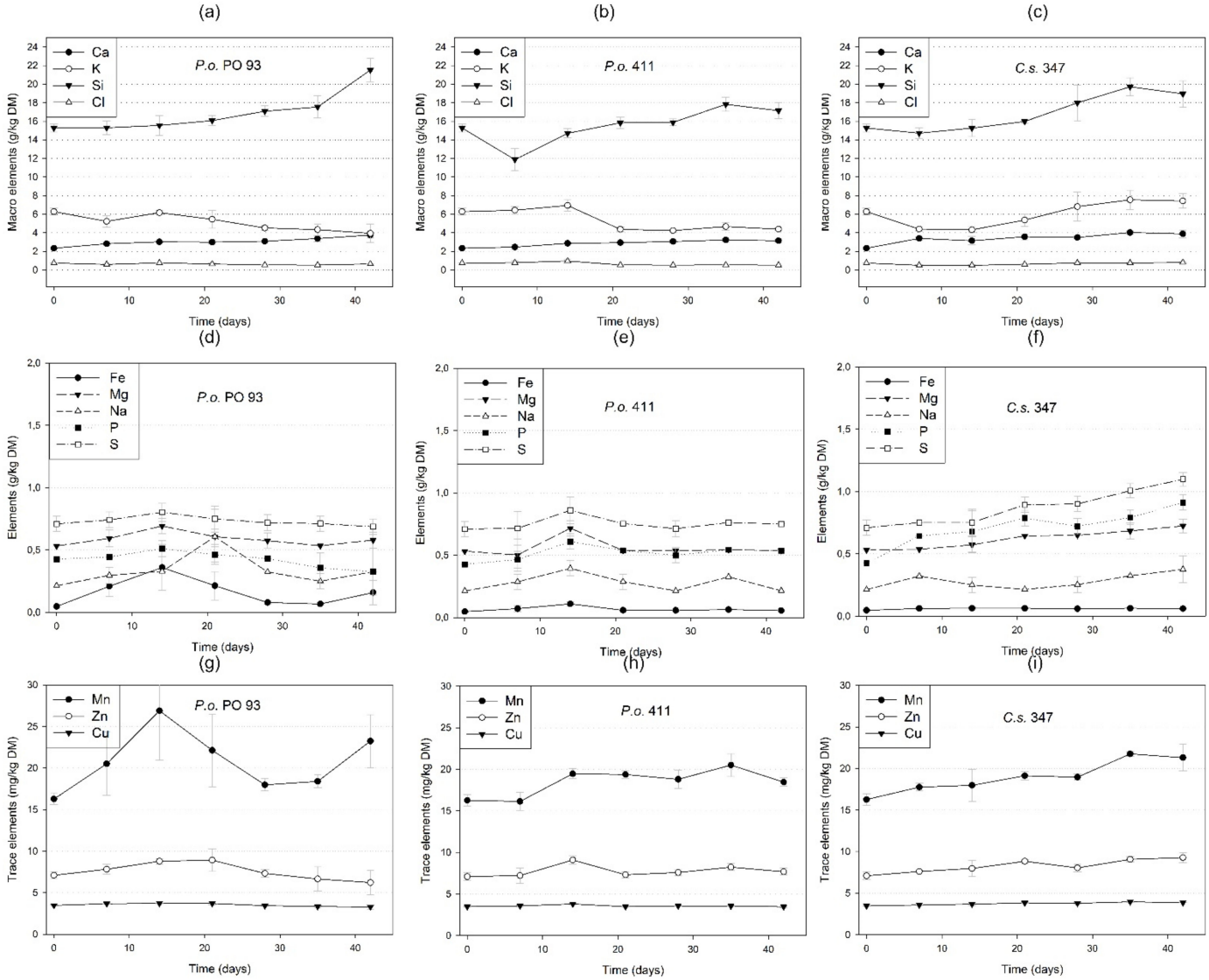

Figure 6. Macro and trace elements in fungi treated drained straw during 42 d of incubation. **Left** (**a**,**d**,**g**): inoculated with *Pleurotus ostreatus* (*P.o.*) PO93, **center** (**b**,**e**,**h**): with *Pleurotus ostreatus* (*P.o.*) CBS 411.71, **right** (**c**,**f**,**i**): with *Ceriporiopsis subvermispora* (*C.s.*) CBS 347.63. First row (**a**–**c**): macro elements, second row (**d**–**f**): elements up to 2 g/kg DM, third row (**g**–**i**): trace elements. Error bars indicate the standard deviation (SD).

In the drained straw, S did not change over time with *P. ostreatus* strains, while it increased steadily with *C. subvermispora* from 0.71 to 1.10 g/kg DM after 42 d (Trial 1, Figure 7d–f). There was no significant effect of time on P content in PO 93 straw and only a slight peak in the second *P. ostreatus* strain CBS 411.71 after 14 d (Figure 6d,e). In contrast, drained *C. subvermispora*-biodegraded straw more than doubled in P from 0.426 to 0.911 g/kg DM within 42 d (Figure 6f). Cl content was the highest (0.966 g/kg DM) after 14 d in straw with *P. ostreatus* CBS 411.71 (Figure 6b) and declined thereafter. Peaks in Mg, Na, and Fe were also observed at that time. The highest sodium content (0.606 g/kg DM) was noticed in PO 93 straw after 21 d (Figure 6d). Moreover, there was a noteworthy multiplication (>7fold) of Fe towards day 14 in the same treatment. Slight changes occurred in the content of the trace elements Cu and Zn over time in all three treatments, while Mn concentrations showed a more pronounced behavior in PO 93 after 14 d, increasing by

165% (Figure 6g–i). Effects of strain, time, and their interaction were most significant in the drained straw except for Ca (Table S3).

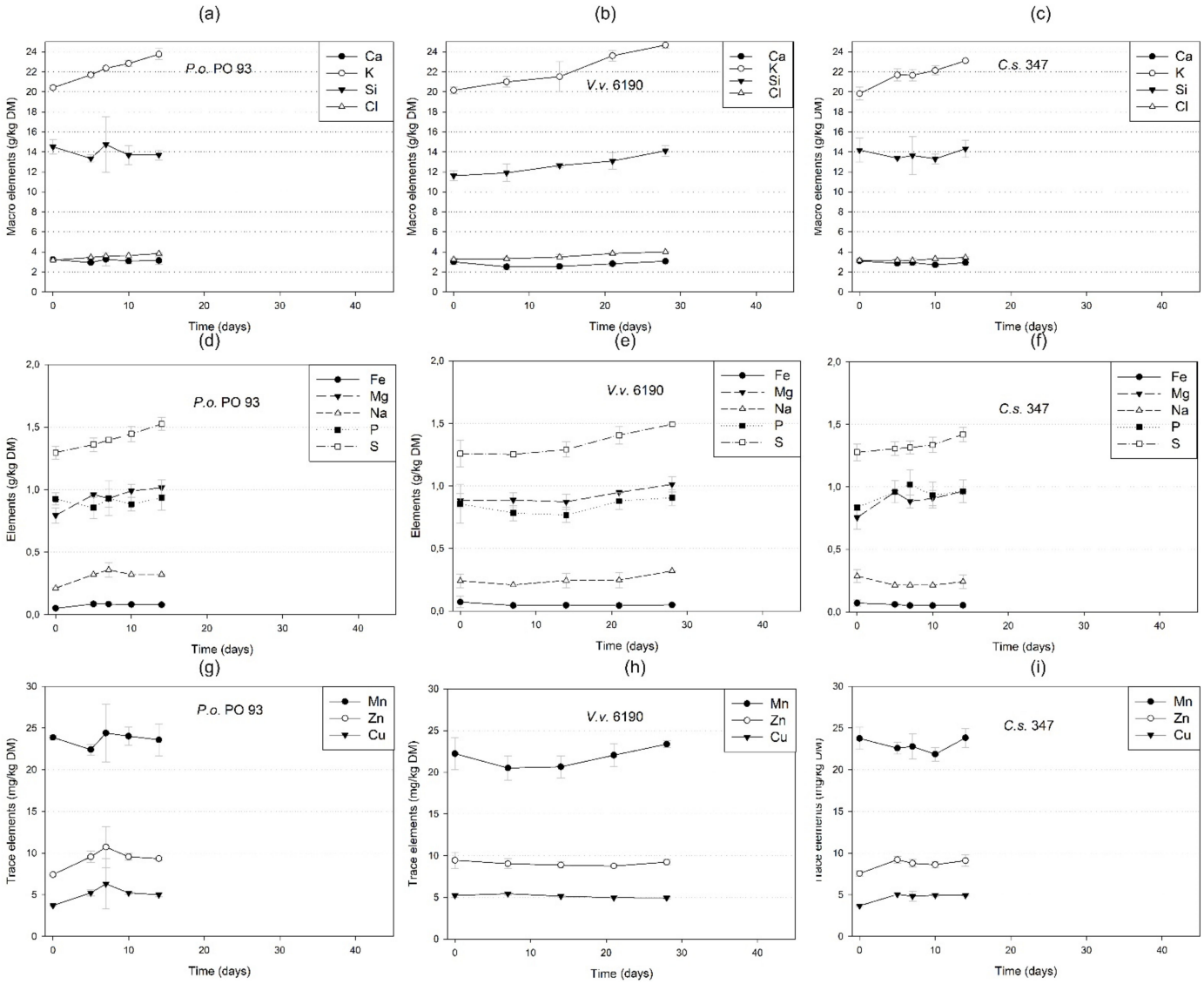

Figure 7. Macro and trace elements in fungi treated remoistened straw during up to 28 d of incubation. **Left** (**a**,**d**,**g**): inoculated with *Pleurotus ostreatus* (*P.o.*) PO93, **center** (**b**,**e**,**h**): with *Volvariella volvacea* (*V.v.*), **right** (**c**,**f**,**i**): with *Ceriporiopsis subvermispora* (*C.s.*) CBS 347.63. First row (**a**–**c**): macro elements, second row (**d**–**f**): elements up to 2 g/kg DM, third row (**g**–**i**): trace elements. Error bars indicate the standard deviation (SD).

In contrast to the drained treatments, the K content was more than three times higher in the remoistened straw and even increased during incubation (Figure 7a–c). Likewise, S content was about double that of the drained treatments (Figure 7d–f). Even Mn started at a higher level (Figure 7g–i). However, in contrast to the drained treatments, the effect of the interaction of time x strain was limited to K, Cl, Mg, Na, Fe, and Zn (Table S4).

In the remoistened uninoculated control straw, no significant change occurred in the analyzed contents of K, Ca, P, Na, Fe, Mn, Zn, and Cu during the incubation time of 28 d (values not shown).

4. Discussion

4.1. Fungal Growth

Mycelial growth was enhanced in remoistened straw treatments. It was at least one week ahead of the drained treatments. This could also be seen in the granules, which appeared earlier in the remoistened straw inoculated with *C. subvermispora*. These "droplets" were interpreted as secreted enzymes by Thomsen et al. [31]. *V. volvacea* growth increased during the first two weeks of fermentation, then stagnated towards day 21 and further decreased. This might be explained by the cellulose/lignin ratio, which was highest during the first 7 days and declined afterward. It is reported to be correlated to mycelial growth rates [32]. Furthermore, the study confirms the lower conversion efficiency of the substrate to fruiting bodies compared to *P. ostreatus* [24]. In our trials, a high pH, which occurred in the remoistened treatments, was no indication of poor fungal growth, as assumed earlier by Nayan [12]. Some authors recommend stabilizing the pH with CaCO3, both in grain spawn production and in the SSF with straw and other substrates [26,33]. A ruminal pH of 6 to 7.5 was optimal for the adhesion of cellulolytic microorganisms to plant cell walls [34]. For *P. ostreatus*, Fernandez-Fueyo et al. [35] found that most of the nine genes of ligninolytic peroxidases worked well at pH 6, while two genes for Mn peroxidase upregulated at pH 8. The best conditions for the production of laccase or Mn peroxidase were at pH 4.8 for different white-rot fungi [36]. For *C. subvermispora* it was at pH 4.5 under laboratory conditions [37]. In general, fungal laccases work well in an acidic pH range [38]. This might be a reason why no lignin degradation was observed in the presented trials, although pH decreased with *P. ostreatus* in the drained treatments.

Enhanced growth may be explained by the availability of NFC, which had about twice the concentration in the remoistened straw. This might also be a reason for the initial heat production from 21 to 32 °C in Trial 2 (remoistened) with *V. volvacea*. Excessive heating was mentioned as one of the main obstacles in upscaling solid-state fermentation [39], while optimal temperature conditions vary according to species [33], and a certain rise in temperature also increased organic matter and partly lignin decomposition [9]. In many trials, *P. ostreatus* and *C. subvermispora* were grown between 24 and 26 °C [40–43]. For *V. volvacea*, the storage temperature differences were more pronounced, between 24 °C [43] and 35 °C [23].

4.2. Fibre and Minerals

Either of the treatments compared to day 0 significantly reduced neither ADL nor cellulose concentration. Van Aken and Agathos [44] stated that nutrient limitation is necessary to induce the ligninolytic system. However, even the drained treatments after 6 weeks of fermentation did not present any ligninolysis in our experiment. This is in contrast to decreasing lignin values found for *P. ostreatus* CBS 411.71 by Salvachua et al. [21] and also for *C. subvermispora* CBS 347.63 by Nayan et al. [12] and Srebotnik and Messner [26]. On the other hand, even alkaline treatment with a clear effect on digestibility did not reveal any variation in lignin structures by ^{13}C spectra [27].

Van Kuijk et al. [22] explained the delignification process of *C. subvermispora* and *L. edodes* fungi as follows: there are two phases: in the first phase, easily fermentable carbohydrates (starch, pectin, and part of hemicelluloses) are degraded; thus in vitro gas production (IVGP) will be decreased, and no significant lignin degradation occurs. The second phase is the delignification phase, which started measurably after 3 weeks with *C. subvermispora* and *L. edodes* and resulted in increasing IVGP in their experiments. Following this sequence, a group of authors found out with wood that some fungi have a high initial selectivity for lignin, while they, later on, change to cellulose degradation [45–48]. On the other hand, the highest laccase activity was measured already after 6 d with the saprophyte *Coprinopsis cinerea*, reducing rapidly thereafter [49]. For *V. volvacea*, laccase is described to dominate during sporophore development [32]. An explanation of the apparently lacking decrease in lignin concentration might be that the accompanying microflora used up the carbohydrates, which were released by the fungi and were more easily metabolized.

A general increase in the concentration of minerals and elements (crude ash) can be explained by losses of organic matter. However, varying changes in the content of single minerals are more complex to interpret. They must be related to the inoculum, as those changes did not happen in the uninoculated straw. It was a relatively closed system, except for the condensed water, where the mycelium was included in the substrate analysis. Only in the case of PO 93, fruiting bodies, which started to appear from day 28 on in Trial 1, were excluded from the analysis. Minerals play varying roles in the metabolism of wood-decaying fungi. Mn, for example, is essential for the glycoprotein manganese peroxidase found in *C. subvermispora*, which allows the oxidation of phenolic substrates such as phenolic lignin compounds and involves Fe in its catalytic reaction [50]. Additional Mn also enhanced lignin degradation in cotton stalks by *P. ostreatus* [51]. In our case, Mn content increased temporarily in straw inoculated with PO 93. Copper atoms form the catalytic center of laccases [38]. Time and fungal strain had a significant effect on copper contents in our study. A high sulfur content limits the lignin utilization by fungi [52]. This might be another explanation for why lignin was not degraded. However, it is no reason for the increase in S content in *C. subvermispora*-biodegraded straw. The temporary increase in Mg in the *P. ostreatus* treatments may point to the adhesion of bacteria to the plant cell wall [53], the same as Ca [54]. The higher level of K, S, and Mn in the remoistened treatments point to their water solubility in the substrate, a reason why they were washed out with soaking and draining. For K, the same effect of leaching was observed by Jenkins et al. [39].

4.3. Sterilization of Substrate

In the attempt to open up the option for on-farm upscaling, the straw was not autoclaved in the presented experiments (see [9]) nor finely milled, and higher volumes were handled in contrast to many laboratory studies on biological fiber treatment (e.g., [41,55,56]). In a manual for hobby cultivation of mushrooms, it was stated that usually competing fungi will be overgrown by the inoculated ones [27], whereas Milenkovic [57] recommended intensive washing to hygienize the straw. In a comparison between the soaked and drained straw and the remoistened straw in the presented trials, the plate count of the accompanying microflora actually showed lower numbers in the former. However, none of the inoculated treatments showed any signs of deterioration by bacteria or molds in the optical and sensory evaluation. Look and smell were completely dominated by the inoculated mushroom. This is also supported by the observations of Wan and Li [58] and Zhang et al. [49].

On the other hand, the effect of autoclaving is not only in sterilization. It changes both the physical [49] and chemical characteristics [43]. Zhang et al. [49] found that mycelial growth was enhanced by preceding autoclaving.

Another option to sterilize the straw was discussed by Akhtar et al. [59] and Thomsen et al. [31]. They suggested chemical disinfection with sodium bisulfite (or metabisulfite or hydrosulfite), while 15 s of atmospheric steaming were also sufficient to outcompete epiphytic bacteria and fungi. Handling and economic viability have to be considered for the on-farm application.

4.4. Accompanying Microflora

High numbers of aerobic bacteria, yeasts, and molds were counted, especially in the remoistened treatments. However, this did not affect the sensory quality of the straw, which developed a typical smell of mushroom in the case of *P. ostreatus* and *V. volvacea* and a characteristic smell with *C. subvermispora*. In no case it smelled moldy. The basidiomycetes dominated the process, and there might have been bacterial-fungal interactions of mutual control and support [60,61]. Blanchette et al. [60] found that some bacteria and yeasts accelerated the decay of wood in combination with white-rot fungi. On the other hand, the accompanying microflora might have used up the easily accessible nutrients which were released by the basidiomycetes, as mentioned before.

4.5. Lactic Acid Bacteria

Independent of the different pH, which developed under "drained" and "remoistened" conditions, a high number of LAB was observed within the first 14 d under humid but aerobic conditions. In the second and third trial some of the colonies which had grown culture dependently could be identified. Although it was not a quantitative analysis, it may be assumed that the colonies randomly picked from the culture medium represented a certain predominance in the original material. Thus, a shift throughout the storage time and depending on the inoculated fungus could be observed. *Weissella cibaria/confusa* was mainly observed directly after remoistening and in the *C. subvermispora* treatment. Zotta et al. [62] found that under aerobic conditions, the growth of *Weissella* improved without acidification. It showed activity up to pH 10 and grew rapidly up to pH 9 in MRS broth [63]. The second most prevalent genus was *Pediococcus* after 14 d in *C. subvermispora*-biodegraded straw and after 7 d in PO 93 straw. This LAB is facultative anaerobic [64]. *Enterococcus* dominated in the uninoculated control after 7 d and was also present after 14 d in PO 93 straw. Wahyudi et al. [65] found that *Enterococcus casseliflavus/gallinarum* could degrade lignocellulolytic substances [66], which would explain its occurrence in straw. From the *P. ostreatus*-biodegraded straw, *Leuconostoc* was identified. In particular, *Leuconostoc citreum* is an aerobic LAB that can act in a wide pH range [67]. Some isolates could not be identified.

5. Conclusions

Growth conditions for mushrooms were optimized by remoistening the straw with a defined amount of water instead of soaking and draining as classically recommended. On the other hand, lignin degradation was not observed with either of the treatments, which might have been related to suboptimal pH conditions or microflora competing for nutrients. The interaction of the different microbial groups during SSF is a field for further studies. For feeding purposes, feasible sterilization options other than autoclaving should be evaluated. Compared to *Pleurotus*, *Volvariella* is a rather demanding fungus less suitable for lignin degradation. If SSF with edible mushrooms such as *P.o.* is practiced for the dual purpose of food and feed, the tested pretreatment of remoistening with a defined volume of water might be a feasible approach for farmers.

Supplementary Materials: The following supporting information (appendix) can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8120695/s1, Figure S1: Flowchart of the experiments carried out in the study; Figure S2: Distribution of LAB genera (in % of isolates) isolated from the remoistened straw inoculated with different fungi after 0, 7, and 14 d of incubation; Table S1: Neutral-detergent fiber content (g/kg DM) in the different fungal treatments after varying storage time (d); Table S2: Acid-detergent fiber content (g/kg DM) in the different fungal treatments after varying storage time (d); Table S3: Significance of effects on chemical parameters and microbial counts in drained inoculated straw (Trial 1); Table S4: Significance of effects on chemical parameters and microbial counts in remoistened inoculated straw (Trials 2 and 3).

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