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Organic Farm Bedded Pack System Microbiomes: A Case Study with Comparisons to Similar and Different Bedded Packs

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Abstract: Animal housing and bedding materials influence cow and farm worker exposure to microbial pathogens, biocontrol agents, and/or allergens. This case study represents an effort to characterize the bacterial and fungal community of bedding systems using an amplicon sequencing approach supplemented with the ecological assessment of cultured Trichocomaceae isolates (focusing on *Penicillium* and *Aspergillus* species) and yeasts (Saccharomycetales). Bedding from five certified organic dairy farms in northern Vermont USA were sampled monthly between October 2015 and May 2016. Additional herd level samples from bulk tank milk and two bedding types were collected from two farms to collect fungal isolates for culturing and ecology. Most of the microorganisms in cattle bedding were microbial decomposers (saprophytes) or coprophiles, on account of the bedding being composed of dead plant matter, cattle feces, and urine. Composition of bacterial and fungal communities exhibited distinct patterns of ecological succession measured through time and by bedding depth. Community composition patterns were related to management practices and choice of bedding material. *Aspergillus* and *Penicillium* species exhibited niche differentiation expressed as differential substrate requirements; however, they generally exhibited traits of early colonizers of bedding substrates, typically rich in carbon and low in nitrogen. *Pichia kudriavzevii* was the most prevalent species cultured from milk and bedding. *P. kudriavzevii* produced protease and its abundance directly related to temperature. The choice of bedding and its management represent a potential opportunity to curate the microbial community of the housing environment.

Keywords: *Aspergillus*; compost-bedded pack; loose-housing systems; *Penicillium*; *Pichia kudriavzevii*; Saccharomycetales; Sanger sequencing; Trichocomaceae

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1. Introduction

Dairy cattle housing and bedding systems influence animal health, reproduction, milk quality, animal well-being, productivity, and farm profitability [1]. Understanding implications of microbiome relationships between the environment and the health of animals offers a holistic perspective on the effects of farm management. Microbiomes may link bedding, teat, rumen, and milk, each of which are affected by myriad other factors including diet, season, and the milking management system. In 2014, tie-stall and free-stall housing systems were the most common housing types for lactating cows among all dairy operations in the USA [2]. Bedded pack barns are receiving increased attention as a type of open communal housing for dairy cows [3,4].

Bedded pack systems use carbon-rich substrates such as wood shavings, sawdust, straw, or wood chips to create a comfortable and clean surface on which animals move freely [5]. Fecal matter is not removed when bedding material is renewed, in contrast with other housing systems [6]. Bedded pack systems can be coarsely grouped into two management strategies. "Deep bedded pack" (DPB) is an untilled system in which strata of bedding and feces and urine accumulate throughout the bedding period [1]. Oxygen necessary for aerobic decomposition is retained in the system by the selection of bedding

material and timing of application. A mechanically aerated bedded pack, commonly referred to as a "compost bedded pack" (CPB), typically uses bedding material with smaller-sized particles and relies primarily upon a tractor-drawn chisel or rotary tiller to maintain oxygen in the system and bring up partially decomposed, heat-dried bedding material to the surface to be re-used.

Bedded pack feedstocks likely contain unique microbiota [7]. The ratios of sawdust, wood chips, and straw vary greatly based on season and differences in farm management practices. In DBP, the absence of mechanical aeration results in a bedding architecture with three distinct zones: a dry top layer of hay or wood shavings, which fosters aerobic organisms, below the dry layer is a thermophilic zone, and below the thermophilic zone is a water-saturated anoxic zone (Figure 1). Aerobic and thermophilic microbes begin to decompose manure and bedding, generating heat and indirectly drying the surface layer [8]. Thus, the type and management of feedstock alters the microbial community of compost, changing rate of decomposition, and heat production [9].

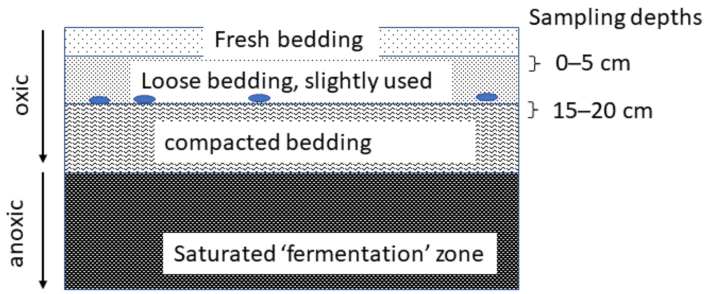

Figure 1. Vertical stratification of compost bedded pack. Loose fresh bedding was removed and two layers of oxic conditions were sampled. The upper (0–5 cm) layer was a relatively dry zone with slightly used, loose bedding. The second layer (15–20 cm) layer was compacted and thermophilic. Temperature and oxygen measurements (ovals) were taken at the interface between two oxic layers, not the saturated 'fermentation' zone.

While there is no standard for bedded pack construction among pasture-based dairy farms, the winter housing season in the Northeast US begins when standing forage is no longer available and temperatures decrease, typically by mid-October. Bedding and feces accumulate throughout the winter until cows can return to pasture, typically by mid-May or later. Pack depth by May ranges between 0.5 and 1.5 m. Strata vary in compaction, degree of decomposition, moisture, and temperature.

The aim of this study was to describe the basic ecology of detrital communities associated with bedded pack systems and determine whether the results could be extrapolated to other organic dairies. The study is presented in two phases. Phase 1 was part of an extensive case study that examined the microbiomes of environments around and within healthy and mastitic dairy cattle through time. To assess the generalizability of the case study, bedding samples were expanded to include additional farms, two with similarly and two with differently constructed bedded packs (Table 1, Table S1). To determine bedding microbiomes through time and between farms, amplicon sequencing enumerated the microbial community. Phase 2 used data from phase 1 to focus on the isolation and culture of two contrasting groups of fungi, Trichocomaceae and Saccharomycetales. Trichocomaceae dominate indoor environments and may impact air quality [10,11]. Furthermore, species in the family represent both the earliest decomposers and later successional communities. Many members of the family produce copious and varied exudates that impact local microbiomes [12]. Most are obligate aerobes, although hemolytic activity is noted in a few *Aspergillus* spp. [13,14]. Determinations on these isolates focused on environmental factors related to colonization and decomposition. In contrast, Saccharomycetales are more closely associated with the presence of cattle. Members of the order represent many facultative anaerobes and several opportunistic pathogens. Ecological characters determined for the yeasts focus on factors that could lead to an opportunistic udder infection.

Table 1. Bedding material, stocking density and median temperature and oxygen for each farm. Wood shavings were dried compressed pine, sawdust was freshly milled pine. Wood chips were unidentified type, except Farm C used hardwood chips. Hay was farm-grown. Straw was purchased (Farm S) or farm-grown (B). Primary bedding represents approximately 75% of bedding, whereas secondary bedding represents 25%. Bedding style is either deep bedded pack (DBP) or compost bedded pack (CBP). More management details for each farm are available in Table S1.

Management Practice	Farm Name				
	C	B	D	L	S
Bedding Style	DBP	DBP	DBP	CBP	DBP
Primary Bedding	hay	hay	hay	sawdust	straw
Secondary Bedding	wood chips	wood chips, straw	wood chips	hay	wood shavings
Breed	Holstein—Jersey cross	Jersey	Jersey	Holstein—Jersey cross	Jersey
Stocking Density (m^2/cow)	8.18	7.43	8.23	9.3	12.01
Bedding (kg/cow/day) [a]	4.14	2.45	12.47	5.33	5.02
Median Temperature °C	30.5 (n = 12)	41 (n = 4)	36 (n = 3)	33.5 (n = 4)	13 (n = 2)
Median Oxygen (%)	8.66 (n = 9)	1.23 (n = 4)	2.3 (n = 3)	2.10 (n = 4)	7.8 (n = 1)

[a] Farmer reported data.

2. Materials and Methods

2.1. Experimental Design

2.1.1. Micobiomes

Bedding was collected from five certified organic dairy farms using a bedded pack system. The farms were located within an 80 km radius of Burlington, VT, USA (Table 1). Samples were collected between October 2015 and May 2016 when pastures were inaccessible (due to cold or wet conditions) to these pasture-based dairies. Bedding was collected on seven dates at a reference farm (Farm C). Farm C is well characterized from a previous study that compared udder microbiomes of cows that were healthy and those with mastitis [15] and is the farm presented in the greater case study. The farm was a 150-cow, certified organic, pasture-based dairy with a mix of Holstein and Jersey breeds. Bedding samples were collected at an additional four organic dairies (B, D, L, S) at six dates between November 2015 and May 2016 (on concurrent days as Farm C) to discern whether the findings could be applied more generally. Farms B and D were managed similarly to Farm C. However, Farm L used tilled wood shavings and Farm S the straw bedded pack was mesophilic and less sheltered. All bedded packs housed lactating animals except Farm S, which dried off during the winter months. Temperatures in the study region reached a mean monthly low of −12 °C in January and mean monthly high of 21 °C in May. The bedding pH in these systems ranged from 5.2 to 8.3 (Figure 2).

2.1.2. Isolations

Herd level samples from bulk tank milk and two bedding types (tie-stall and bedded pack) were collected in December 2019, January 2020, and February 2021 from two farms. One of the farms (C) was the same sampled for amplicon sequencing (Table 1). The second farm (M) relates to the student-run dairy at University of Vermont. Farm M is a tie-stall barn with sawdust on rubber mattresses, with bedding renewed daily. Farm M was chosen as an accessible source of milk for yeast isolations, not the bedding.

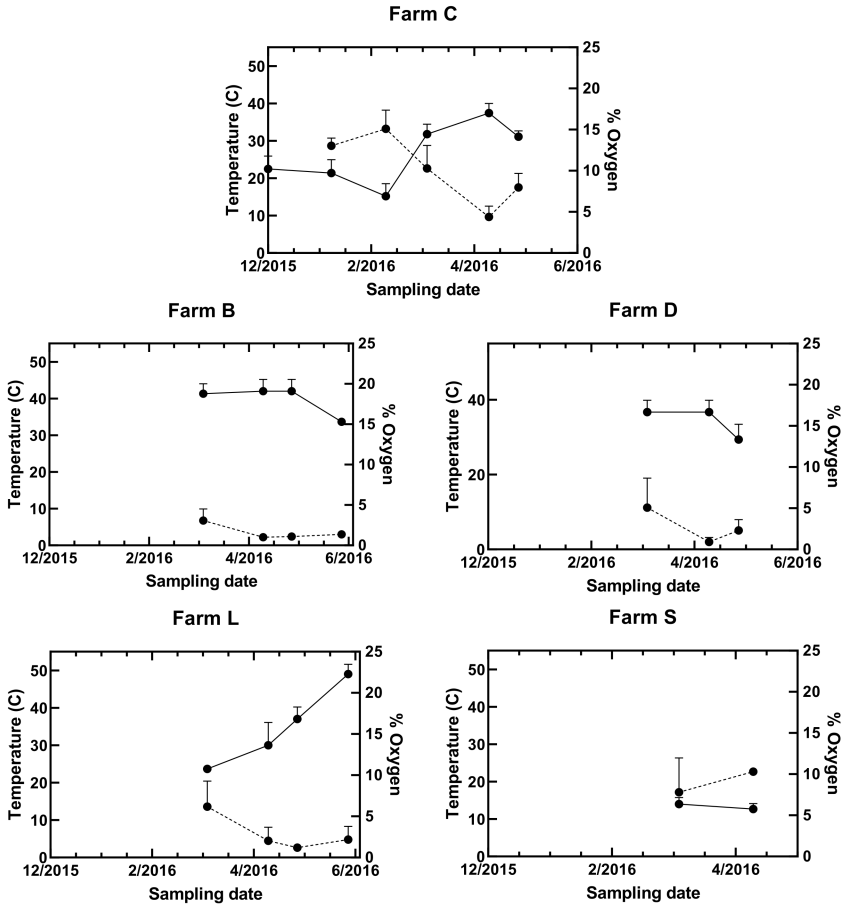

Figure 2. Temperature (solid line) and percent oxygen (dashed line) in the oxic zone in bedded pack at five farms. Means (±standard error) are illustrated. Farm C was the reference farm (n = 10) and four additional farms were compared (n = 3 replicate measurements per time). Some error bars cannot be seen because they are smaller than the symbols, except for the 4/2016 measurement at farm S that had only 1 sample.

2.2. Part 1: Amplicon Sequencing

2.2.1. Sampling

Five 3.75 L samples were collected at 5-paces apart along a systematic transect with a random starting point within each bedded pack barn at each sampling date. Fresh surface hay was brushed away to sample layers that had been exposed to cows for at least three days. Samples were collected from the top layers of pack that were unsaturated and, thus, oxic (Figure 1). The top loose straw layer was separated from the compacted layer below. In untilled bedded packs, sampling the compacted layer required a mattock to penetrate. The wet anoxic layer below (fermentation layer) was not sampled. Occasionally, when the pack was less than 15 cm deep, layers were not divided into separate composite samples. Subsamples were pooled to form a composite sample of each of two layers for each barn at each sampling time. Temperature and percent oxygen were also measured at time of sampling using an OxyTempTM (Reotemp, San Diego, CA, USA) probe at the middle of the oxic zone (Figure 1). Temperature tended to be greatest at the surface 10–15 cm (35–35.5 °C) and decline progressively with depth, i.e., 32–34.5 °C at 38 cm, and 29.5–31 °C at 91.4 cm at reference farm C. Composite samples were transported to the lab in insulated containers for same-day processing. Using clean nitrile gloves, each composite sample was hand homogenized, and five subsamples removed for a total of 25 mL that were trimmed to 1 mm length pieces using ethanol flamed stainless steel shears. The pieces were again homogenized by hand and approximately 1 mL was sampled and frozen at −80 °C until DNA was extracted.

2.2.2. DNA Extraction and Sequencing

Extraction of DNA was performed using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA). Manufacturer's instructions were augmented by heating bead tubes to 65 °C for 10 min, and then shaking horizontally for 2 min at maximum speed with the MoBio vortex adapter [16]. Subsamples of 0.15–0.25 g were placed into the bead tube of the PowerSoil kit. Subsample mass varied due to density differences between bedded pack samples; less dense samples filled the bead tube at a smaller weight. Extracted DNA samples were frozen at −80 °C until being shipped to the University of Colorado Next Generation Sequencing Facility (Boulder, CO, USA) for PCR amplification, sequencing, initial data filtering, and taxonomic reference mapping [17].

Detailed amplification, sequencing and taxonomic mapping methods were described previously [15]. Briefly, amplification was performed in triplicate using 515F and 806R primers targeted for the V4 region of bacterial/archaeal 16S rRNA genes and ITS1F and ITS2 primers targeting the fungal ITS1 region. Sequencing was performed on the Illumina MiSeq platform. Cleaned and quality filtered reads were dereplicated before clustering at 97% nucleotide identity into representational OTU sequences via UCLUST (version 7) [18]. Sequences were referenced to the Greengenes (bacteria) [19] and UNITE (fungi) [20] databases to produce combined Operational Taxonomic Unit (OTU) count and taxonomic information tables.

Library size (read counts per sample) was determined, and depth of sequencing relative to unique OTUs on a sample basis was compared using the *rarecurve* function within the *vegan* package for R software [21] (Figures S1 and S2). Samples with fewer than 100 sequences were removed prior to proportional scaling [22]. OTU counts within each sample were expressed as a proportion of total sample counts and multiplied by the mean of all sample counts. This scaled value was rounded to obtain a whole number, eliminating OTUs with a scaled proportion less than 1.0. Bacterial and fungal sequence counts were normalized separately. Normalizations were performed for each of two discrete subsets of data for both bacterial and fungal sequence counts: Farm C bedding (over one year, Figure S1), and all farms bedding (over four months, Figure S2). No comparisons were made between discrete datasets.

2.2.3. Statistical Analysis

OTU counts were converted to proportion of total sample counts (Relative Abundance; RA) and mean OTU RA was calculated among farms and dates. The core microbiome of Farm C was defined as OTUs that existed in 100% (bacteria) and at least 45% (fungi) of all samples collected at Farm C.

Bray–Curtis dissimilarity matrices were calculated to compare community composition among farms. Permutational multivariate analysis of variance (permanova) was used to test a three-way model describing contribution of farm, date, and depth to variation in OTUs observed at all farms and to further assess differences between sample depths at farms with similar bedding practices. Permanova post hoc tests were performed to assess pairwise differences in microbial DNA sequences between farms. Median RA of the 100 most abundant genera across dates for each of the five farms were computed (Tables S1 and S2). Statistical analyses were conducted in R version 4.02 (R Core Team, Vienna, Austria). Distance-based redundancy analysis was performed using the *capscale* function in *vegan* [21].

2.3. Part 2: Ecology

2.3.1. Culturing and Characterization by Morphology

Subsamples of bedding cultured to isolate Trichocomaceae and supplemental bulk milk samples were collected to isolate Saccharomycetales. Bulk tank milk was collected using sterile dippers (Sterilin™ Dippa™, ThermoFisher Scientific, Waltham, MA, USA). The milk was transported to the lab in a cooler. The samples were inverted 30 times for homogeneity and pipetted to 50 mL plastic conical tubes. Several handfuls of the top few

inches of bedding were taken from various locations in the same barn. Bedding samples were hand-homogenized and placed in freezer bags. Subsamples of milk (2 mL) and bedding (0.5 g) were removed for culturing of isolates and functionality assays. Milk and bedding samples were frozen at −80 °C and –20 °C, respectively.

A bedding subsample (0.5 g) in 50 mL sterile milli-Q water, was macerated at 4000 rpm for 30 s in a Polytron 3100. A 1 mL aliquot of each bedding isolate culture or 100 µL milk was pipetted from each onto 11 different culture media including Czapek Yeast Autolysate Agar (CYA), blood agar media, Acidified Weak Potato Dextrose Agar with Yeast Extract (AWPY), Sabouraud Dextrose Agar (SDA), Corn Meal Agar (CMA), Caffeic Acid Agar (CAF), Brain Heart Infusion Agar (BHI), Yeast Sodium Agar (YNG), Yeast Lactose Agar (YLA), Rose Bengal Chloramphenicol (RBA), and Artificial Rumen Agar (RUM) (File S1) for yeasts. Isolates of Trichocomaceae fungi were obtained through an integrated approach of using both agar plates and natural substrates (Table 2) to identify species using current taxonomy [23–25]. Taxonomic identification was based on growth on different media and various macro- and micro-morphology [23,25,26]. Macromorphology characters were qualitatively based on colony texture, degree of sporulation, color of conidia, mycelia properties (abundance, texture and color), presence and color of soluble pigments and exudates, colony reverse color, growth rate, and acid production [25]. Micromorphology allowed further identification based on differences of conidial heads, stipes, hyphae, vesicles, metulae and phialides, conidia, sclerotia and hilal cells, ascomata, and ascospores. Yeast isolates were differentiated from bacteria by measuring cells under 40X magnification. Other features such as cell shape (ellipsoid, subglobose, and globose) and bud scars were also noted.

Table 2. Trichocomaceae isolates from bedding material collected at Farm C between October 2015 and May 2016. Species were identified by Sanger sequencing of the amplicon generated by NS7-F and ITS2-R primers and contigs assembled using CAP3 [27]. Nucleotide sequences were blasted in NCBI, all yielding E values of 0. Percentage nucleotide matching for the assembled contig sequence is illustrated.

Isolate Contig	Sample Source [a]	Isolation Media [b]	Culture Temperature (°C)	Species	% Match
1	BP	CZA	22	*Aspergillus niger*	99.67
5	BP	CZA	22	*Penicillium solitum* strain 20-01	99.83
6	BP	CZA	22	*Penicillium solitum* strain 20-01	99.83
9	TS	CYA	35	*Aspergillus fumigatus* strain cy018	99.65
11	TS	CYA	35	Uncultured fungus CMH603	99.78
14	BP	Unknown	22	*Penicillium solitum* strain 20-01	99.82
16	TS	CYA	22	*Penicillium solitum* strain 20-01	99.82
17	TS	CYA	22	*Penicillium solitum* strain 20-01	99.82
19	TS	CZA	22	*Penicillium solitum* strain 20-01	99.83
21	BP	DG18	22	*Penicillium solitum* strain 20-01	99.83
27	BP	YE20S	22	*Penicillium chrysogenum* strain ZJ-T2	99.65
32	BP	YE20S	22	*Aspergillus fumigatus* strain cy018	99.65
33	TS	DG18	22	*Penicillium janthinellum* series	99.65
37	BP	DG18	22	*Penicillium solitum* strain 20-01	95.65
39	BP	Humidity Chamber	22	*Penicillium solitum* strain 20-01	99.82
41	TS	CZA	22	Hypocreales uncultured	99.26
47	TS	CYA	22	*Talaromyces radicus* (*Penicillium radicum*)	96.91
95	BP	CMA	22	*Aspergillus luchuensis*	100
129	BP	Humidity Chamber	34	*Talaromyces verruculosus* (*Penicillium verruculosum*)	99.13
CG48	BP	YES	34	*Aspergillus fumigatus* strain cy018	99.82
CH4	TS	DG18	35	*Aspergillus fumigatus* strain cy018	99.83

[a] Source (BP: bedded pack, TS; tie-stall); [b] CMA: Cornmeal Agar, CYA: Czapek Yeast Autolysate agar, CZA: Czapek-Dox Agar, DG18: Dichloran Glycerol 18% agar, MEA: Blakeslee Malt Extract Autolysate agar, YES: Yeast extract sucrose agar, YE20S: Yeast Extract Sucrose 20% Salt agar.

2.3.2. Carbon Utilization

Niche differentiation of 20 *Aspergillus* and *Penicillium* spp. isolates was measured as range and overlap of carbon utilization. An isolate of Hypocreales (Ascomycota) was used as a reference outlier representing another organism with a taxonomy replete with anamorphs and teleomorphs [27]. Carbon utilization profiles for each of 20 isolates were

determined using Filamentous Fungal identification plates (BIOLOG FF Microplate™ Biolog Inc., Hayward, CA, USA). Instead of following manufacturer instructions, we opted to follow more specific ecological guidelines for *Aspergillus* and *Penicillium* developed in the 1980s and 1990s [23]. Briefly, each isolate was grown on malt extract agar for 4 days at 22 °C. A viscous solution of 0.3% of type II carrageenan was poured on top of the fungal colony that was gently scraped with a plastic inoculating loop to suspend the conidia uniformly. Turbidity of the suspension was measured on a spectrophotometer at OD590 and then diluted with a sterile 0.7% saline solution to achieve a final transmittance of 0.022. The final conidia solution was pipetted into the 96-well plate, adding 100 µL per well. The microplates were incubated at 22 °C and read every 24 h until sporulation was observed [23]. Optical density readings measured 24 h before sporulation were used for analysis. Response data were transformed as log (x + 0.01) and carbons were centered and standardized. A redundancy analysis was performed constrained by species as an explanatory variable and carbon metabolism activity as response variables using Canoco version 5 software (Microcomputer Power, Ithaca, NY, USA) [28].

2.3.3. Indicators of Colonization and Pathogenicity

Yeast isolates were tested for their ability to grow at 35 °C, hemolysis, protease, and two oxidases (catalase, peroxidase) as indicators of virulence/pathogenicity. Growth at 35 °C for 48 h was a measure of ability to live within a cow [29]. Yeast hemolysis was evaluated by streaking isolates on blood agar and incubated at 28 °C for 72 h. Positive hemolytic activity was indicated by the presence of a translucent halo zone around the colony. The protease assay was performed using Thermo Scientific Pierce Fluorescent Protease Assay (Waltham, MA, USA) with a negative control of 100 µL of casein tagged with fluorescein and 100 µL of suspended in buffer. Catalase and peroxidase were determined by suspending 1 mL of cells with 250 µL of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 20 µL of 3% hydrogen peroxide. Cultures were left to incubate at room temperature and checked for color change at 15 min, 1 h, and 24 h. Bubbles generated by reaction with hydrogen peroxide provided evidence of catalase activity [30]. Bubble generation and color change were evidence of peroxidase activity [31]. Raw milk was used as a positive control given it contains lactoperoxidase [32].

2.3.4. DNA Extraction, PCR and Sanger Sequencing

DNA was extracted from 20 isolates of Trichocomaceae (Ascomycota) and 46 isolates of Saccharomycetales (Ascomycota) using the Qiagen DNeasy Powermax Soil Extract Kit as described for Part 1. Extracted DNA was PCR-amplified using NS7F and ITS2R primers targeted for the first rRNA internal transcribed spacer region (ITS1). NS7F provides a longer sequence read than ITS1F, has fewer mismatches, and less bias toward Basidiomycota [33]. Reactions were held at 94 °C for 3 min to denature the DNA, with amplification proceeding for 35 cycles at 94 °C for 45 s, 54 °C for 60 s, 72 °C for 90 s, and 10 min at 72 °C, followed by a final extension of 10 min at 72 °C. Amplicons were shipped to GENEWIZ (South Plainfield, NJ, USA) for purification and Sanger Sequencing. Taxonomy was assigned to each OTU via the NCBI BLASTn database (https://blast.ncbi.nlm.nih.gov, accessed on 30 May 2022) with criteria as 0.0 E and nucleotide match of at least 97% (Tables 2 and 3). Contigs were assembled using CAP3 software [27].

Table 3. Sanger Sequencing results for cultured yeast isolates and their respective physiological characteristics. Samples collected in December 2019, January 2020, and February 2020 at Farms M (milk only) and C (milk and bedding). Species were identified by Sanger sequencing of the amplicon generated by NS7-F and ITS2-R primers and contigs assembled using CAP3 [27]. Nucleotide sequences were blasted in NCBI, all yielding E values of 0. Percentage nucleotide matching for the assembled contig sequence is illustrated.

Isolate Contig	Farm	Sample Source [a]	Isolation Media [b]	Species	% Match	Protease	Hemolytic	35 °C	Catalase	Peroxidase
1	M	BTM	RBC	*Diutina rugosa*	95.7	1	0	1	1	0
2	M	BTM	RBC	*Diutina rugosa*	95.7	1	0	1	1	0
3	M	BTM	RBC	*Diutina rugosa*	95.7	1	0	1	1	0
5	M	BTM	RBC	Uncultured Tremellomycetes	98.9	1	0	0	0	0
6	C	High SCC	CAF	Uncultured Tremellomycetes	99.8	1	0	0	0	0
7	C	BTM	CAF	*Pichia holstii*	97.2	1	0	1	0	0
8	C	BTM	AWPY	*Pichia kudriavzevii*	99.8	1	0	1	0	0
9	C	BTM	AWPY	*Wickerhamomyces anomalus*	99.8	0	0	1	0	0
10	C	BTM	CYA	*Wickerhamomyces anomalus*	82.4	1	0	1	0	0
11	C	BTM	CYA	*Wickerhamomyces anomalus*	99.7	1	0	1	0	0
12	C	QM	Blood Agar	*Diutina rugosa*	96.0	1	0	1	0	0
13	C	BP	BHI	*Diutina catenulata*	100.0	1	1	1	0	0
14	C	BP	BHI	*Wickerhamomyces anomalus*	99.7	1	0	1	0	0
15	C	BP	RUM	*Pichia fermentans*	99.8	1	1	1	0	0
16	C	BP	RUM	*Pichia fermentans*	99.6	1	0	0	1	0
17	C	BP	CAF	*Pichia fermentans*	99.6	1	0	1	0	0
22	C	BP	YLA	(*Candida*) *glaebosa*	99.3	1	0	0	0	0
23	C	BP	BHI	*Wickerhamomyces anomalus*	100.0	1	0	0	0	0
25	C	BP	RUM	(*Candida*) *glaebosa*	99.0	1	0	0	0	0
26	C	BP	CAF	*Hyphopichia pseudoburtonii*	99.6	1	0	0	0	0
27	C	TS	SDA	(*Candida*) *glaebosa*	98.7	1	0	0	0	0
28	C	TS	SDA	(*Candida*) *glaebosa*	98.4	1	0	1	0	0
29	C	TS	SDA	*Debaryomyces hansenii*	100.0	1	0	0	0	0
30	C	TS	RUM	*Debaryomyces hansenii*	99.8	1	0	0	1	0
31	C	TS	RUM	*Debaryomyces hansenii*	100.0	1	0	0	1	0
32	C	TS	RUM	*Hyphopichia burtonii*	99.8	1	0	1	0	0
33	C	TS	CAF	*Diutina catenulata*	99.4	1	0	1	1	0
34	C	TS	CAF	*Debaryomyces hansenii*	100.0	1	0	0	1	0
35	C	TS	BHI	*Debaryomyces hansenii*	100.0	1	0	0	0	0
36	C	TS	BHI	*Debaryomyces hansenii*	99.8	1	0	0	0	0
37	C	TS	BHI	*Diutina catenulata*	100.0	1	0	1	0	0
38	C	TS	BHI	*Debaryomyces hansenii*	99.7	1	0	1	0	0
39	C	TS	YLA	*Wickerhamomyces anomalus*	99.7	1	0	1	0	0
40	C	TS	YNG	*Hyphopichia burtonii*	99.8	1	0	1	0	0
41	C	BP	SDA	*Debaryomyces hansenii*	99.7	1	0	1	0	0
42	C	BP	CAF	*Wickerhamomyces anomalus*	100.0	1	0	1	0	0
43	C	BP	YLA	*Debaryomyces coudertii*	99.5	0	0	1	0	0
44	C	BP	BHI	*Diutina catenulata*	100.0	1	0	1	0	0
45	C	BP	YLA	*Wickerhamomyces anomalus*	99.7	1	0	1	0	0
46	C	BP	SDA	*Diutina catenulata*	100.0	1	0	1	0	0

[a] Source (BTM: bulk tank milk, QM: quarter milk, BP: bedded pack, TS: tie-stall, High SCC: high somatic cell count); [b] Media (AWPY: Acidified Weak Potato Dextrose Agar with Yeast Extract, BHI: Brain Heart Infusion Agar, CAF: Caffeic Acid Agar, CYA: Czapek Yeast Autolysate Agar, RBC: Rose Bengal Chloramphenicol, RUM: Artificial Rumen Agar, SDA: Sabouraud Dextrose Agar, YLA: Yeast Lactose Agar, YNG: Yeast Sodium Agar).

3. Results

3.1. Most Common Taxa at Farm C and Their Distribution with Season and Depth

Bacteria within γ-Proteobacteria, Bacteroidetes, Firmicutes, and β-Proteobacteria dominated the bacterial community (Figure 3), whereas Ascomycota fungi represented 88% of all OTUs within the fungal community (Figure 4). Common genera within γ-Proteobacteria included *Acinetobacter*, *Pseudomonas*, and *Cellvibrio*, whereas *Comamonas* represented β-Proteobacteria (Table S2). Bacteroidetes was represented by *Ruminofilibacter*, *Sphingobacterium* and *Flavobacterium*. Firmi-

cutes was represented by greater relative abundance of orders MBA08, OPB54, and Clostridiales. Fungal microbiomes were dominated by Saccharomycetales yeasts classified as *Candida*, the anamorph stage, associated with teleomorphs Pichiaceae (especially *Pichia*), Debaryomycetaceae (*Yamadazyma*, *Debaryomyces*, *Kurtzmaniella*, CTG clade), and Phaffomycetaceae (*Wickerhamomyces*, *Cyberlindnera*) (Table S3).

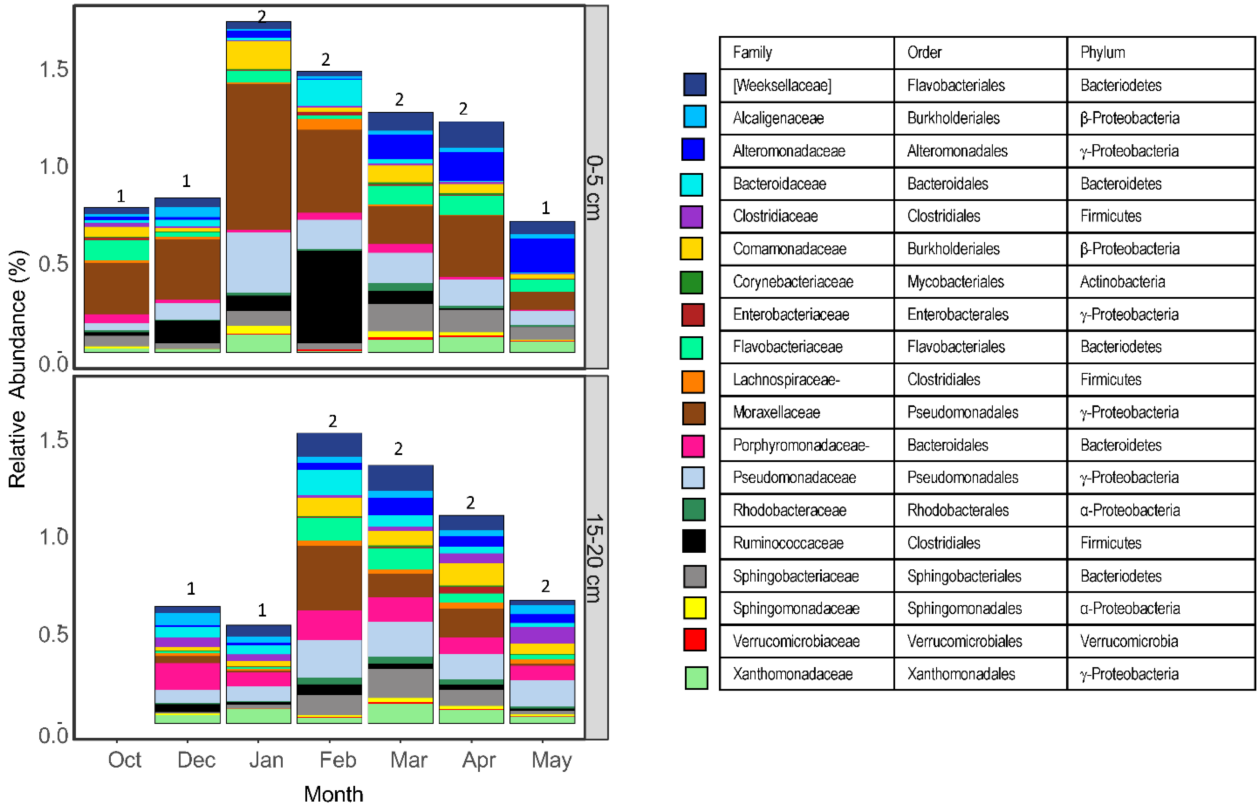

Figure 3. Relative abundance of bacterial taxa (out of 220) that are represented in all 100% samples split by depth and month at Farm C. The first date (October) is just the top layer because it is the beginning of the pack building. Sample size is illustrated above each bar. Microbiome varied by time (pseudo-F = 1.57, p = 0.002) and depth (pseudo-F = 1.55, p = 0.031).

Composition of both bacterial and fungal communities at Farm C varied through time (Figures 3 and 4). Bacterial communities also varied with depth but not fungal communities. For example, Moraxellaceae and Ruminoccaceae had greatest relative abundance in mid-winter of the 0–5 cm layer. Pseudomonadaceae was similarly abundant in both depths. Porphyromonadaceae was more abundant in 15–20 than 0–5 cm layers. The only time showing depth differentiation for fungal communities was in February (Figure 4). Ascobolaceae was abundant at the beginning of the bedded pack formation in October, and appeared in the 15–20 cm layer by January and persisted through May. Saccharomycetales was dominant at both depths beginning in November. Trichocomaceae (*Aspergillus* and *Penicillium* spp.) was equally abundant across depths.

3.2. Cultured Filamentous Fungi and Yeast Isolates

Filamentous fungi isolated and cultured from bedded pack samples included 36, 11, 4, and 9 isolates of *Aspergillus*, *Penicillium*, *Paecilomyces*, and *Trichoderma*, respectively. Tie-stall samples yielded 11, 9, 3, 3, 2, and 2 isolates of *Aspergillus*, *Penicillium*, *Paecilomyces*, *Fusarium*, *Byssochlamys*, and *Trichoderma*, respectively. Among the Trichocomaceae were identified three *Aspergillus* species and five *Penicillium* species (Table 2).

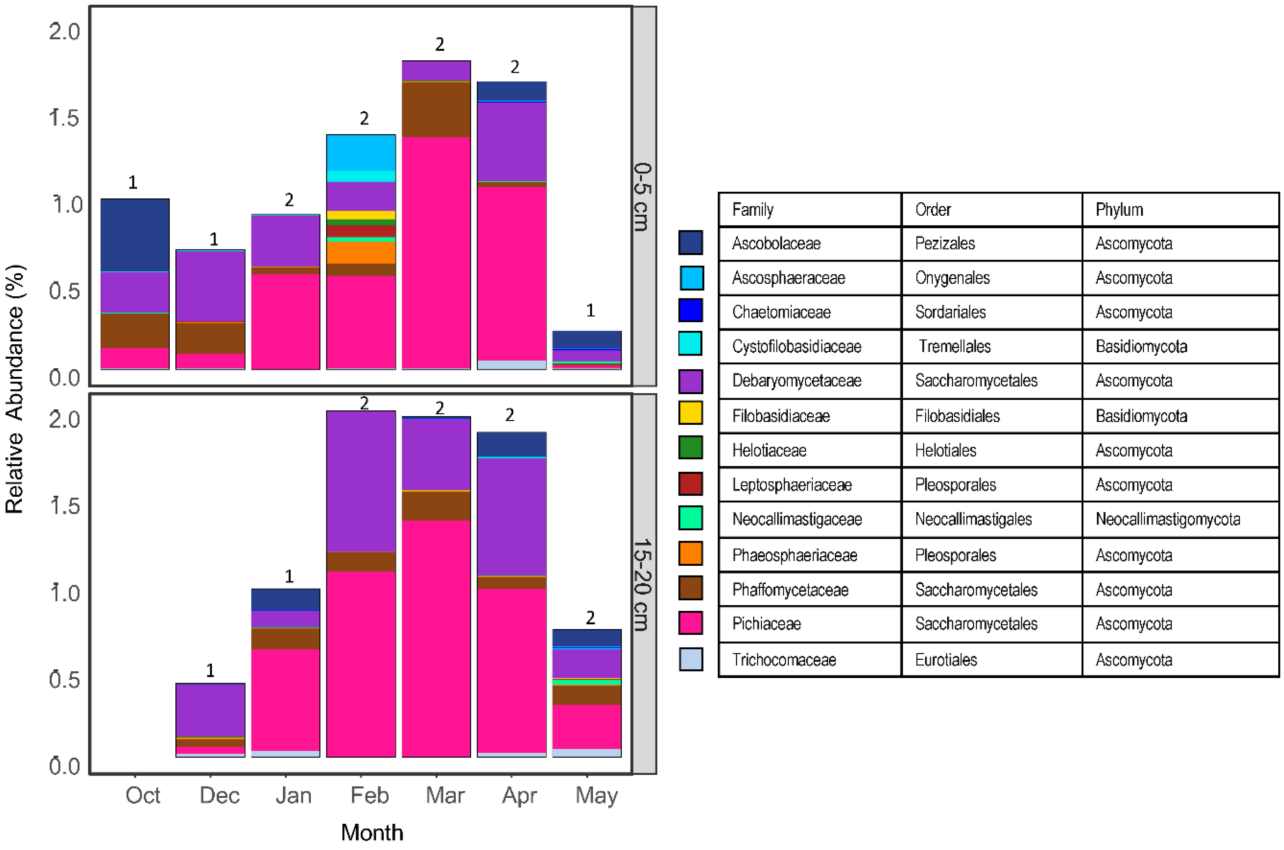

Figure 4. Relative abundance of fungal taxa (out of 82) that are represented in 45% of all samples split by depth and month at Farm C. The first date (October) is just the top layer because it is the beginning of the pack building. Sample size is illustrated above each bar. Microbiome varied by time (pseudo-F = 1.27, p = 0.003).

There was no obvious specificity of isolation media and taxonomic species of yeast from bedding (Table 3). For example, *Debaryomyces hansenii*, *Wickerhamomyces anomalus*, *Diutina catenulata*, and (*Candida*) *glaebosa* were isolated on at least three different media. Different species of the same genus were isolated on contrasting media. For example, *D. hansenii* was isolated on four media (SDA, RUM, CAF, BHI), and its congeneric species *Debaryomyces coudertii* was isolated on YLA. Both CAF and RUM media were more general isolation media, yielded 5 and 4 species, respectively.

Three species of yeast cultured from milk were also present in bedding samples (Table 4). *Pichia kudriavzevii* was the most prevalent. Two other prevalent species were cultured from one but not both substrates. *D. rugosa* was present in milk but not bedding. *D. hansenii* (anamorph *Candida famata*) was present in bedding but not milk. *Diutina rugosa* (anamorph *Candida rugosa*) was isolated from milk on RBC media. A previously uncultured Tremellomycetes was isolated from bulk tank milk cultured on RBC, and from high SCC milk of a single animal cultured on CAF. *W. anomalus* was isolated both using AWPY and CYA. *Pichia holstii* (synonym *Nakazawaea holstii*) and *P. kudriavzevii* (anamorph *Candida krusei*) were isolated using CAF and AWPY, respectively. All milk isolates were cultured subsequently on CAF media. *P. kudriavzevii* relative abundance tends to be associated positively with increased temperature (Figure 5).

Table 4. Milk isolates cultured from milk also present in bedded pack samples from each of the five farms in phase 1 of the case study (n = 40). Bedding samples were estimated by amplicon sequencing [15]. Sequence counts were converted to a proportion of total within-sample counts (relative abundance) and median relative abundance of OTUs with matching binomial epithets were calculated on a species basis among samples in which the matching taxa were present.

	Pichia kudriavzevii	***Pichia holstii***	***Wickerhamomyces anomalus***
Samples in which isolate is present	90%	8%	3%
Median relative abundance among samples in which isolate is present	12.2%	0.1%	0.9%

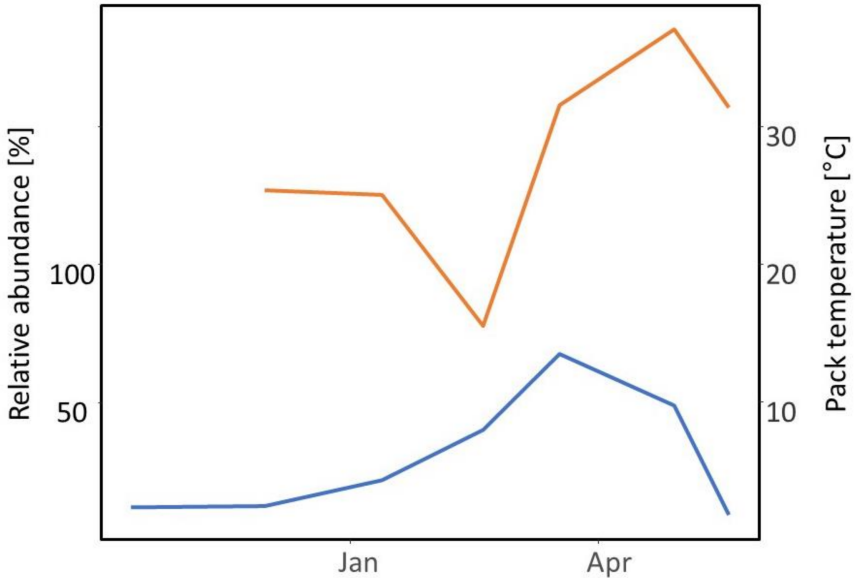

Figure 5. Mean monthly relative abundance of *Pichia kudriavzevii* (blue line, left *y*-axis) as a function of bedding temperature (orange line, right *y*-axis) in the oxic zone through time at Farm C.

3.3. Indicators of Carbon Utilization and Pathogenicity

The genera *Aspergillus* and *Penicillium* and their respective species varied in substrate requirements (Figure 6). For example, *A. fumigatus* were associated positively with N-acetyl-D-mannosamine and D-arabitol, and *A. luchuensis* was associated positively with a media depletion of carbon–nitrogen substrates (Figure 6). Hypocreales and *Penicillium janthinellum* series were associated positively with a multitude of carbon–nitrogen substrates and were orthogonal to the other isolates.

Thirty five percent (14 of 40) of the yeast isolates did not grow at 35 °C. Twenty percent (8 of 40) of the isolates produced catalase (1 *Pichia fermentans*, 3 *D. hansenii*, 2 *D. rugosa*, 1 *D. catenulate*, 1 unknown). Variation of production of hemolytic or catalase was apparent between species of *Pichia*, *Debaryomyces* and *Diutina* and within species of *D. hansenii*, *D. catenulate*, *D. rugosa* and *W. anomalus*. One isolate of each *D. catenulate* and *P. fermentans* were hemolytic. All isolates produced protease with two exceptions. The single isolate of *D. coudertii* did not produce protease, and one of the eight *W. anomalus* isolates did not produce protease. None of the isolates produced peroxidase.

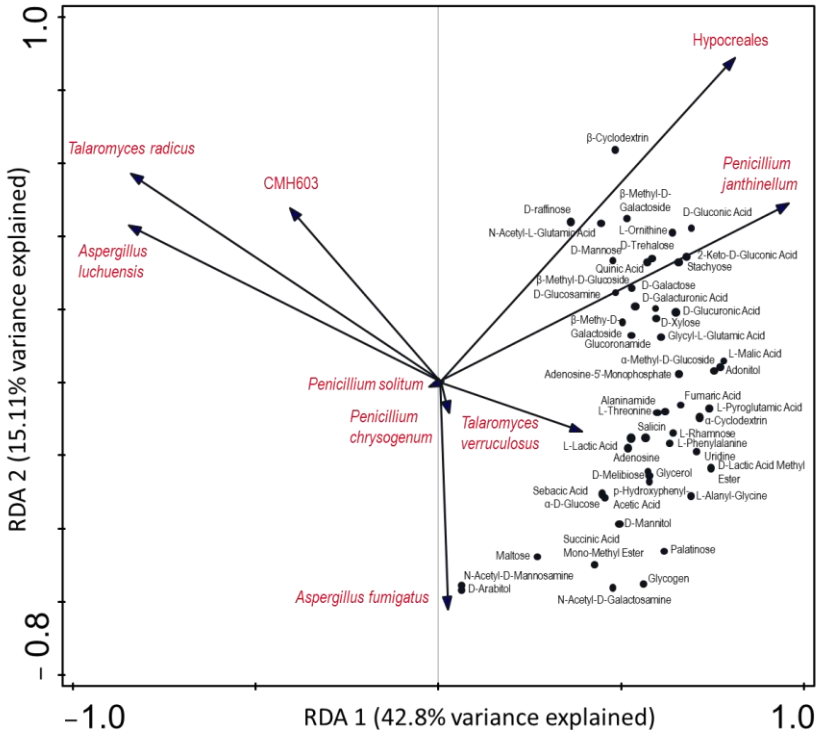

Figure 6. Redundancy Analysis biplot of carbon utilization by Trichocomaceae species constrained by genus. Points represent 50 best-fitting (out of 95) carbon substrates shown using data compiled from 40 isolates from the results of the Filamentous Fungal identification plates. Response values are transformed as log (x + 0.01) and carbons were centered and standardized. An uncultured Hypocreaceae is included as a reference outlier. Eigenvalues are 0.2458, 0.0921, 0.0731, and 0.0405 for the x-, y-, third, and fourth axes, respectively (pseudo-F = 2.2, p = 0.004). Analysis performed with Canoco Ver 5 software.

3.4. Comparison among Farms

Farms B and D used a DBP such as Farm C. Farm S used a DBP, but it had a lower temperature than other DBP farms. Farm L used a unique material, green pine sawdust, and was the only farm that tilled its pack. All farms except S had similar median bedding temperature and stocking density (Table 1, Figure 2). However, bedded pack farms varied in bedding replenishment rate and application of woodchips (Table S1). Farm S stocking density was the least of all the farms, and cows were not restricted to the barn: they could range into the snowy, frozen, or muddy pasture. Less fecal matter and bedding accumulated in the barn, and microbial decomposition was not well insulated from freezing temperatures, possibly explaining why the lowest bedding temperatures were observed on Farm S. Farm L and S fed cows outside the barn, whereas other farms fed cows in rings placed on the surface of the bedded pack, likely increasing cow time spent on the pack, as well as the volume and concentration of manure and urine. Composition of bacterial communities found in bedding at Farms B and D were similar to Farm C. Farms L and S communities were dissimilar (Figure 7a). Common to all farms was γ-Proteobacteria *Acinetobacter* and *Pseudomonas*. There was greater relative abundance of Clostridia (Orders MBA08 and OPB54) and Bacteroidetes (e.g., *Ruminofilibacter*, *Sphingobacterium*) at Farms C, B, D and L than Farm S (Table S2). In contrast, Farm S was enriched in different members of Bacteroidetes (Marinilabiaceae, Sphingobacteriaceae) and γ-Proteobacteria (Pseudomonadaceae) than the other farms. Bacterial community composition varied by sample depth (F = 3.417, p = 0.003) on farms that had a median bedding temperature greater than 30 °C (Table 1, Figure 2).

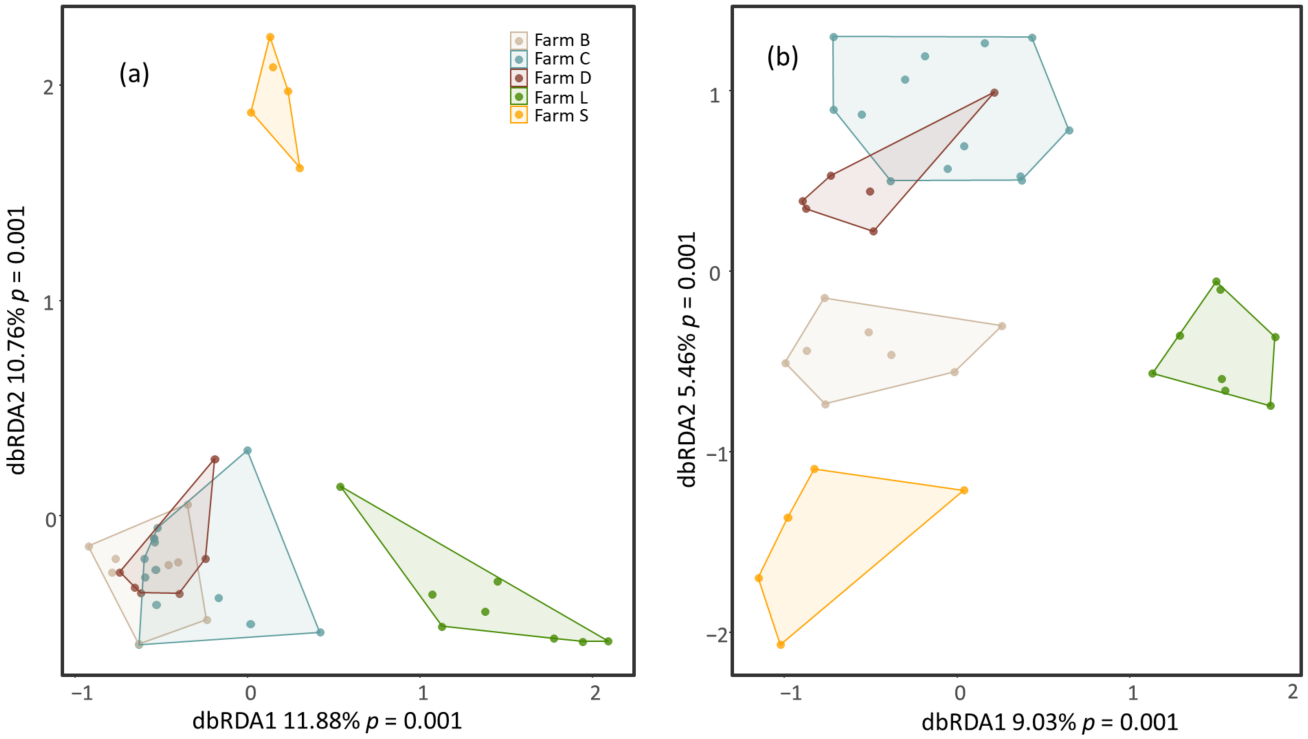

Figure 7. Distance-based Redundancy Analysis biplot of Bray–Curtis dissimilarity of bacterial (**a**) and fungal (**b**) communities constrained by farm (tan: Farm B, blue: Farm C, red: Farm D, green: Farm L, yellow: Farm S). Points represent monthly samples for each farm. Bacterial microbiome varied by farm (pseudo-F = 4.09, p = 0.001), time (pseudo-F = 1.72, p = 0.001), and depth (pseudo-F = 2.37, p = 0.001). Fungal microbiome varied by farm (pseudo-F = 2.52, p = 0.001), and time (pseudo-F = 1.55, p = 0.001). Post hoc comparison among farms: C and D were similar, and B and D were similar, but all other comparisons were different for both bacteria (p fdr < 0.03) and fungi (p fdr < 0.04). Ordination calculated via capscale and model terms compared via adonis2 in vegan package for R software version 4.0.2 (R Core Team, Vienna, Austria).

The composition of fungal communities found in bedding at Farms D was similar to Farm C. Farms B, L and S communities were dissimilar (Figure 7b). Most of the fungi across all farms belonged to the phylum Ascomycota (median 83.6%), whereas 13.6% belonged to the Basidiomycota (Table S3). Ascomycota order Saccharomycetales, especially *Pichia* spp. and *Wickerhamomyces* spp., were common at all farms (Table S3). Farm B was the only farm to have greater relative abundance of *Thermomyces* (Eurotiales) and *Wallemia* (Wallemiales).

4. Discussion

The focus of this study was to examine detrital microbial communities associated with organic bedded pack systems and temporal aspects of community assemblage. To further understand assemblages enumerated through DNA, the Trichocomaceae and yeasts were targeted for isolation due to their relationship to early decomposition or as opportunistic pathogens, respectively. The bedding microbiome of case study, Farm C, was most similar to the bedding microbiome of farms that used similar bedding material and managed for a warm deep bedded pack. This suggests generally that bedded pack microbial communities are similar only as far as the feedstock and management strategy create analogous environmental conditions. Culture work performed in this study suggests the fungal community is represented by two broad niches, early colonizers without specific vitamin requirements and later successional taxa that have vitamin or other nutrient requirements. Furthermore, yeasts exhibit traits associated with host colonization and pathogenicity suggesting a link between bedding microbiome and udder health.

4.1. Core Bedding Microbiome

Prevalent genera within the γ-Proteobacteria, Bacteroidia, Sphingobacteriia and Flavobacteria are reported for composts made with dairy cow feces [9,34,35], and grow well in oxic environments [36] and within temperature ranges found on compost bedded pack. Bacteroidetes classes Bacteroidia, Flavobacteriia, Sphingobacteria and Cytophagia contain gut species [37] that are common gut microbiota of cows [38].

There is increasing evidence that Bacteroidetes play a crucial role in producing polymer-degrading enzymes that metabolize a diverse array of polysaccharides, proteins, and chitin related to the cell walls of plants and fungi [37,38]. The variation in the polysaccharides that environmental Bacteroidetes can degrade is a measure of habitat breadth [37]. Bacteroidetes orders Flavobacteriales and Sphingobacteriales metabolize a variety of polysaccharides and proteins as carbon sources [39]. *Spingobacterium* and *Flavobacterium* demonstrate copiotrophic lifestyles given their ability to thrive on abundant and labile nitrogen content of dairy feces [40]. Porphyromonadaceae are also associated with copiotrophy, suggesting that consistent renewal of excess nutrients in the bedding habitat favors organisms that thrive in conditions of excess and instability [41].

Acinetobacter and *Pseudomonas* (γ-Proteobacteria) are also associated with copiotrophy lifestyles as created by nutrient-rich fecal material deposited on bedding. *Acinetobacter guillouiae* (Moraxellaceae) is common in sewage and sequesters copper and degrades microcystin [42,43]. *Pseudomonas* is an extremely diverse genus genetically, physiologically, and spans functions that range from pathogen to growth promoter of plants [44]. *P. umsongensis* metabolizes aromatic substrates [45]. *Cellvibrio* has the capacity to metabolize cellulose and glucose [46], and *Penicillium janthinelium* series is a cellulose degrader. *Luteimonas* spp. (Xanthomonadaceae) are mostly found in marine and freshwater environments, but one species was isolated from food waste compost [47].

OTUs from the Alcaligencaceae family exhibited a narrow range of variation through time suggesting that they do not respond rapidly to environmental conditions or availability of substrate. Instead, Alcaligenaceae and *Comamonas* increased abundance through time to become the dominant β-Proteobacteria in cowpats [48], behaving as copiotrophs.

4.2. Trichocomaceae

Within the filamentous fungi, the Trichocomaceae exhibit traits of early colonizers of plant material used in bedding and niche differentiation. Trichocomaceae are among the most abundant conidia found on spore traps in anthropogenic indoor environments [10,11] where animals have contact with high density of spores. However, some species of the family require specific nutrients and vitamins. This suggests assemblages from amplicon sequencing comprise a successional range of taxa [12]. Trichocomaceae are primary colonizers of substrates associated with cattle feed or bedding material such as silage, hay, and corn [49]. This suggests the taxa that colonize the surface of bedding early in the season originate from the bedding itself. The farms in this study used mostly sawdust, wood chips, and straw as bedding (Table 1), which is common [3]. Cell walls of herbaceous plants are rich with cellulose, so one would expect the microbiome to be rich with organisms that produce cellulases, e.g., Trichocomaceae genera *Aspergillus* and *Penicillium*. Cell walls of woody plants will also include lignin fibers for rigidity. Wood substrates would attract inhabitants such as jelly fungi (e.g., Tremellomycetes), and brown rot fungi (e.g., Microbotryomycetes and Agaricomycetes). These taxa produce copper-based laccases and manganous peroxidases. However, more research is necessary to demonstrate the impact of oxidative competition on opportunistic yeasts. The material used as bedding defines the matrix carbon sources which can be utilized by decomposer communities.

Conceptually, isolations attempted to select conditions that may favor later successional species by including vitamin sources such as yeast extract while using other environmental factors to limit competing isolates including submerged and unsaturated enrichment with sodium chloride and sucrose solutions at 23 °C and 35 °C. Morphological consistencies of the isolates and growth characters indicate that all isolates were early suc-

cessional species. All *Aspergillus* and *Penicillium* isolates obtained from bedding, grow on minimal media without specific vitamin or amino acid requirements. The isolates reduced nitrate to nitrite by producing the enzyme nitrate reductase [50]. Nitrate utilization is significant due to being somewhat uncommon in decomposer communities. These genera can produce structural proteins and enzymes in environments high in carbon, but scarce of other nitrogen sources, which is the case for cattle bedding. Class Eurotiomycetes (enriched in farms B, D, and L) consisted mostly of family Trichocomaceae within order Eurotiales. However, Farm D had an elevated abundance of *Aspergillus*. Both *Aspergillus* and *Penicillium* of Trichocomaceae are early ecological colonizers in decomposition environments, with a large breadth of carbon and nitrogen utilization [36]. In addition, *Aspergillus* and *Penicillium* exude antifungals, enzymes, organic acids, immunosuppressive compounds, and mycotoxins [51–53]. These extrolites allow *Aspergillus* and *Penicillium* to be highly competitive in their environments due to the inhibition of other organisms [36,54] and may structurally impact community composition.

Studies investigating their ecological role in animal bedding decomposition are underrepresented although these genera are researched extensively due to their applications in food safety, fermentation, pharmaceuticals, and bioremediation [55]. Further studies should explore the relationship between barn air quality parameters and growth of species associated with allergic hypersensitivities. Surface growth, in culture, indicates that most isolates are obligate aerobes and likely detected as transient conidia in milk by DNA methods. However, *Aspergillus* can co-occur on the skin with *Alternaria* and *Cryptococcus* [15]. Results from this study suggest that early colonizers in the family Trichocomaceae partition their ecological niche chemically and the ability to utilize nitrate N versus requirements of amino acids and vitamins separate the Trichocomaceae into colonizers and later successional niches.

4.3. Yeasts

Fungal yeasts are prevalent in bedding and milk [15]. Initial BLAST results returned fungal microbiomes dominated by yeasts classified as *Candida*, the anamorph stage. However, recent studies link anamorphs and teleomorphs and resulted in their reclassification as *Pichia*, *Wickerhamomyces*, *Cyberlindnera*, *Nakazawaea*, *Yamadazyma*, *Debaryomyces*, *Barnettozyma*, and *Kurtzmaniella*. The anamorph *Candida* (associated with teleomorphs *Debaryomyces*, *Diutina*, *Pichia* and *Wickerhamomyces*) is a commensal yeast that can become pathogenic when host defense breaks down [56], and has been isolated from cases of bovine mastitis [57]. Yeasts did not dominate the fungal microbiome until about one month of cow presence (Figure 4). This suggests that prevalence of yeast is related to cumulative effects of cow presence on bedding. Two yeasts found in the bedding environment were capable of hemolysis that suggest they may be competent opportunists in the mammary environment. *Debaryomyces* is a facultative aerobe with optimal temperature of 22 °C yet tolerates freezing conditions and metabolizes diverse carbon substrates [58–60]. *Wickerhamomyces* has diverse strain morphology and can survive a wide range of environmental conditions. Both *Wickerhamomyces* and *Debaryomyces* produce mycotoxins to compete with other organisms [58]. *Debaryomyces hansenii* was only from bedding in this study, but also isolated from milk isolated in other studies [15,61] that isolated from bulk tank milk in contrast to individual animals [62]. *D. hansenii* is a facultative aerobe and known as an extremophilic yeast that metabolizes diverse carbon substrates [59]. Strains of *D. hansenii* produce myocins that suppress in vitro growth of common pathogens including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes* [63].

Diutina rugosa and *Pichia kudriavzevii* have known associations with bovine dairies [64,65] and cases of mastitis [61]. *D. rugosa* has been isolated from dung of a scouring cow, soil, cheese, and considered a rare human pathogen linked to invasive medical procedures or previous exposure to broad spectrum antibiotics [64]. *P. kudriavzevii is* thermotolerant [66] and exhibits antibacterial activity against pathogens including *Staphylococcus aureus* [67]. It follows that *P. kudriavzevii* is prevalent in mastitic milk samples [65]. *P. holstii* demonstrates antifungal

activity with applications in the wine industry [68]. The Tremellomycetes isolate had not been cultivated prior to this study; however, sequences of this taxon have been associated with milk from organic dairies [69] and detected in air samples from five dairy farms using high-throughput sequencing [70].

4.4. Indicators of Pathogenicity

Hemolysins are virulence factors which help pathogens to survive and persist in the host [14]. A hemolysis test is an indicator used to determine whether a yeast isolate is potentially pathogenic [61]. Hemolysins are often defined as extracellularly produced mycocins and are understudied in fungi compared to bacteria [13]. Hemolytic activity in *Candida* and *Cryptococcus* species are associated with iron uptake and regulation required for a variety of mechanisms including phenotype switching, metabolism, and enzyme production [13,71]. Notable iron containing pathogenic enzymes produced by yeasts are peroxidase and catalase which were evaluated in this study where *P. fermentans* and *Diutina catenulate* isolates exhibited hemolytic properties, but not *D. rugosa.* This contrasts with another study that demonstrated that *D. rugosa* exhibited hemolytic properties [72]. Related species *P. kudriavzevii* and *D. hansenii* have been implicated in hemolysis [61].

Proteases are hydrolytic enzymes that are produced by organisms to degrade proteins and can be implemented to break down free intercellular or intracellular proteins for metabolic purposes. Proteases can also be produced pathogenically or defensively to degrade proteins which maintain the structure of cell membranes [26]. Most isolates in this study produced proteases, which supports reports of *D. hansenii*, *W. anomalus*, *D. rugosa*, *P. holstii* and *P. fermentans* all producing proteases in consumable food products [73–77]. Yeast mycocins are being touted as having potential applications in promoting health as antimicrobials, with its main mechanism of action being the inhibition of β-glucan synthesis in the cell wall of sensitive strains [78].

Extracellular oxidases are deployed by both bacteria and fungi to mitigate the toxicity of phenolic molecules and metal ions, and aid in antimicrobial defense [79]. Oxidative enzymes are a basic fungal response to hostile chemical conditions [80]. Peroxidases play a key role in the defense against oxidative stress in bacteria by catalyzing the decomposition of hydrogen peroxide [30] with peroxidases [31]. In this study, catalase oxidases were apparent but not peroxidases capable of reacting with ABTS. However, in pure culture, these genes might not be up-regulated due to an absence of oxidative stress brought on by competition. Many of the wood substrates inhabitants in bedding, particularly Basidiomycetes, produce laccases and manganous peroxidases. However, more research is necessary to demonstrate the impact of oxidative competition on opportunistic yeasts. Finally, none of the isolates produced both hemolytic and peroxidase capabilities in pure culture.

4.5. Source of Yeasts

Teat surface is a transmission point of butyric acid bacteria (BAB) originating in silage to the mammary gland and then to bulk tank milk [81,82]. Similarly, the same genotype of *Pichia kudriavzevii* was present in bulk tank milk, teat surface, and the feed of sheep [83]. Furthermore, both BAB and *P. kudriavzevii* are normal flora of the healthy rumen [53,84]. In this study, *P. kudriavzevii* was cultured from both milk and bedding, suggesting a link between bedding, teat, rumen, and milk microbiomes. A longitudinal study of bulk tank milk microbiota suggests that milk microbiomes respond more to weather and feeding than milking system, number of cows and quality of milk [85]. One can hypothesize that in pasture-based dairies, management response to seasonal changes, for example, feeding preserved hay in the winter versus fresh grass in the summer and use of seasonal winter bedding changes the environmental microbial population, impacting exposure and transmission of potential cow symbionts such as *P. kudriavzevii.*

4.6. Bedding Management and the Microbiome

The bacterial and fungal microbiome of the bedding was related to farm management practice. Management factors including bedding replenishment rate, stocking density, tillage, and cow time spent on pack likely impact microbial habitat and the introduction of organisms [4,7]. For example, tillage homogenizes bedding material and creates a physical zone of aeration, whereas an untilled bedded pack has intact fecal pats that may remain anoxic throughout the bedding period. Farm L was the only farm that mechanically aerated bedding. *Thermomyces lanuginosus*, a thermophilic hemicellulose degrader [86], was greater in farms B and L than farm C, all of which were thermophilic packs. Farm B replenished the bedding most frequently and this indicates that both mechanical aeration and bedding rates promote elevated temperature [4]. Temperature, moisture and oxygen are intercorrelated and impact microbial community composition [4,39]. The microbiome on farms with warmest bedded packs varied between the slightly used (0–5 cm) and compacted strata (15–20 cm) of the oxic bedding zone. Vertical gradients create environments for niche differentiation, with deeper levels being older and more compacted and/or anoxic, representing a later stage of ecological succession.

4.7. Bedding Material and the Microbiome

A unique feature of the bedded pack habitat is constant replenishment with bedding, fresh cattle feces, and urine [3]. Feces and urine contribute a large amount of nitrogen to bedding environment, which varies based on what the cows are fed [87].

Aspects of community structure appeared more dependent on bedding material than management. While bedding management practices differed between Farm S and Farms C, B and D, all four farms used cellulose-based bedding material. Farms that used cellulose bedding had the greatest abundance of *Cellvibrio*. *Cellvibrio* is an aerobic bacterial species with the capacity to degrade cellulose. *Cellvibrio japonicus* is a model organism for utilizing nearly all components of plant cell wall polysaccharides [46]. The cellulose-to-lignin ratio of straw is typically 50% greater than wood [88,89]. Likewise, relative abundance of Basidiomycota was more prevalent in Farm L, which used primarily wood sawdust bedding. Basidiomycota contain white-rot fungi known for their ability to produce oxidative enzymes that degrade lignin [79].

Wallemia is a xerophilic mold that has been isolated from hay and some species are associated with "Farmers Lung Disease" [90]. Farm L, which did not use dried grass bedding, was void in this genus, suggesting that *Wallemia* is a bedding related taxon. However, divergent abundance among farms primarily using hay and straw bedding suggests that *Wallemia* is also sensitive to conditions related to management practices.

5. Conclusions

In the case study, the bedded pack contained a temporally dynamic detrital microbial community structured by temperature and bedding architecture. Comparison to other bedded packs suggested that similar pack management and bedding materials yield similar microbial communities among farms, though more detailed environmental measurements and a review of management practices would be required to assess specific questions related to producer decision making. Bedding may contain a mixture of pathogens and biological control organisms that directly affect the microbiomes of cow udders and indirectly impact health status. Understanding whether the greater microbiome can mediate organisms that impact mammary gland infections is critical to managing mastitis. The choice of bedding and its management represent a potential opportunity to curate the microbial community of the housing environment. There is need for further investigation of how management practices affect the transmission of microbes between the bedding and mammary gland. Some bedding OTUs were associated with key ruminal organisms, raising questions about the relationship between bedding and the gut microbiomes and feeding management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dairy3030042/s1, File S1: Detailed methods for isolation and culture of Trichocomaceae and yeasts. File S2: Figure S1: Normalization curves for bacterial amplicons; Figure S2: Normalization curves for fungal amplicons; Table S1: Farm Management Practices; Table S2: Most abundant bacterial genera; Table S3: Most abundant fungal genera.

Author Contributions: Conceptualization, D.A.N., T.R.W. and J.W.B.; Data curation, D.A.N. and T.D.A.; Formal analysis, D.A.N., T.D.A. and T.R.W.; Funding acquisition, D.A.N. and J.W.B.; Investigation, D.A.N., T.R.W. and A.H.; Methodology, D.A.N., T.R.W. and A.H.; Project administration, D.A.N.; Supervision, D.A.N. and J.W.B.; Visualization, T.D.A.; Writing—original draft, D.A.N. and T.D.A.; Writing—review and editing, D.A.N., T.D.A., T.R.W., A.H. and J.W.B. All authors have read and agreed to the published version of the manuscript.

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