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Microbiome Evolution of Brewer's Spent Grain and Spent Coffee Ground Solid Sidestreams Under Industrial Storage Conditions

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Featured Application: Storage conditions for solid sidestreams for improved shelf-life and safety of upcycled products such as food, feed, and value-added products.

Abstract: Brewer's spent grain (BSG) and spent coffee ground (SCG) are solid sidestreams from beverage production increasingly being upcycled into food, feed and other value-added products. These solid sidestreams are prone to microbial spoilage, negatively impacting their upcycling potential. Three samples each of BSG and SCG were obtained from generators and recycling facilities in Singapore, and their chemical, elemental, and microbial composition was characterized. The spoilage mechanisms were investigated during storage under anaerobic and aerobic conditions. Bacterial loads of sidestreams were low from craft brewery and café sources (<1 and 3.53 ± 0.03 $\log_{10}$ CFU/g) and high from recycling facilities (>6 $\log_{10}$ CFU/g). The microbiome of BSG from recycling facilities was dominated by *Bacilli*, and *B. coagulans* was identified as the most prevalent species. SCG from recycling facilities was dominated by lactic acid bacteria, with *L. panis* being the most prevalent species. Storage up to 14 days under anaerobic conditions led to further bacterial proliferation mainly by *Bacilli*, lactic acid bacteria, and acetic acid bacteria, while aerobic storage led to extensive fungal contamination, including potential aflatoxin-producing *Aspergillus flavus*. The results shed light on the spoilage mechanisms, while highlighting the short shelf-life and food safety risks of BSG and SCG to inform valorization strategies.

Keywords: food waste; brewer's spent grain; spent coffee ground; microbiome; spoilage; shelf-life

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1. Introduction

Approximately one-third of all produced food is lost or wasted, amounting to a staggering 1.3 billion tons in 2007 [1], a figure estimated to almost have doubled to 2.5 billion tons in 2021 [2]. Industrialized Asia and South and Southeast Asia together account for about 50% of global food waste [1], and the city-state of Singapore generated 755,000 tons of food waste in 2023, with only 18% being recycled [3]. Minimizing and valorizing food waste is receiving increasing attention to prevent the associated greenhouse gas emissions and to improve water, energy, and food security [4–6]. Solid sidestreams from beverage production, such as brewer's spent grain (BSG) from beer brewing and spent coffee ground (SCG) from coffee brewing are prevalent in industrialized countries and urban environments. An estimated 36.4 million tons of BSG and 15.3 million tons of SCG are produced annually on a global scale [7,8].

BSG is nutritionally rich in dietary fiber and protein, containing arabinoxylan and β-glucan and a high proportion of essential amino acids, and is a promising source of human nutrition [7]. BSG protein predominantly contains hordeins, which can be extracted and

processed to have functional properties such as emulsifying and foaming capacity suitable for a range of food formulations [9]. A recent market survey identified 125 products containing BSG-derived ingredients, the majority being snacks and bakery products, but also pasta, cereal, meat alternatives, and more [7]. BSG also has potential use in biotechnological processes, such as bioethanol, enzyme production, and other fermentation processes [10]. However, BSG has a high moisture content and is prone to spoilage; therefore, it is typically valorized for animal feed and biogas, while the remainder is landfilled [11,12]. The shelf-life has variously been reported to be 2 days [13], 3–5 days [11], and 7–10 days on average [14], due to differences in definitions of shelf-life and storage conditions [15]. Spoilage is caused by excessive microbial growth, leading to dry matter and nutritional losses, unpleasant odors, low palatability, and even toxin formation [11].

The culturable microbiome of BSG has previously been investigated by Robertson et al. [16] and found to predominantly consist of spores of aerobic thermophilic bacteria capable of surviving the mashing temperatures between 65 °C and 76 °C [17], while microaerophilic (e.g., LAB) and anaerobic bacteria (e.g., *Clostridia*) are thought to proliferate post-production. Robertson et al. [18] also investigated the microbiome and storage of BSG at different temperatures under aerobic conditions, but without environmental exposure. They found bacteria total plate counts to reach approximately 8 $\log_{10}$ CFU/g for mesophilic bacteria and 6 $\log_{10}$ CFU/g for thermophilic bacteria by day 16 at 20 °C, with most of the growth happening before day 3 and stabilizing by day 7 [18]. Fungal genera such as *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, and *Rhizopus* capable of mycotoxin production have all been found in barley grains, and the associated mycotoxins have been found in beer, malted barley, and BSG after 30 days of storage in gunnysacks [7,15,19]. Now, with the advent of advancements in molecular profiling and metagenomics, additional insights may be gleaned from the microflora biology on these economically important residues.

With respect to coffee, SCG predominantly comprises insoluble dietary fiber, proteins, lipids, and a variety of phenolic compounds [20,21] and is rich in bioactive compounds, including prebiotics, which can promote gut health [22]. In the food industry, SCG is being upcycled into functional foods and dietary supplements, tapping into the growing trend of sustainable and health-focused consumption [23,24]. The lipids present in SCG can be extracted for biodiesel production, offering a sustainable energy alternative [8,25]. Additionally, SCG extracts contain potent bioactive compounds with anti-inflammation, anti-cancer, anti-microbial, and anti-fungal properties making them promising candidates for nutraceuticals, cosmetics, preservatives, and biocides [26–29]. At the time of writing, there are no published reports on the shelf-life or microbiome of SCG.

Fresh coffee cherries contain a natural microbiome, which may be harnessed in spontaneous fermentation to remove the cherry pulp surrounding the bean [30]. Such spontaneous fermentation shows a highly diverse bacterial microbiome comprising 160 genera, but with a majority of LAB, especially *Leuconostoc* and *Lactobacillus*, with over 60% relative prevalence [30]. The fungal microbiome is more homogeneous, with *Pichia*, *Candida*, and *Dipodascus* being the prominent genera, with over 90% of the fungal relative prevalence. Yeasts, such as *Saccharomyces*, *Torulaspora*, and *Candida*, have been used as starter cultures for coffee fermentation [31]. However, most of the natural or introduced microbiome of green coffee beans is eliminated in the roasting process, with the exception of some spores, especially of molds, which can survive the roasting temperatures [32]. Therefore, the microbiome of SCG can be expected to be distinct from that of coffee products. Molds, or filamentous fungi, have been found to be the major spoilage-causing microorganisms in SCG, with a prevalence of genera such as *Aspergillus*, *Fusarium*, and *Penicillium* known to potentially produce mycotoxins [32]. Such fungal contamination also represents an occupational risk factor in coffee roasting, milling, and packaging facilities, where *Aspergillus* is widespread in the environment, and *Cladosporium*, *Penicillium*, *Chrysosporium*, *Mucor*, and *Rhizopus* have been observed on coffee grains [33]. Typical microbial loads in roasted coffee are around 2 $\log_{10}$ CFU/g for fungi and around 3 $\log_{10}$ CFU/g for bacteria [33]. After brewing in hot water at 90 °C, the microbial load reduces significantly to an average of 3 CFU/mL in

brewed coffee [32]. Cold brew coffee is brewed at a lower temperature but still has a low CFU/mL, typically <25 CFU/mL, and microbial spoilage is not a concern [34].

As these solid sidestreams are increasingly being used for food, feed, and cosmetic purposes, understanding their shelf-life and spoilage characteristics becomes important, and metagenomic analysis tools can provide new insights into the microbial dynamics in these residues. This study investigates the properties and microbiome of three different samples of BSG and SCG, as received and after industrially relevant storage in tropical conditions in Singapore. The findings highlight issues in the logistics and storage of solid sidestreams and inform choices on suitable handling before the valorization of solid sidestreams in industrialized Asia.

2. Materials and Methods

2.1. Solid Sidestreams

Brewer's spent grain (BSG) was procured from a craft brewery (Alive Brewing Company, Singapore) and two recycling facilities (Tiong Lam Supplies and Envcares) in Singapore. Spent coffee ground (SCG) was procured from a café (Little Big Coffee Roasters, Singapore) and a recycling facility (Tiong Lam Supplies, Singapore). Samples from the craft brewery and café were collected in the evening on the same day as the BSG and SCG were produced, while samples from the recycling facilities were collected in the morning of the day subsequent to production, as the solid sidestreams are stored in bins in ambient conditions during the day before collection and transport to the recycling facility at night. Samples weighing 5 kg to 25 kg were collected in clean plastic ziplock bags, and fresh samples were immediately taken for analysis of moisture content, pH measurement, and microbiome characterization, with the remainder being either frozen at −80 °C or freeze-dried (Martin Christ, Osterode, Germany) for compositional analysis of total carbon and total nitrogen. The time from production to analysis and freezing of the solid sidestreams from the craft brewery and café is estimated to be 3 h to 6 h, and for recycling facility samples, 12 h to 24 h. The sample codes, source and collection is summarized in Table 1.

Table 1. Sample code, source, and description of the brewer's spent grain (BSG) and spent coffee ground (SCG) investigated in this study.

Sample	Source	Description	Collection Time
BSG-1	Alive Brewing Company	Craft brewery	Evening same day
BSG-2	Tiong Lam Supplies	Recycling facility	Morning next day
BSG-3	Envcares	Recycling facility	Morning next day
SCG-1	Little Big Coffee Roasters	Café	Evening same day
SCG-2	Tiong Lam Supplies	Recycling facility	Morning next day
SCG-3	Tiong Lam Supplies	Recycling facility	Morning next day

2.2. Chemical and Elemental Analysis

The moisture content of the materials was measured by the difference in weight before and after the freeze-drying of samples to complete dryness (no additional weight loss) after 2–3 days. Following that, the solution pH was measured by adding an equivalent mass of distilled water to the freeze-dried sample, equilibrating, and measuring the pH of the resultant slurry. Total carbon (TC) was measured on freeze-dried samples on a TOC-LCPH (Shimadzu, Kyoto, Japan) equipped with a solid-state module SSM-5000A (Shimadzu, Kyoto, Japan). Total nitrogen (TN) was measured on freeze-dried samples using Kjeldahl methodology (FC212 Kjeldahl, AOAC Official Methods; ALS, Singapore), and a Kjeldahl factor of 6.25 was applied to calculate crude protein from total nitrogen.

2.3. Total Plate Counts, Microbial Isolation and Identification

Total plate counts (TPCs) and microbial isolates were obtained from 10 to 25 g samples diluted 10-fold in sterile phosphate-buffered saline (PBS) solution and mixed for 1 min in a BagMixer 400 (Interscience, Saint Nom la Bretêche, France). A volume of 50 mL of filtered liquid was aliquoted and subjected to serial dilution in PBS, obtaining dilution ratios of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. A volume of 100 µL of diluted liquid was spread-plated onto various agar types (Table 2; Sigma-Aldrich, Burlington, MA, USA) and incubated for 72 h under the conditions for each respective agar medium. TPCs were obtained from duplicate colony counts on non-selective plate count agar (PCA) after 24 h for bacteria and 120 h for fungi, enumerating morphologically distinct filamentous fungi, following ISO 4833-2 [35]. Streak plating was performed on selective agar to isolate morphologically distinct colonies, and a single colony was suspended in 20 µL of sterile water and subjected to polymerase chain reaction (PCR; Platinum Taq DNA Polymerase, Invitrogen, Waltham, MA, USA) using the primers and conditions described in Table 2. Gel electrophoresis was performed on the PCR product with loading dye (DNA Gel Loading Dye, Thermo Scientific, Waltham, MA, USA) and a DNA ladder (GeneRuler 1 kb DNA Ladder, Thermo Scientific, Waltham, MA, USA). On confirmation of positive reaction, the PCR product was cleaned up using E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, GA USA), and the DNA yield and purity were assessed by 260/280 nm and 260/230 nm absorbance ratios with a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Sanger sequencing (Bio Basic Asia Pacific, Singapore) of PCR samples was conducted via capillary electrophoresis of fluorescent-labeled DNA fragments, with read lengths of up to 800 bp (typically 500–600 bp). The sequence of each isolate was reviewed, and low-quality bases were trimmed. Subsequently, trimmed sequences were subjected to a Basic Local Alignment Search Tool—Nucleotide (Blastn) search against the NCBI nucleotide database using highly similar sequences (megablast) to ascertain the identity of each isolate, with a threshold of at least 99% percent identity for identification.

Table 2. Experimental design including agar media and incubation conditions for total plate counts (TPCs) and microbial isolation, PCR primers and run conditions for Sanger sequencing of microbial isolates, and description of storage containers, datalogging, sampling, and storage conditions.

Agar Medium	Selectivity	Incubation Conditions
Luria–Bertani agar (LB)	Aerobic bacteria	Aerobic; 30 °C; 72 h
Luria–Bertani agar (LB)	Anaerobic bacteria	Anaerobic; 30 °C; 72 h
Potato dextrose agar (PDA)	Fungi	Aerobic; 30 °C; 72 h
Yeast extract peptone dextrose agar (YPD)	Yeasts	Aerobic; 30 °C; 72 h
De Man–Rogosa–Sharpe agar (MRS)	Lactic acid bacteria	Anaerobic; 37 °C; 72 h
Plate count agar (PCA)	Bacteria	Aerobic; 30 °C; 24 h
Plate count agar (PCA)	Fungi	Aerobic; 30 °C; 120 h
Primer and 5′–3′ Sequence	**Target Region**	**Run Conditions**
27F: AGAGTTTGATCCTGGCTCAG 1494R: CTACGGCTACCTTGTTACGA	Bacterial 16S rRNA gene	94 °C for 5 min, 31 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and then 72 °C for 8 min, and finally 4 °C for 30 min
NS1: GTAGTCATATGCTTGTCTC ITS2: GCTGCGTTCTTCATCGATGC	Yeast 18S gene, the internal transcribed spacer (ITS) segment, and most of the 5.8S gene in ribosomal (r)DNA	95 °C for 3 min; 35 cycles of 60 s at 95 °C, 60 s at 55 °C, and 90 s at 72 °C, and finally an extension step of 5 min at 72 °C
Storage Containers	**Datalogging and Sampling**	**Storage Conditions**
2 × Trays (aerobic) 100 cm × 100 cm × 12 cm 40 kg wet weight	Temperature and humidity: Middle, bottom Sampling: Day 0, 3, 7, 14	Ambient conditions at Tiong Lam Supplies (~30 °C; 70% to 80% RH)
2 × Drums (anaerobic) ∅58 cm × 95 cm 100 kg wet weight	Temperature and humidity: Front, middle, back Sampling: Day 0, 3, 7, 14	Ambient conditions at Tiong Lam Supplies (~30 °C; 70% to 80% RH)

2.4. Amplicon Metagenomic Sequencing

To analyze non-culturable microbiome composition, 100 mg of freeze-dried BSG solid sidestream was added directly to the lysis tube from the DNA extraction kit, DNeasy PowerFood Microbial Kit (Qiagen, Hilden, Germany), and lysed using TissueLyser II (Qiagen, Hilden, Germany) at 25 Hz for 15 min × 2 cycles. DNA clean-up was performed using Mag-Bind® TotalPure NGS (Omega Bio-tek, Norcross, GA, USA). DNA yield and purity were determined using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). SCG samples were sent for extraction at a commercial lab (NovogeneAIT Genomics, Singapore), where 200 mg freeze-dried solid of each SCG sample was used for extraction using the FastDNA Spin kit (MP Biomedical, Santa Ana, CA, USA). Extracted DNA samples from BSG and SCG were submitted for 16S rRNA and fungal ITS amplicon metagenomics sequencing (NovogeneAIT Genomics, Singapore). 16S rRNA and ITS gene amplicon library construction and sequencing were carried out on the Illumina Novaseq PE250 platform (San Diego, CA, USA) at 100 K raw tags/sample with a focus on the V3–V4 hypervariable region and ITS1-5F region for bacteria and fungi, respectively. Data QC and analysis were carried out using QIIME 2.

2.5. Storage of Solid Sidestreams

Storage trials of BSG and SCG were carried out in duplicate High-Density Polyethylene (HDPE) trays or drums to simulate large-scale storage conditions, with trays intended to facilitate the establishment of aerobic conditions and drums intended to facilitate the establishment of anaerobic conditions. Drums were outfitted with three sampling ports, top, middle, and bottom, by cutting a square hole and outfitting a hinge and bolt-lock. Three small holes were drilled near the sampling port to fit temperature and relative humidity (RH) sensors from a GSP-6, model datalogger (Elitech, San Jose, CA, USA) placed at the center of the drum. The top sample port and datalogger sensor were above the level of the solid sidestream, and therefore, top samples could not be collected, and the sensors were placed on top instead of inside of the solid sidestreams. Three water locks were installed on the lid to maintain anaerobic conditions within the drum. Trays were outfitted with three dataloggers to measure temperature and humidity at the front, middle, and back of the tray, inserted into the solid sidestream. Trays were covered with fine-masked nets secured by magnets to deter pests. Containers were cleaned with 2.5 wt% bleach solution, washed with distilled water, and air-dried prior to loading 40 kg or 100 kg of freshly obtained solid sidestreams from Tiong Lam Supplies. For consistent comparison between aerobic and anaerobic treatments, BSG-2 was used for both tray and drum storage trials, while SCG-2 was used for tray storage and SCG-3 was selected for drum storage. Samples of 100 g wet weight were collected in clean ziplock bags on days 0, 3, 7, and 14 using the sample ports for the drums and digging a sample for the trays. The ambient conditions at Tiong Lam Supplies were measured every morning with a separate thermometer and humidity meter placed in the trial area. The experimental setup is summarized in Table 2, and photographs can be found in the Supplementary Materials Figure S1.

3. Results and Discussion

3.1. Chemical and Elemental Analysis of BSG and SCG

The chemical and elemental analysis of the three sources of BSG and SCG are reported in Table 3. The chemical and elemental properties show relatively low variation between the three samples of BSG and SCG.

BSG has a moisture content of 69 ± 4%, somewhat lower than the range of 70.6% to 82.6% reported by Coronado et al. [36] and the typical value of 80% [14], while the TC of 48.3 ± 6.3% is in the reported range of 43.6% to 51.6% [36], and the TN of 3.0 ± 0.7% (corresponding to 19 ± 4% crude protein by TN × 6.25) is below the reported range of 3.5% to 4.2% [36] and typical crude protein content of 25% to 30% [14]. The measured pH of 4.8 ± 1.1 is typical of BSG, reported as a range of 3.8 to 6.9 [37].

Table 3. Chemical and elemental analysis and total plate counts for BSG and SCG solid sidestreams.

	Chemical Analysis (fwb [1])		Elemental Analysis (dwb [2])		Total Plate Count (log_{10} CFU/g (fwb) [1])	
Sample	**Moisture**	**pH**	**TC [3]**	**TN [4]**	**Bacteria**	**Fungi**
BSG-1	65%	6.0	43.3%	2.43%	3.53 ± 0.03	<1
BSG-2	70%	4.7	46.1%	2.80%	7.42 ± 0.02	<1
BSG-3	72%	3.7	55.4%	3.76%	7.07 ± 0.08	<1
Average	69 ± 4%	4.8 ± 1.1	48 ± 6.3%	3.0 ± 0.7%	6.0 ± 1.9	<1
SCG-1	64%	5.1	55.1%	2.14%	<1	<1
SCG-2	56%	5.8	50.6%	2.38%	7.70 ± 0.02	<1
SCG-3	48%	4.8	43.2%	1.71%	6.16 ± 0.02	1.94 ± 0.00
Average	56 ± 8%	5.2 ± 0.5	49.6 ± 6.0%	2.1 ± 0.3%	4.6 ± 3.6	0.65 ± 1.00

[1] Fresh weight basis. [2] Dry weight basis. [3] Total carbon; [4] Total nitrogen.

SCG has a somewhat variable moisture content of 56 ± 8%, higher than the range of 38% to 48% reported by Bejenari et al. [38] but within the wider range from 42% to 65% and a typical value of 60% reported by Johnson et al. [25], presumably due to differences in grinding, roasting, and brewing conditions. The TC of 49.6 ± 6% is in the reported range of 45% to 69% [25], and the TN of 2.1 ± 0.3% is in the reported range of 1.2% to 2.7%, or even up to 4% [25]. In SCG, calculating crude protein by TN × 6.25 will likely overestimate actual protein content due to the presence of nonprotein nitrogenous compounds such as alkaloids, amines, amino acids, and their reaction products [21]. Indeed, the measured crude protein of 13 ± 2% falls in the higher end of the reported range of 6.7% to 14% [21]; however, higher values of 13% to 17% have also been reported [39]. The measured pH of 5.2 ± 0.5 falls within the reported range of 5.0 to 6.0 [39].

3.2. Microbiome Characterization of BSG and SCG

As can be seen in Table 3, the total plate counts (TPCs) depend strongly on the source of the solid sidestream, with those obtained directly from the craft brewery and café having low bacteria TPCs, 3.53 ± 0.03 log_{10} CFU/g for BSG and not detectable at a level of 1 log_{10} CFU/g for SCG, and no detectable fungi. This is in stark contrast with the solid sidestreams from recycling facilities especially having high bacterial TPCs of more than 6 log_{10} CFU/g and one SCG sample having a fungal TPC of 1.94 ± 0.00 log_{10} CFU/g. The presumed cause is the longer overnight storage time under ambient conditions at the recycling facilities prior to collection and testing and may be accelerated by opportunistic colonization of microorganisms from the environment, such as during transport from the production site to the recycling facility.

The pH of BSG spans a range from 3.7 to 6.0 and decreases in proportion to the measured TPC. This indicates that BSG is only slightly acidic on lautering but is rapidly colonized by acid-producing bacteria, such as lactic acid bacteria (LAB), further lowering the pH. The TPC of BSG-1 from the craft brewery is about 3 orders of magnitude lower than that from the recycling facilities and therefore is expected to be more representative of the microbial load expected from BSG fresh from the brewery lauter tun. The pH of SCG falls in a narrow range of 4.8 to 5.9, without a clear link between pH and TPC. As stated, high bacterial TPCs of 6.16 ± 0.02 and 7.70 ± 0.02 log_{10} CFU/g were found in the SCGs from the recycling facility, while SCG-1 from the café had significantly lower TPCs below the limit of detection of 1 log_{10} CFU/g.

The microorganisms isolated and identified from BSG are listed in Table 4. BSG contains the LAB genera *Pediococcus*, *Enterococcus*, and *Limosilactobacillus*, all facultative anaerobic thermotolerant non-spore-forming bacteria [40]. *Pediococcus* spp. are associated with beer spoilage [41], with *P. acidilacti* specifically found in a craft brewery [42], while *E. durans* is a sour milk starter culture [43] and *L. fermentum* is a common spoilage microorganism in tomato products [44]. Facultative anaerobic spore-forming bacteria of the genus *Bacillus* and non-spore-forming but desiccation-resistant *Chronobacter* [45] were also

identified. *B. subtilis* is found in soil and fermented and dried foods such as *nattō* and milk products [46–48]; similarly, *B. rugosus* [46] is found in soil, and *C. malonaticus* in milk products [45]. Fungi were not prevalent, as evident also from the TPC in Table 3, yet three yeasts were isolated: *Candida tropicalis*, *Pichia kudriavzevii*, and *Kluyveromyces marxianus*. *C. tropicalis* is prevalent in Asia and is one of the most common species of *Candida* in bloodstream infection [49]. *P. kudriavzevii* (formerly *Candida krusei*) has also but less frequently been linked with hematological malignancies [49] and is applied in the food industry as a starter for coffee and cocoa fermentation [31,50]. *K. marxianus* is also known as *Candida kefyr* and is commonly found in dairy products [51] but can also be used for the production of natural flavors, such as 2-phenylethanol [52]. The three yeasts have in common that they are thermotolerant and pentose-assimilating, and therefore, they are suited for growth on hemicellulosic sugars, such as those present in BSG [53].

Table 4. Identification of isolated microorganisms in BSG by Sanger sequencing.

Type of Microorganism	Identified Species
Aerobic bacteria	*Bacillus subtilis* *Bacillus spizizenii* *Bacillus rugosus*
Anaerobic bacteria	*Enterococcus durans* *Cronobacter malonaticus*
Lactic Acid Bacteria	*Pediococcus acidilactici* *Enterococcus faecium* *Limosilactobacillus fermentum*
Yeast	*Candida tropicalis* *Pichia kudriavzevii* *Kluyveromyces marxianus*
Fungi	-

The freshest BSG-1 from the craft brewery has the lowest microbial load of 3.53 ± 0.03 $\log_{10}$ CFU/g, similar to the lowest TPC reported by Robertson et al. [16]. The identified microbial species, with the exception of *Bacilli*, are not spore-formers and would not survive mashing temperatures. Nor are they the species typically found in or applied for beer brewing [54], even considering the unique case of sour beers [55]. Instead, these microorganisms are associated with beer spoilage, or wild fermentation, or are prevalent in the environment, such as in other food products likely occurring in the environment at the production site and contaminating the BSG post-production.

3.3. Chemical Analysis During Storage of BSG and SCG

The results of the chemical analysis of pH and moisture content of BSG and SCG subjected to storage for up to 14 days in aerobic trays and anaerobic drums are shown in Figure 1.

The pH of BSG in trays continuously increases from the initial value of 4.7 to 6.9 ± 0.1, with no significant difference between the two trays at day 14, as seen in Figure 1a. The increasing pH in trays is thought to be linked to the observed fungal growth, as fungi with high proteolytic activity can cause ammonia formation on extended solid-state fermentation [56]. In contrast, BSG stored in drums exhibits a slight drop in pH already at day 3 to 4.2 ± 0.5 to 4.3 ± 0.6, which appears to be stable over the course of the storage up to 14 days. The rapid decrease in pH by day 3 is thought to be caused by the proliferation of LAB and the generation of lactic acid from the metabolism of fermentable sugars, similar to many traditional fermented foods [57]. The higher variability of pH in drums reflects larger differences in measurements between the vertically separated sampling positions, in contrast to the laterally separated samplings for trays.

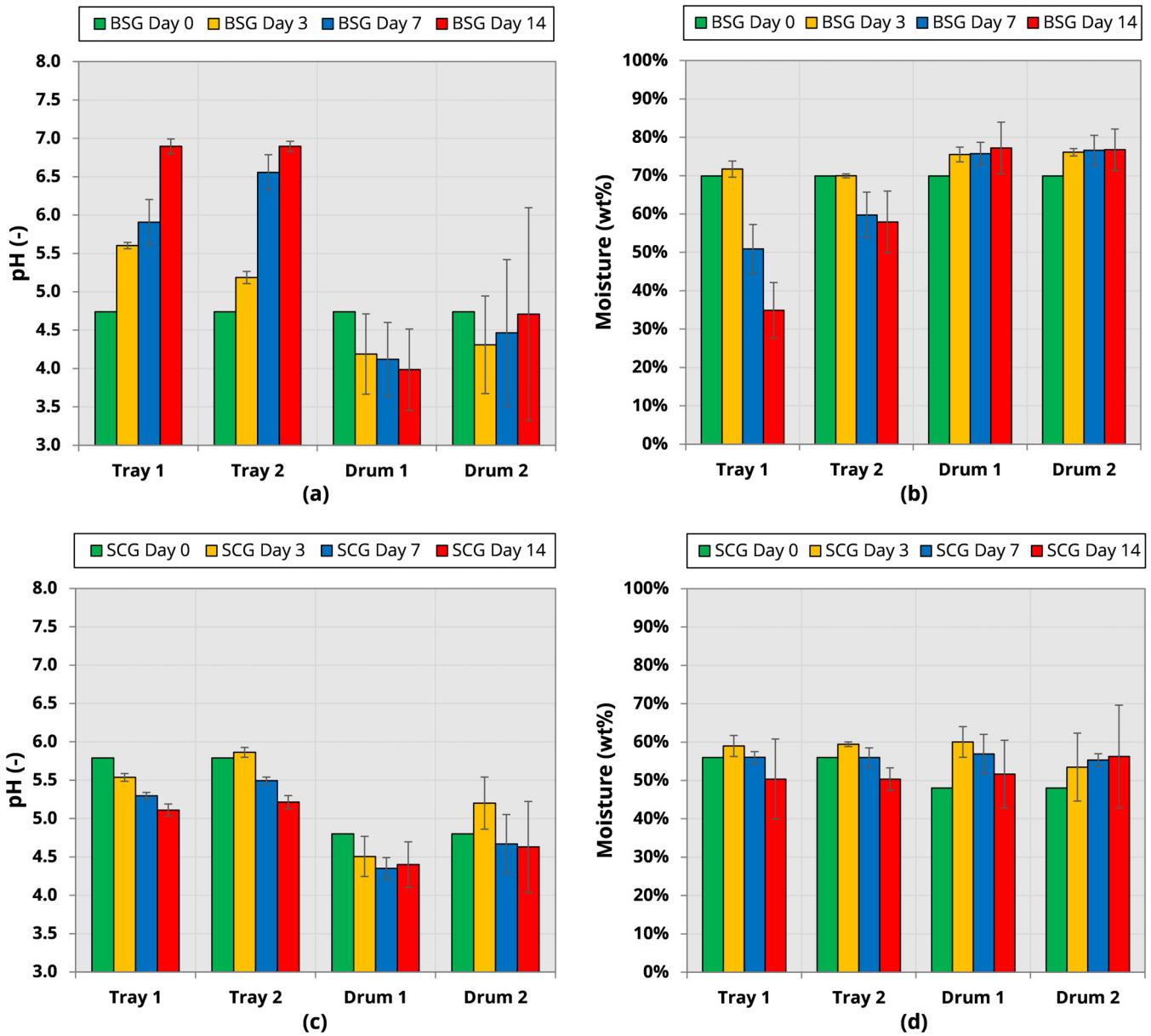

Figure 1. Chemical analysis on wet matter basis of BSG and SCG during storage in trays (BSG-2 and SCG-2) and drums (BSG-2 and SCG-3): (**a**) pH of BSG in a 50 wt% solution; (**b**) moisture content of BSG; (**c**) pH of SCG in a 50 wt% solution; (**d**) moisture content of SCG.

The moisture content for BSG is shown in Figure 1b, and storage on trays shows no significant difference from the initial 70% value at day 3, but then the moisture content lowers over the course of storage with significant differences between the two trays of 35 ± 7% and 58 ± 8%. The BSG in trays apparently dries out under ambient conditions (ca. 30 °C; 70% to 80% RH). In contrast, the BSG stored in drums shows no significant difference in moisture content during storage, maintaining a stable moisture content across the trials of 76 ± 3%. The slightly elevated moisture content compared to the initial 70% is likely caused by free water draining towards the bottom of the drum due to gravity flow and may be the fundamental cause of the observed differences in pH.

Figure 1c,d show the pH and moisture content of SCG stored in trays and drums. The moisture content in trays declines slightly from the initial value of 56% to 50 ± 3%, suggesting this material is more resistant to drying compared to BSG. SCG stored in drums show a slight but insignificant increase from 48% to 52 ± 9% and 56 ± 13%, similar to BSG

in drums. In trays, the pH at day 3 is similar to the initial value of 5.8 but then gradually drops to finally reach 5.1 ± 0.1 to 5.2 ± 0.1, presumably due to the production of organic acids such as acetate and lactate by bacteria. With respect to pH, SCG in drums had a lower initial pH of 4.8 and showed larger variability in final values compared to BSG, with a final pH of 4.4 ± 0.3 and 4.6 ± 0.6. The SCG in trays did not show the same trend of increase in pH and decrease in MC as BSG. A possible interpretation is that SCG is better able to retain water while being less prone to mold contamination than BSG, which could be caused by phenolic compounds with anti-fungal activity known to be present in SCG [27].

In addition to the chemical analysis reported here, the measurements of temperature and moisture content with dataloggers can be found in the Supplementary Materials.

3.4. Total Plate Count After Storage of BSG and SCG

The microbial growth by TPC at the beginning and end of 14 days of storage was investigated for the different storage conditions. The bacteria and fungi TPCs for BSG stored in trays and drums are shown in Figure 2a,b. In trays, bacterial TPCs in the range of 9 to 10 $\log_{10}$ CFU/g were found at the end of storage, representing a 100-fold increase compared to an initial value of 7.42 ± 0.02 $\log_{10}$ CFU/g. Fungal TPCs show a more striking change, with the initial value below the limit of detection (1 $\log_{10}$ CFU/g) and a final value of 7 to 8 $\log_{10}$ CFU/g. This suggests that aerobic storage of BSG shows substantial vulnerability to fungal contamination. In contrast, BSG in drums exhibits no detectable fungal TPC, while bacterial TPCs are similar to or slightly lower than those in trays in the middle position, but much lower than those in the bottom position with 6.12 ± 0.27 and 6.66 ± 0.07 $\log_{10}$ CFU/g. Overall, these studies suggest that enclosed storage or ensilage will help to protect BSG from contamination with fungal contaminants that may give rise to mycotoxins and the degradation of feedstock quality.

With respect to coffee grounds, the bacteria and fungi TPCs for SCG stored in drums are shown in Figure 2c,d. Bacterial TPCs were found to be in the range of 8 to 9 $\log_{10}$ CFU/g for all positions in both replicates, representing over a 100-fold increase compared to the initial value of 6.16 ± 0.02 $\log_{10}$ CFU/g. Although SCG is less nutritious than BSG, high counts of bacteria are still found at the end of storage. This could be due to high surface area and porosity of SCG, which provide a favorable microenvironment for microbial colonization [58]. Indeed, even if nutrients are limited, the structure of the SCG can retain moisture, making residual nutrients present in the SCG available for microbial consumption. Conversely, fungi were only detected in the bottom sample of one of the replicates, with a TPC of 2.60 ± 0.07 $\log_{10}$ CFU/g, compared to 1.94 ± 0.00 $\log_{10}$ CFU/g fungi initially. SCG stored in drums thus appears prone to bacterial spoilage, even though reported literature on the spoilage of coffee products has focused on fungal spoilage [32–34]. A low fungal TPC of 1.94 ± 0.00 $\log_{10}$ CFU/g was found in the SCG-3 as received, and also in one sample from the bottom of the drum with 2.60 ± 0.05 $\log_{10}$ CFU/g, indicating a risk of fungal spoilage even in drum storage. These results highlight that SCG is microbially unstable towards both bacteria and fungi, with likely implications on the quality of products such as food ingredients and lipid and bioactive extracts that can be produced from SCG after industrially relevant storage.

In addition to the TPCs reported here, bacterial and fungal abundance measured by quantitative PCR can be found in the Supplementary Materials Figure S10. These supplementary results indicate that the bacterial growth occurs rapidly, stabilizing around day 3 for BSG and day 7 for SCG, while fungal growth increases more gradually, with the exception of BSG under anaerobic storage, in which the fungal presence decreased.

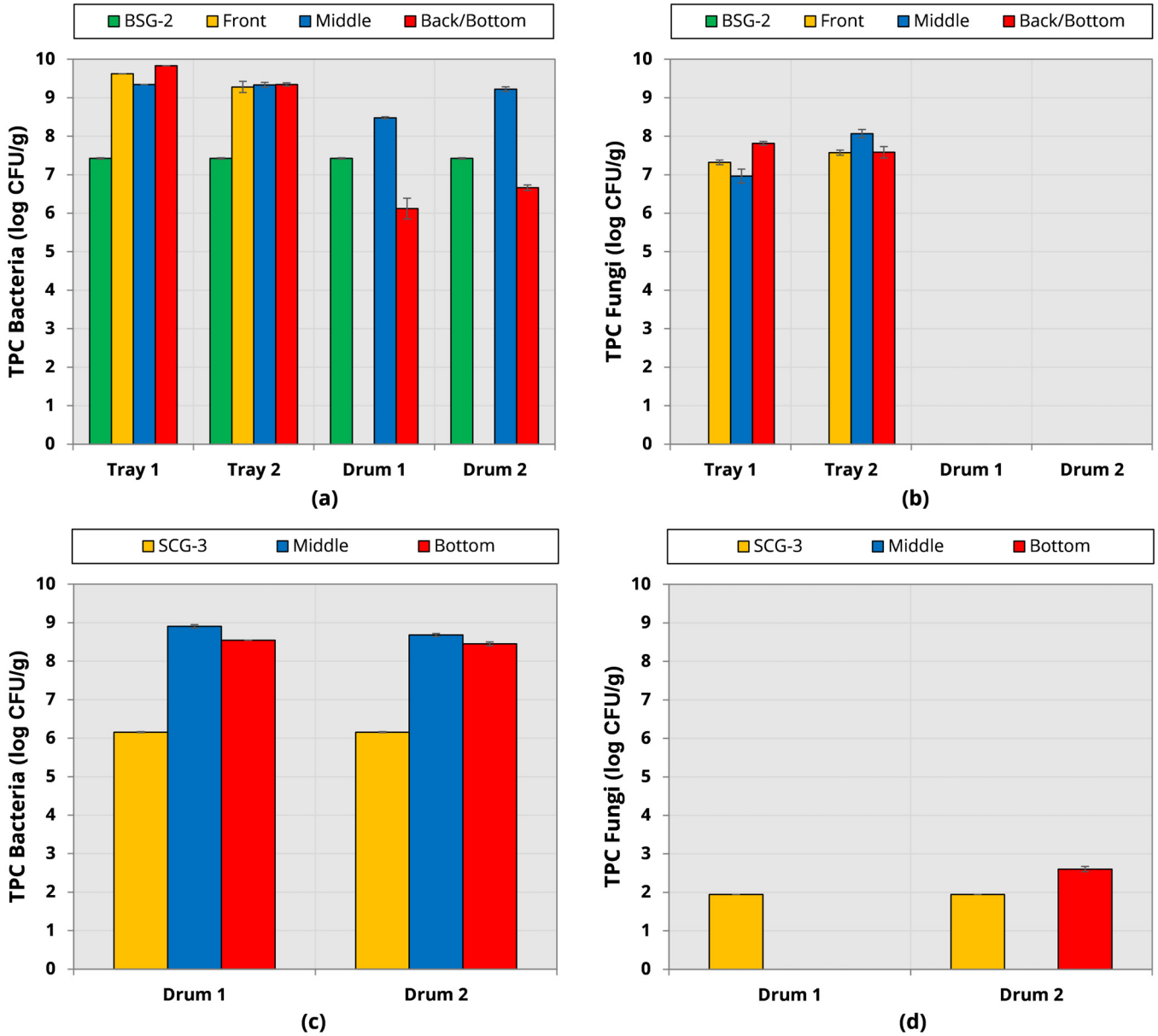

Figure 2. Total plate count (TPC) on wet matter basis of BSG and SCG before and after 14 days of storage in trays (BSG-2) and drums (BSG-2 and SCG-3): (**a**) $\log_{10}$ TPC of bacteria in BSG; (**b**) $\log_{10}$ TPC of fungi in BSG, (**c**) $\log_{10}$ TPC of bacteria in SCG; (**d**) $\log_{10}$ TPC of fungi in SCG.

3.5. Amplicon Metagenomic Sequencing After Storage of BSG and SCG

In order to characterize the microbiome, bacterial 16S rRNA and fungal ITS amplicon metagenomics sequencing was conducted on the solid sidestreams as received and after 14 days of storage. The metagenomic sequencing results at the genus level are summarized for BSG in Figure 3, showing the top 10 genera and species of bacteria and fungi identified by amplicon sequencing.

BSG-1, from the craft brewery, has a very different bacterial metagenomic sequencing result from BSG-2 and BSG-3 from the recycling facilities. The dominant results from BSG-1 are *chloroplast* and *mitochondria*, arising from the BSG material itself and indicating a very low microbial load as also found by TPC. In contrast, significant bacterial loads were found by TPC for BSG-2 and BSG-3, and the metagenomic sequencing found *Bacillus* (*B. coagulans*, *B. smithii*, and *B. thermoamylovorans*) to be the dominant genus. *Paenibacillus* (*P. tumbae*, *P. cookie*, and *P. barengoltzii*), *Lactobacillus* (*L. amyloticus* and *L. delbrueckii*), and *Acetobacter* (*A. lovaniensis*) are also prominent in BSG-3, while other genera have minor

contributions to the BSG microbiome. The fungal microbiome characterization of as-received BSG was inconclusive, with only BSG-3 having *Kluyveromyces* (*K. marxianus*) and *Pichia* (*P. kudriavzevii*) identified at the species level, as seen in Figure 3a,c. These results generally agree with those obtained by microbial isolation and identification and shown in Table 4, but with some differences in the assigned genus and species identified.

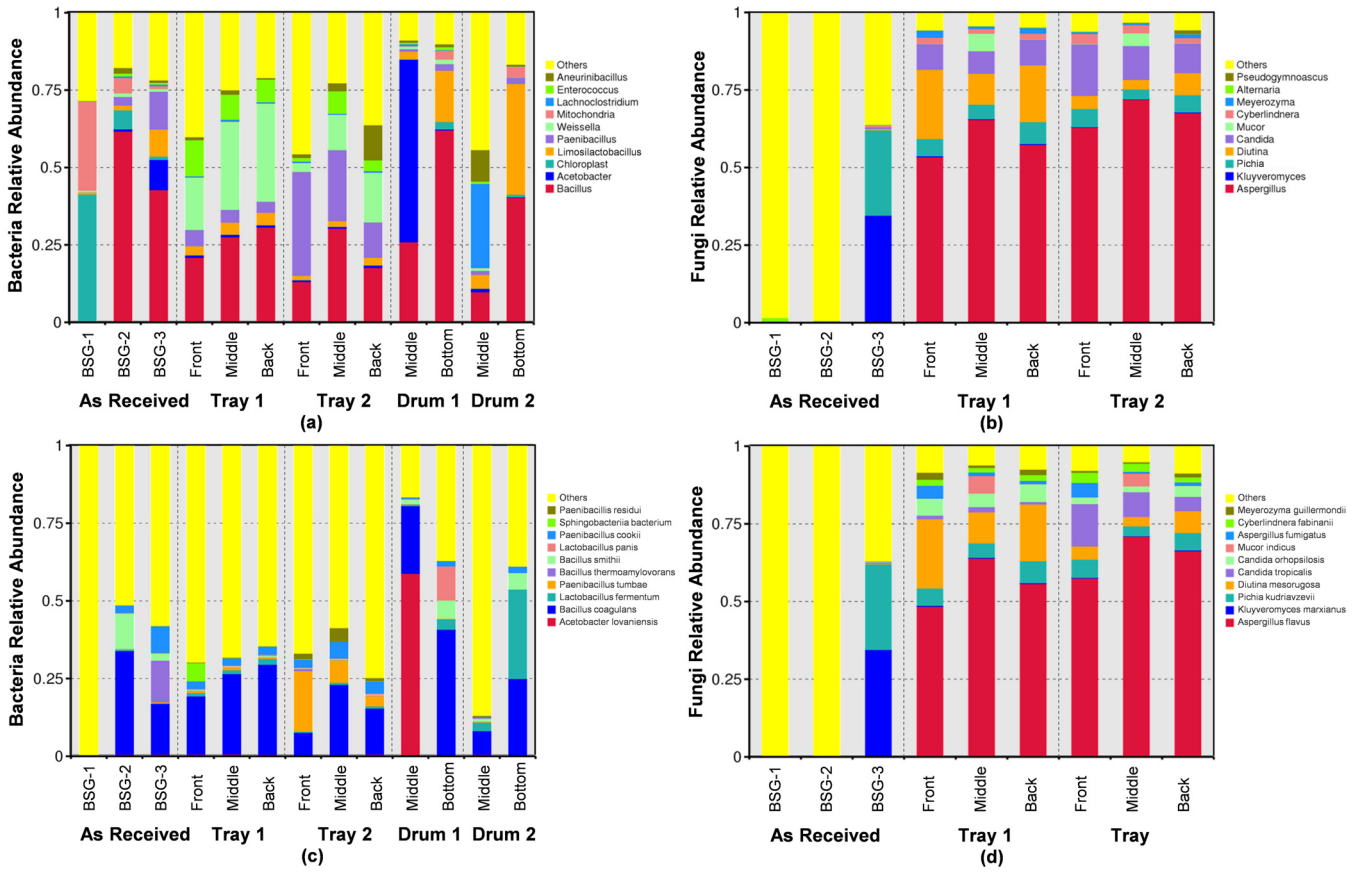

Figure 3. Amplicon metagenomic sequencing of BSG-1, BSG-2, and BSG-3 as received and BSG-2 after 14 days of storage in trays and drums: (**a**) top 10 genera of bacteria; (**b**) top 10 genera of fungi; (**c**) top 10 species of bacteria; (**d**) top 10 species of fungi identified by 16S rRNA and ITS amplicon sequencing. Note that due to negligible fungal TPC, drum samples were not ITS-sequenced.

Storage of BSG in trays substantially altered the microbiome, with the dominance of *Bacillus* bacteria being supplanted by *Enterococcus*, *Weisella*, and *Paenibacillus*, with some differences between the two trays and sampling positions, especially for the species identified, as per Figure 3. The fungal microbiome on storage in trays is dominated by filamentous fungi of the genus *Aspergillus* (*A. flavus* and *A. fumigatus*), with over 50% relative abundance in all tray samples. Aspergilli, especially *A. flavus*, are known spoilage microorganisms in BSG, known to potentially produce highly toxic compounds known as aflatoxins [59]. This taxon was dominant in all tray samples on storage as shown in Figure 3b. The other major filamentous fungus identified was *Mucor* (*M. indicus*), which is found in a number of traditional Asian fermented foods [60]. *Alternaria* and *Pseudogymnoascus* genera were also found with low prevalence. *Alternaria* spp. have previously been associated with BSG spoilage [59]. A number of yeasts were identified, including all three yeasts isolated and identified previously: *Kluyveromyces* (*K. marxianus*), *Pichia* (*P. kudriavzevii*, *Candida* (*C. tropicalis* and *C. orthopsilosis*), *Diutina* (*D. mesorugosa*), *Cyberlindnera* (*C. fabianii*), and *Meyerozyma* (*M. guilliermondii*). In conjunction with the high bacterial and fungal TPCs for BSG under aerobic storage, it is evident that growth of

Enterococcus (*E. durans* and *E. faecium*) and *Aspergillus* (*A. flavus* and *A. fumigatus*) is a key concern not just for food safety, but also for animal feed safety and quality due to aflatoxins.

Compared to the aerobic conditions present in trays, under anaerobic conditions, the BSG stored in drums showed a more variable yet distinctively fermentative bacterial microbiome. Bacilli are present in all samples and are especially prevalent in the bottom samples. This is surprising as bacilli are typically aerobic, and the drum bottom is expected to have an anaerobic environment. This may be due to *B. coagulans*, the most prevalent *Bacillus* species, having been reclassified as the facultative anaerobe *Heyndrickxia coagulans*, originally isolated from spoiled canned milk products [61]. The middle sample from drum 1 is dominated by the acetic acid bacterium (AAB) *A. lovaniensis*, while drum 2 has a high proportion of LAB, specifically *Limosilactobacillus fermentum* (formerly known as *Lactobacillus fermentum*). The middle of drum 2 instead has a significant presence of *Lachnoclostridium*, a proposed genus in the class of Clostridia, described as obligate anaerobes producing acetate as major end-products from mono- and disaccharides [62]. The difference in microbiomes is believed to be due to differences in oxygen prevalence—AABs require the presence of oxygen to metabolize their substrates, while Clostridia lack aerobic respiration and do not tolerate oxygen.

The microbiome dynamic observed in drum 1 is a metabolic synergy between LAB and AAB. LAB produce lactic acid either by a homofermentative glycolytic pathway, or a heterofermentative phosphoketolase pathway. The genus *Lactobacillus*, after the taxonomic revision, now contains exclusively homofermentative species producing lactic acid as the major product from hexoses [40]. The genus *Limosilactobacillus* was split from *Lactobacillus* and contains only heterofermentative species, such as *L. fermentum*, producing lactic acid, CO_2, and acetate or ethanol as major products [40]. AAB are named for their ability to convert ethanol to acetic acid, while *Acetobacter* are distinguished by being able to oxidize lactate and acetate into CO_2 and H_2O [63]. AAB such as *A. lovaniensis* are therefore able to metabolize the end-products from LAB such as *L. fermentum*. Therefore, LAB and AAB jointly proliferate under microaerobic conditions, as evidently present in drum 1. In addition to the metagenomic results reported here, a taxonomic heatmap for the top 35 bacterial species identified can also be found in Figure S11 in the Supplementary Materials.

With respect to coffee grounds, the microbiome of SCG-2 used in tray storage and SCG-3 used in drum storage, both from the recycling facility, contain similar genera, but in different proportions. Figure 4 shows the top 10 genera and species of bacteria and fungi identified in SCG by amplicon sequencing.

SCG-2 has over 50% relative abundance of *Limosilactobacillus*, with *L. panis* being the major identified species, while the SCG-3 microbiome is dominated by *Lactiplantibacillus*. *Acetobacter* is more prevalent in SCG-2, and *Ligilactobacillus* (*L. acidipiscis*) is more prevalent in SCG-3 but is present in both. Genera such as *Lactobacillus*, *Bacillus* (*B. coagulans*), *Pseudomonas*, *Liquorilactobacillus* (*L. nagelii* and *L. vini*), *Lacticaseibacillus* (*L. camelliae*), *Enterobacter*, and others have minor abundance in the SCG as received. The fungal microbiomes in Figure 4b,d similarly show the same genera, but with differences in their prevalence. SCG-2 is dominated by *Pichia* (*P. kudriavzevii* and *P. manshurica*), with about 50% abundance, while *Candida* (*C. orthopsilosis*) is prevalent in SCG-3, with about 35% abundance. A number of filamentous fungi from the genus *Aspergillus*, including *A. flavus*, *A. welwithschiae*, and *A. piperis*, were identified. Note that SCG-1 was not analyzed by metagenomic sequencing due to difficulties in extracting high-quality DNA, likely due to the low TPC.

SCG storage in trays led to a proliferation of *Acetobacter* at over 60% relative abundance in all samples, followed by *Ligilactobacillus* (*L. acidipiscis*) and *Bacillus* (*B. coagulans*), especially in tray 2. In drums, *Acetobacter* also generally dominate the bacterial microbiome, followed by LAB. The only exception is the drum 2 bottom, with low *Acetobacter* but high *Ligilactobacillus* (*L. acidipiscis*) and *Bacillus* (*B. coagulans*). The bacterial dynamics are similar to those described for BSG stored in drums, with the co-existence of AAB and LAB under aerobic conditions, while this dynamic likely broke down in drum 2 due to anaerobic conditions, as evidenced by the proliferation of anaerobes. The anaerobes *Lentilactobacillus*

buchneri, *Roseburia intestinalis*, and *Ruminococcus bicirculans* are present in low abundance in drum bottom samples as seen in Figure 4c.

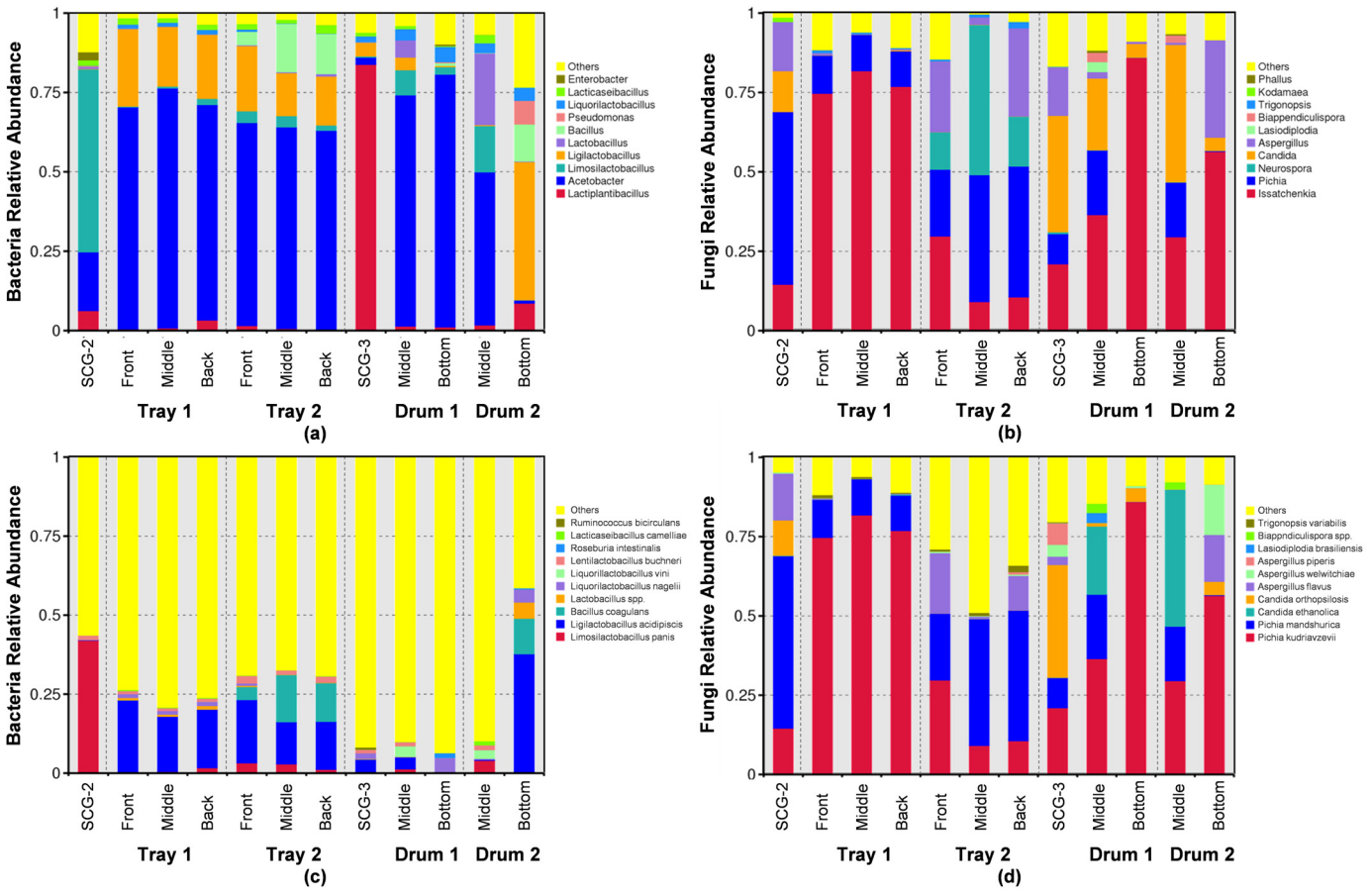

Figure 4. Amplicon metagenomic sequencing of SCG-2 and SCG-3 as received and after 14 days of storage in trays (SCG-2) and drums (SCG-3): (**a**) top 10 genera; (**b**) top 10 genera of fungi; (**c**) top 10 species of bacteria; (**d**) top 10 species of fungi identified by 16S rRNA and ITS amplicon sequencing. Note that SCG-1 was not analyzed by metagenomic sequencing due to difficulties in extracting high-quality DNA.

For SCG, there is less difference between the tray and drum microbiome at the genus level, possibly due to the ability of SCG to better retain moisture during storage in trays, consistent with a lower reported effective moisture diffusivity for SCG than BSG [38,64]. The bacterial microbiome is generally dominated by synergistic AAB and LAB, while the fungal microbiome is dominated by *Pichia* in both trays and drums, with relative abundance ranging from about 50% to 90%. The most common species is *P. kudriavzii*, classified under the genus *Issatchenkia* (with the heterotypic synonym *I. orientalis*) in Figure 4. In addition to the metagenomic results reported here, a taxonomic heatmap for the top 35 bacterial species identified can also be found in Figure S12 in the Supplementary Materials.

The results reported here complement those of Robertson et al., who investigated the microbiome and storage of BSG at different temperatures under aerobic conditions, but without environmental exposure [18]. They found bacteria TPC to reach approximately 8 $\log_{10}$ CFU/g for mesophilic bacteria and 6 $\log_{10}$ CFU/g for thermophilic bacteria by day 16 at 20 °C, with most of the growth happening before day 3 and stabilizing by day 7 [18]. Robertson et al. previously identified the presence of microaerobic bacteria (presumably LAB), anaerobic bacteria (presumably *Clostridia*), yeast, and mold at the site of production [16], but they evidently did not proliferate or were not culturable upon aerobic storage. Metagenomic sequencing provides deeper insight into the microbial ecology,

identifying the dominant genera and key species both at the time of production and after storage. Mesophilic aerobic bacterial genera such as *Bacillus* and *Paenibacillus*, as well as the facultative anaerobes *Weisella* and *Enterococcus*, proliferate in aerobic storage, while AAB and LAB proliferate in anaerobic and microaerobic storage. The most prevalent bacterial species in BSG, but also found in SCG, is the thermophilic facultative anaerobe *B. coagulans*, recently reclassified as *Heyndrickxia coagulans* [61]. Extensive growth of the yeast genera *Kluyveromyces*, *Pichia, Diutina*, and *Candida* and molds *Aspergillus* and *Mucor* occurs in aerobic storage. The bacterial microbiome of SCG on storage shows similarities with BSG at the genus level, with AAB and LAB prevalent in both aerobic and anaerobic storage conditions, and *Bacillus* present in conditions, but with differences in identified species. The yeast genus *Pichia* is extensive in all storage conditions, while *Candida* proliferate in anaerobic conditions, and the molds *Neurospora* and *Aspergillus* are found especially in aerobic storage.

4. Conclusions

BSG and SCG solid sidestreams at the point of generation have low bacterial loads (3.53 ± 0.03 and <1 $\log_{10}$ CFU/g) while lacking a distinct microbiome and are suitable for valorization into food, feed, cosmetics, or other applications. However, these solid sidestreams undergo rapid chemical and biological changes during logistics, and after collection by recycling companies, they have high bacterial loads (>6 $\log_{10}$ CFU/g) and a distinct microbiome, dominated by *Bacilli* for BSG and LAB for SCG, arising from environmental contamination. This highlights the significant risk of contamination with food-borne contaminants such as *B. cereus*, although it was not identified in this study. The microbiome changed significantly during storage under industrially relevant aerobic and anaerobic conditions. Rapid bacterial growth occurred, stabilizing around day 3 for BSG and day 7 for SCG, with over 100-fold increases in bacterial loads (>8 $\log_{10}$ CFU/g) by day 14 under aerobic conditions. *B. coagulans* (reclassified as *Heyndrickxia coagulans*) was identified as the most prevalent bacterial species in BSG on storage and was also found in SCG. Extensive fungal growth (>7 $\log_{10}$ CFU/g) was observed for BSG, with potential aflatoxin-producing *A. flavus* being the dominant species, which was also identified in SCG stored aerobically. Aflatoxin contamination is a major food and feed safety concern and must be managed if BSG and SCG are valorized for such applications. Anaerobic storage inhibited fungal growth and led to changes in the bacterial microbiome. A dynamic of LAB and AAB co-dependency was identified, likely under microaerobic conditions, while facultative anaerobes such as *Pseudomonas*, *Enterococcus*, and *Enterobacter*, and even obligate anaerobic *Clostridia*, were also identified in anaerobic conditions. These findings highlight critical food safety concerns while providing essential information for establishing shelf-life and storage guidelines for BSG and SCG, with the aim of promoting the further valorization of these solid sidestreams in industrialized Asia.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14219759/s1: Figure S1: Experimental setup used for storage trials. Figures S2–S5: Physical measurements of temperature and humidity by dataloggers for each tray and drum containing BSG; Figures S6–S9: Physical measurements of temperature and humidity by dataloggers for each tray and drum containing SCG. Figure S10: Quantitative polymerase chain reaction (qPCR) of BSG and SCG during 14 days of storage in trays (BSG-2 and SCG-2) and drums (BSG-2 and SCG-3). Figure S11: Taxonomic abundance cluster heatmap hierarchical clustering of samples for the top 35 bacterial species of BSG-1, BSG-2, and BSG-3 as received and after 14 days of storage in trays (BSG-2) and drums (BSG-2). Figure S12: Taxonomic abundance cluster heatmap hierarchical clustering of samples for the top 35 bacterial species of SCG-2 and SCG-3 as received and after 14 days of storage in trays (SCG-2) and drums (SCG-3). Table S1: PCR primers and run conditions for qPCR of BSG and SCG samples. References [17,65] are cited in the Supplementary Materials.

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Draft, C.H.; Writing—Review and Editing, M.W. and E.C.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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